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Research

Phylogeographically concordant chloroplast DNA divergence in sympatric *Nothofagus s.s.* How deep can it be?

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Summary

- Here, we performed phylogenetic analyses and estimated the divergence times on mostly sympatric populations of five species within subgenus *Nothofagus*. We aimed to investigate whether phylogenetic relationships by nuclear internal transcribed spacer (ITS) and phylogeographic patterns by chloroplast DNA (cpDNA) mirror an ancient evolutionary history that was not erased by glacial eras. Extant species are restricted to Patagonia and share a pollen type that was formerly widespread in all southern land masses. Weak reproductive barriers exist among them.
- Fifteen cpDNA haplotypes resulted from the analysis of three noncoding regions on 330 individuals with a total alignment of 1794 bp. Nuclear ITS data consisted of 822 bp. We found a deep cpDNA divergence dated 32 Ma at mid-latitudes of Patagonia that predates the phylogenetic divergence of extant taxa. Other more recent breaks by cpDNA occurred towards the north.
- Complex paleogeographic features explain the genetic discontinuities. Long-lasting paleobasins and marine ingressions have impeded transoceanic dispersal during range expansion towards lower latitudes under cooler trends since the Oligocene.
- Cycles of hybridization–introgression among extant and extinct taxa have resulted in widespread chloroplast capture events. Our data suggest that *Nothofagus* biogeography will be resolved only if thorough phylogeographic analyses and molecular dating methods are applied using distinct genetic markers.

Introduction

Phylogeographic analyses are being widely used to locate refugial areas for species' persistence during ice ages around the world. Surprisingly, a growing body of evidence is showing that many plant species were able to survive in multiple glacial refugia, some of which were located in areas of supposedly larger ice extent, that is, towards north and south latitudinal distributional ends for Northern and Southern Hemispheres, respectively (Premoli, 1998; Premoli *et al.*, 2000; Stewart & Lister, 2001; McLachlan *et al.*, 2005; Bhagwat & Willis, 2008; Jakob *et al.*, 2009). For such species, phylogeographic evidence would then reflect the *in situ* survival of genetic variants that were established sometime in the past and endured the ice ages of the Neogene in long-lasting stable areas. As a result, geographic structures of extant populations can be traced back to the early Tertiary (Paleogene) history (Lumaret *et al.*, 2002; Magri *et al.*, 2007; Mathiasen & Premoli, 2010). Hence, a critical issue in the plant biogeography of such lineages is to understand ancient physical scenarios, including the spatial configuration of suitable areas and potential corridors, that is, paleogeographic features, driving early patterns of vicariance and dispersal.

The southern *Nothofagus*, currently distributed in land masses that were once united in Gondwana, is considered to be a key genus in plant geography, and has been the focus of numerous biogeographic reconstructions. This is mainly a result of the fact that the *Nothofagus* fossil record, particularly pollen, is relatively extensive and very well known (Hill, 2001), especially in southern South America (Romero, 1986a,b). Pollen diversity within *Nothofagus* consists of clearly distinct morphological groupings (Dettmann *et al.*, 1990) that match the four extant subgenera *Fuscospora*, *Lophozonia*, *Nothofagus* and *Brassospora* (Hill & Read, 1991). They are monophyletic and related as: (*Lophozonia* (*Fuscospora* (*Nothofagus*, *Brassospora*))) (Manos, 1997). By the Oligocene at the latest, all the subgenera of *Nothofagus* were present and, since then, the amount of change, particularly for microfossils, has been relatively minor (Hill, 1991). As a result, distinct species within each of the individual pollen types, except those of subgenus *Brassospora*, are indistinguishable from others in the same pollen grouping (Hanks & Fairbrothers, 1976). In addition, the homoplasious nature of the leaf architectural traits in *Nothofagus* suggests that the identification of taxa based on macrofossils, such as leaf impressions, should be treated with caution

(Jordan & Hill, 1999). Therefore, restrictions exist in *Nothofagus* for biogeographic reconstructions based on fossils, particularly at lower taxonomic levels.

The first appearance of *Nothofagus* pollen was recorded at 83.5 million yr ago (Ma) in southern Australia and Antarctica (Dettmann *et al.*, 1990) and is remarkably consistent with molecular estimates (Cook & Crisp, 2005). Conversely, some inconsistencies occur for the radiation dates of extant lineages, estimated as 71–73 Ma for pollen attributed to all four subgenera (Dettmann *et al.*, 1990; Swenson *et al.*, 2001a) and no more than 58 Ma for the earliest node in the *Nothofagus* phylogeny (Cook & Crisp, 2005). Discrepancies may arise from interpretations of the fossil record, given that the occurrence of a given pollen type would not necessarily signal the appearance of the subgenus (Hill, 2001) or the origins of extant taxa (Knapp *et al.*, 2005). Similarly, DNA regions analyzed by molecular studies may have distinct mutation rates (Manos, 1997 and references therein) and/or may show intraspecific polymorphisms which can alter phylogenetic reconstructions (Acosta & Premoli, 2010). As a consequence, biogeographic analyses of *Nothofagus* combining phylogenies with fossils have yielded ambiguous results.

Traditionally, *Nothofagus* was considered as an ancient genus not suited for ocean dispersal, which reinforced the hypothesis of vicariance and land-based dispersal to explain the transoceanic distributions in the Southern Hemisphere. However, combined phylogeny and molecular dating evidence by chloroplast DNA (cpDNA) put forward scenarios of recent divergence within subgenera *Fuscospora* and *Lophozonia* (Cook & Crisp, 2005; Knapp *et al.*, 2005). Yet, phylogenetic and phylogeographic analyses of subgenus *Nothofagus* in southern South America showed that, although nuclear DNA illustrates relationships among delimited species (Acosta & Premoli, 2010), cpDNA is region specific. Hence, the use of cpDNA in phylogenetic analyses needs to be revisited, given that neglected intraspecific polymorphisms may bias biogeographic interpretations.

Estimates of divergence times within and among species have always been a relevant topic of discussion in evolutionary biology. Abundant molecular evidence shows that most temperate taxa were profoundly affected by Quaternary events (Hewitt, 2000). However, a cpDNA phylogeography of the widespread cool-temperate rainforest species *Nothofagus cunninghamii* from southeastern Australia and Tasmania yielded a deep chloroplast divergence which was interpreted as long-term *in situ* occupation that almost certainly significantly predates the ice ages (Worth *et al.*, 2009). Similarly, a phylogeographic analysis of the South American cold-tolerant *Nothofagus pumilio* showed that the current genetic structure is the result of regional processes that took place during the early Tertiary (Paleogene) that were enhanced by contemporary local effects of drift and isolation in response to Quaternary glaciations in Patagonia (Mathiasen & Premoli, 2010; Premoli *et al.*, 2010). In addition, although relatively recent among-species' divergence was suggested for congener species within subgenus *Nothofagus* (Romero, 1986a), population genetic studies provided evidence of a more ancient evolutionary history than previously hypothesized (Premoli, 1997). Nevertheless, the question is still open on how old are extant cold-hardy species and if the remains of an ancient genetic structure that were not erased by glacial eras can be traced by molecular dating methods on nuclear and chloroplast genomes, respectively.

Here, we dated a multispecies' phylogeographic analysis using DNA sequences of three noncoding regions of the chloroplast genome and the complete internal transcribed spacer (ITS) region. Such extended nuclear and chloroplast genealogy was based on samples collected along the entire distributional range of the five extant species within subgenus *Nothofagus* – *N. antarctica*, *N. betuloides*, *N. dombeyi*, *N. nitida* and *N. pumilio* – in southern Argentina and Chile. The aim of our study was to analyze the spatially explicit evolutionary history of *Nothofagus* lineages in order to: evaluate whether spatially concordant phylogeographic patterns exist among populations of all species; provide a time frame from estimates of divergence times for the combined intra- and interspecific gene genealogies by nuclear and chloroplast regions; and reconstruct the biogeographic history in relation to paleofeatures of the landscape significantly distinct from those at present.

Materials and Methods

Study species

Most trees and shrubs within subgenus *Nothofagus* are commonly found nowadays along *c.* 20° latitude in temperate South America. They can grow in sympatry, but within highly distinct habitat types. Natural regeneration within the subgenus is dependent on disturbances occurring at different spatial and time scales (Veblen *et al.*, 1996). Subgenus *Nothofagus* was widespread during the early Tertiary (Paleogene) (Dettmann *et al.*, 1990; Hill, 1991) and its extinction outside South America is suggested to have occurred as a result of a decline in catastrophic disturbances in these areas (Hill, 1991). Therefore, gene pools of such widespread lineages have been shaped by disturbance (Premoli & Kitzberger, 2005; Millerón *et al.*, 2008; Premoli & Steinke, 2008; Mathiasen, 2010). Although species within subgenus *Nothofagus* are ecologically distinct and clearly identifiable wherever they occur in sympatry, hybrids are locally produced between almost all species' pairs (Premoli, 1996; Stecconi *et al.*, 2004; Quiroga *et al.*, 2005), and have been so in the past (Acosta & Premoli, 2010).

Paleogeography of the Patagonian Cordillera

Since the early work of Cande & Leslie (1986), it has been well established that the Andean margin of Patagonia was modified by an important collision of oceanic ridges during Eocene times against the subduction zone between 42° and 43° southern latitude (Ramos & Kay, 1992). Ridge collision is one episodic process that can cause significant tectonic effects when a thermally active oceanic spreading ridge approaches, collides with and potentially subducts below a continental margin. Such oceanic ridge collision produced mountain uplift, which segmented the Patagonian Cordillera into two different paleogeographic realms (Fig. 1). Therefore, very different scenarios were established by 40 Myr that generated different basins and landscapes (Fig. 2). North of 43°S, the main cordillera was characterized along

Fig. 1 Paleogeographic realms of the Patagonian Andes at 40 million yr (Myr) ago. North of 43°S the terrain consists of low-elevation mountains with an active volcanic arc; the southern segment is characterized by higher mountains associated with extensive plateau basalts. Based on Cande & Leslie (1986) and Ramos (2005).

hundreds of kilometers by low-elevation mountains with an active volcanic arc. Important explosive eruptions by stratovolcanoes resulted in up to 1500 m thick volcaniclastic layers, some interspersed with marine deposits with a Pacific provenance (Spalletti, 1983). As a result of this mountainous relief, the marine transgressions produced in the Miocene after 22 Myr were controlled by the previous topography. A series of isolated basins partially connected with the Pacific along the present Chilean margin characterized the region. The volcanic ashes and pyroclastic flows that were absent in the southern segment produced an important stress on the vegetation. South of 43°S, the paleogeography was entirely different from Eocene times. The ridge collision produced an important uplift and deformation along the Patagonian Cordillera. The volcanic arc was not active, and the Paleogene Andes in the southern segment was characterized by higher mountains associated with extensive plateau basalts (Ramos, 2005). These are large outflowings of basalts that cover most of extra-Andean Patagonia which erupted east of the Cordillera and the present volcanic arc throughout the Tertiary. The westerly winds were interrupted along the axis of the cordillera, and therefore areas in the south were probably more arid than the equivalent northern areas. The Eocene basins were filled in this southern segment by continental fluvial deposits. During the Oligocene and Miocene, southern areas were invaded by marine transgression from the Atlantic Ocean. In addition, Pacific marine ingressions occurred at those latitudes of Patagonia from the Oligocene onwards into the Miocene (Bechis *et al.*, 2011). Remarkable paleogeographic differences between north and south of 42°–43° southern latitude persisted until late Miocene times, with new uplifts, until reaching the Present with a continuous geological environment. Therefore, latitudinally distinct paleogeographic settings of the southern Andes have most probably affected the phylogeographic patterns of ancient lineages such as *Nothofagus*.

cpDNA analysis

We sampled fresh leaf material for cpDNA analyses from 330 individuals of 200 populations of the five extant species of the subgenus *Nothofagus* (*N. antarctica*, *N. betuloides*, *N. dombeyi*, *N. nitida* and *N. pumilio*) along their entire range (Table 1, Fig. 3). We collected nine other *Nothofagus* species belonging to the subgenera *Lophozonia* and *Fuscospora*, as well as one individual of *Betula pendula*, to be used as outgroups. Voucher specimens were deposited in the herbarium of Centro Regional Universitario Bariloche, Universidad Nacional del Comahue, Argentina (BCRU). DNA extraction was performed using the DNeasy Plant

Fig. 2 Paleogeography of southern South America during the Oligocene–Miocene. Note that the western Patagonian Cordillera preserved offshore facies that could be an important barrier to *Nothofagus* distribution (modified from Ramos, 1982).

Mini Kit (Qiagen) following the manufacturer's instructions, and concentrations were assessed by electrophoresis on agarose gels and comparisons with a 1-kb DNA ladder (Fermentas). Reactions with 1–2 μl of DNA extract (*c.* 10 ng) and 4–6 μl of GeneReleaser® (BioVentures, Murfreesboro, Tennessee, USA) were performed prior to PCR, which segregate the inhibitors released during lysis, together with preservation agents that may interfere with amplification to facilitate DNA release (conditions: 15 min at 85°C, hold at 32°C). Noncoding regions of cpDNA were amplified by PCR using three universal primer pairs: *psb*B–*psb*H (BH) (Hamilton, 1999), *trn*L–*trn*F (LF) (Taberlet *et al.*, 1991) and *trn*H–*psb*A (HA) (Hamilton, 1999). The PCR mix contained 10 ng DNA, 5 μl 5 × Green GoTaq® Reaction Buffer (Promega, Madison, WI, USA), 0.25 mM of each deoxynucleoside triphosphate (dNTP), 0.3 μM of each primer and 1.25 U of GoTaq® DNA polymerase (Promega) in a total volume of 25 μl (conditions: 4 min at 95°C; 35 cycles of 1 min at 94°C, 1 min annealing at 57°C for BH, 54°C for LF and 56°C for HA, and 1.5 min at 72°C; and a final 6 min at 72°C). In addition, the complete ITS region, including the 5.8S rRNA gene, was amplified using the primers CY1 and CY3 (Wright *et al.*, 2006). We sequenced only one individual from each species for the ITS region as a result of the highly conserved DNA sequences of this nuclear region for any given species (Acosta & Premoli, 2010). The PCR mix contained 1 μl of template DNA (10 ng), 0.625 U GoTaq® DNA polymerase (Promega), 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 was 4 min at 95°C; 30 cycles of 30 s at 94°C, 1 min at 56°C and 2 min at 72°C; a 10-min extension at 72°C; and a final hold at 15°C. The PCR products were purified using 2.5 U of Exonuclease I (USB) and 0.25 U of shrimp alkaline phosphatase (USB, Santa Clara, California, USA) 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) at Universidad Nacional del Comahue using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The cycle sequencing reactions were performed following the manufacturer's protocols. The amplified regions were sequenced in both directions for at least one individual from each population and/or species. Sequences of each sampled cpDNA haplotype and nuclear ITS ribotype were deposited in GenBank (Accession Numbers: **BH**: GQ863274–GQ863275, GQ863285–GQ863286, GQ863367–GQ863370, GQ863397–GQ863399,

Table 1 Origin, distribution, haplotypes and voucher numbers of *Nothofagus* samples used in the phylogenetic analyses

Subgenus	Species	Source	South latitude	Haplotype	Herbarium voucher
Nothofagus	*N. antarctica* (Forst) Oerst.	Argentina/Chile	37°49′	H1	MCA & ACP 157
			36°49′–37°29′	H2	MCA & ACP 166
			37°28′	H3	MCA & ACP 163
			37°49′–39°34′	H4	MCA & EK 137
			38°49′–38°55′	H5	MCA 150
			40°19′–40°41′	H6	MCA *et al.* 171
			39°36′–40°41′	H7	MCA & EK 87
			40°59′–42°42′	H8	MCA & EK 49
			41°30′	H9	MCA *et al.* 126
			42°21′–42°42′	H10	MCA & ACP 95
			42°21′–42°42′	H11	MCA & ACP 95
			42°31′	H12	MCA *et al.* 122
			42°41′–44°54′	H13	MCA *et al.* 116
			45°34′–46°02′	H14	MCA & LG 81
			46°53′–54°52′	H15	MCA & LG 57
	N. betuloides (Mirb.) Oerst.	Argentina/Chile	40°44′–40°47′	H6	MCA & ACP 50
			41°07′–41°44′	H8	MCA 132
			42°20′	H11	MCA & ACP 99
			50°27′	H14	MCA & LG 72
			51°05′–54°52′	H15	MCA & LG 66
	N. dombeyi (Mirb.) Oerst.	Argentina/Chile	36°54′–38°27′	H2	MCA & ACP 160
			38°55′–39°36′	H4	MCA & EK 135
			40°09′–40°41′	H6	MCA & ACP 111
			40°09′–40°41′	H7	MCA & EK 142
			41°04′–42°41′	H8	MCA & EK 47
			42°00′	H10	MCA & ACP 101
			42°21′	H11	MCA & ACP 97
			42°12′–42°21′	H12	MCA *et al.* 123
			42°39′–42°53′	H13	MCA *et al.* 118
	N. nitida (Phil.) Krasser.	Chile	40°46′	H6	MCA & ACP 52
			40°46′–42°42′	H8	MCA & ACP 94
			41°53′	H10	MCA & ACP 103
	N. pumilio (Poeppl. & Endl.) Krasser.	Argentina/Chile	37°49′	H1	PM 7
			35°34′–37°49′	H2	PM 1
			36°53′–36°54′	H3	PM 3
			36°52′–39°35′	H4	PM 17
			38°48′–39°00′	H5	PM 13
			40°36′–40°44′	H6	PM 20
			40°12′	H7	PM 18
			39°14′–41°58′	H8	MCA & EK 40
			41°15′–41°31′	H9	PM 28
			42°30′–46°04′	H13	PM *et al.* 30
			45°28′–51°28′	H14	PM 47
			46°56′–55°03′	H15	PM 61
Fuscospora	*N. alessandri* Espin.	Chile	South America	*N. alessandri*-H1	MCA 224
	N. fusca (Hook.) Oerst.	Cultivated Tasmania, Australia	New Zealand	*N. fusca*-H1	MCA 183
	N. solandri (Hook.) Oerst.	Cultivated Tasmania, Australia	New Zealand	*N. solandri*-H1	MCA 184
Lophozonia	*N. cunninghamii* (Hook.) Oerst.	Cultivated Tasmania, Australia	Australia	*N. cunninghamii*-H1	MCA 185
				N. cunninghamii-H2	MCA 225
	N. glauca (Phil.) Krasser.		South America	*N. glauca*-H1	MCA 222
	N. menziesii (Hook.) Oerst.	Cultivated Tasmania, Australia	New Zealand	*N. menziesii*-H1	MCA 186
	N. moorei (Muell.) Krasser.	Cultivated Tasmania, Australia	Australia	*N. moorei*-H1	MCA 223
	N. nervosa (Phil.) Dim. & Mil.	Argentina	South America	*N. nervosa*-H1	MCA & EK 85
	N. obliqua (Mirb.) Oerst.	Argentina	South America	*N. obliqua*-H1	MCA & EK 140
Outgroup	*Betula pendula* Roth	Cultivated Bariloche, Argentina	Northern Hemisphere	*Betula pendula*-H1	MCA 221

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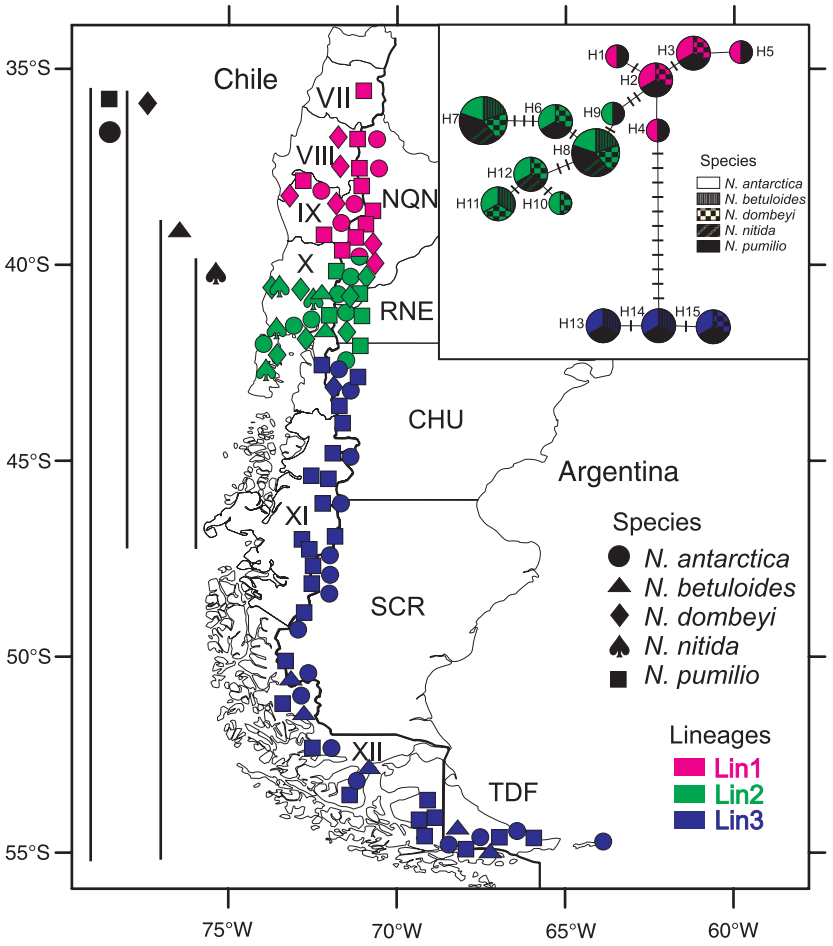

Fig. 3 Sampled locations for populations of the five species within subgenus *Nothofagus* in southern South America. Each point in the map represents a region in which one to five populations of distinct species were sampled. Colors indicate lineages for three combined noncoding chloroplast DNA (cpDNA) regions according to Fig. 4. Provinces and regions of Argentina and Chile are shown by letters and roman numbers, respectively. The distribution ranges of all species are depicted by black lines. Inset: median-joining network showing the relationship among 15 chloroplast haplotypes of subgenus *Nothofagus* species based on the combined cpDNA sequences of *psb*B–*psb*H, *trn*L–*trn*F and *trn*H–*psb*A intergenic spacers. The circle size is proportional to the number of species that present the haplotype. The colors correspond to the lineages identified in the phylogenetic tree.

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; **ITS**: GQ863229–GQ863232, GQ863237–GQ863238, GQ863240, GQ863265–GQ863268, JN247411–JN247413). Sequences for ITS of *N. alessandri* were obtained from GenBank (Accession Number: U96854). Sequencing data for all regions were aligned with MEGA4 (Tamura *et al.*, 2007) with gap additions where needed, and concatenated manually into a single combined dataset for the analyses. The cpDNA analysis resulted in a total of 15 haplotypes which were shared between at least two of the five species within subgenus *Nothofagus*.

Phylogenetic reconstruction

One sequence of each haplotype and ribotype was used to perform the phylogenetic analyses. Maximum likelihood (ML) and Bayesian inference (BI) were used to reconstruct the phylogenetic relationships among sequences, employing PAUP* 4.0b10 (Swofford, 2003) and MrBayes v3.1.2 (Ronquist & Huelsenbeck, 2003), respectively. Gap characters were coded following the 'simple indel coding' method (Simmons & Ochoterena, 2000). The most appropriate models of nucleotide substitution identified under the Akaike information criterion (AIC) by MODELTEST v3.7 (Posada & Crandall, 1998) and MRMODELTEST v2.3 (Nylander, 2004) were implemented in the ML and BI analyses, respectively. Evolution models that best fit the chloroplast data for the ML and BI analyses were K81uf + I + G (Kimura, 1981) and GTR + I + G (Rodríguez *et al.*, 1990), respectively; and for the ITS data was GTR + G (Rodríguez *et al.*, 1990) for both analyses. The ML analysis was performed using a heuristic search with 1000 random replicates, based on branch swapping with tree bisection–reconnection. Characters were considered as unordered and weighted equally. Nodal support was performed by ML bootstrap analysis (Felsenstein, 1985) with 100 replicates. The BI analysis consisted of two independent runs of two million generations with four chains each (three heated and one cold), and trees were saved every 1000 generations in each run. The run length was determined using a convergence diagnosis, that is, until the average standard deviation of split frequencies between runs approached zero. 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. To visualize the relationships among cpDNA haplotypes, a median-joining network was constructed using Network v. 4.1.1.2 (Bandelt *et al.*, 1999).

Molecular dating

The split of the stem lineage *Nothofagus* from the outgroup *B. pendula* was considered as the root of the tree. The calibration point of this node (i.e. base of the *Nothofagus* stem) was set to 84 Myr, according to the first appearance of the *Nothofagus* 'ancestral' pollen type (Dettmann, 1994). Additional time constraints were set for the different subgenera: at 73 Myr, as that has been recognized as the minimum age of pollen attributed to all four subgenera (Dettmann *et al.*, 1990; Swenson *et al.*, 2001b), and a minimum age of 31.5 Myr for the appearance of subgenera based on macrofossil records (Hill, 1991, 2001) (Table 2). In addition, for ITS dating, we used the mean substitution rate of 0.5×10^{-9} substitutions per site per year estimated for *Nothofagus* in Kay *et al.* (2006). Divergence times were estimated using two different Bayesian approaches which implement relaxed molecular clock models: the program MULTIDIVTIME (Thorne *et al.*, 1998), following the protocol from the manual by Rutschmann (2005), and BEAST v1.6.1 (Drummond & Rambaut, 2007). The parameters to run MULTIDIVTIME were set as follows: the Markov Chain was sampled 10 000 times every 100 cycles after a burn-in period of 100 000 cycles. We used an 84 Myr (SD = 10 Myr) prior for the expected number of time units between tip and root, and a 200 Myr prior for the highest possible number of time units between tip and root. The prior for the rate was obtained by dividing the median branch length by the time from the ingroup root to the tips, resulting in 0.0002 substitutions per site per time unit. The Brownian motion constant (v) describing the rate variation was set so that the magnitude of the time units from root to tip multiplied by v was approximately 1.0, as recommended in the manual. We checked for convergence of the Markov chains by building a histogram of all obtained values, which were located around a stable plateau. In addition, a total of 10 runs with the same settings, but different seeds, was performed to check for consistency in the results. The settings to run BEAST with node constraints were as follows: the substitution model was HKY with a Gamma site heterogeneity model with four categories; the clock model was set as an uncorrelated log-normal relaxed model; and we selected the Yule process as a prior for the distribution of divergence dates. The Monte Carlo Markov Chain was set to run for 1 000 000 generations sampling every 200 cycles. Each analysis (MULTIDIVTIME and BEAST) was run using three different fossil combinations as calibration points. In all cases, we set up the root age as 84 Myr (SD = 10 Myr), according to the appearance of the *Nothofagus* ancestral pollen type. The three combinations of calibration points were based on our prior fossil knowledge: first, we used two calibration points based on the first appearance of all subgenera pollen (i.e. *Lophozonia* and *Fuscospora*/*Nothofagus*), set at 73 Myr (microfossils); second, we used three calibration points combining the first appearance of *Lophozonia* and *Fuscospora*/*Nothofagus* pollen at 73 Myr and of macrofossils of different species from subgenera *Lophozonia* and *Fuscospora* at 31.5 Myr (microfossils + macrofossils, respectively); third, we used only two calibration points according to the first appearance of species from subgenera *Fuscospora* and *Lophozonia* based on macrofossils at 31.5 Myr (macrofossils) (see calibration points in Table 2 and Figs 4, 5). In addition, the settings to run BEAST to estimate the divergence time of ITS sequences using a known mutation rate were as follows: an HKY substitution model with four categories; the strict clock model with a fixed mean substitution rate of 0.5×10^{-9} substitutions per site per year (Kay *et al.*, 2006); and we selected the coalescent constant size process as a prior for the distribution of divergence dates.

Results

DNA analyses

Alignment of the final matrix consisted of 1794 bp for the three concatenated cpDNA regions and 822 bp for ITS data, including all five species within subgenus *Nothofagus* and an outgroup consisting of all most distinct *Nothofagus* subgenera *Lophozonia* and *Fuscospora* and *B. pendula.* The length of the sequences varied between 1591 and 1673 bp for the noncoding cpDNA regions *psb*B–*psb*H, *trn*L–*trn*F and *trn*H–*psb*A, and from 790 to 814 bp for the ITS1–5.8S–ITS2 region within *Nothofagus* and the outgroup. For cpDNA data, a total of 76 variable sites was found, 28 of which were indels and the other 48 were substitutions; in the nuclear data, 204 sites were polymorphic, 58 of which were indels and 193 were substitutions. The cpDNA sequence analysis of 330 individuals from 200 populations belonging to subgenus *Nothofagus* yielded 15 different geographically structured haplotypes which were shared between at least two of the five species present at any one location (Fig. 3, Table 1).

Phylogenetic reconstruction

Chloroplast haplotypes of subgenus *Nothofagus* were distributed in three major lineages, two north of 43°S and the third widely

Table 2 Fossil calibration points (ages in million yr (Myr))

Node	Fossil type	Age	Reference
R: base of the *Nothofagus* stem	Microfossil	84	Dettmann (1994)
L/F/N: minimum age of different subgenera	Microfossil	73	Dettmann *et al.* (1990); Swenson *et al.* (2001b)
F/N: minimum age of different subgenera	Microfossil	73	Dettmann *et al.* (1990); Swenson *et al.* (2001b)
L4: minimum age of different species from subgenera	Macrofossil	31.5	Hill (1991, 2001)
F2: minimum age of different species from subgenera	Macrofossil	31.5	Hill (1991, 2001)

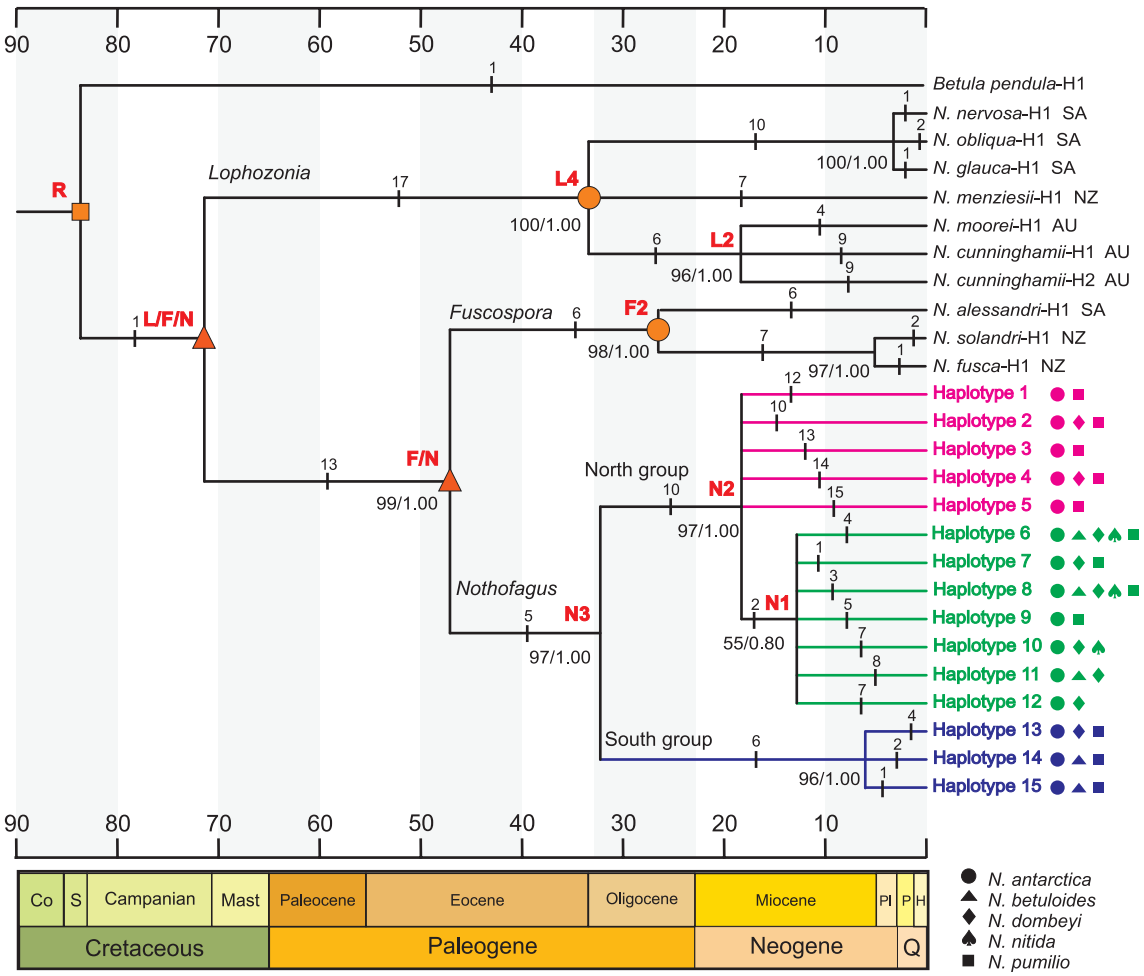

Fig. 4 Chronogram indicating the evolutionary relationships among chloroplast haplotypes found within subgenus *Nothofagus* in southern South America. The number of changes (substitutions) is indicated by the numbers above the lines along each branch. Numbers below the branches indicate bootstrap and posterior probability values for maximum likelihood and Bayesian inference analyses, respectively. Red identifiers correspond to the estimated divergence age for nodes as in Table 3. The root node was fixed and is indicated by an orange square. Additional time constraints are indicated on the nodes by orange triangles (microfossils) and orange circles (macrofossils). Colored branches identify distinct haplotype lineages within subgenus *Nothofagus*. Symbols following the haplotype names indicate which haplotypes occur in each species. The distributions for related species within subgenera *Lophozonia* and *Fuscospora* are indicated: AU, Australia; NZ, New Zealand; SA, South America. Co, Coniacian; H, Holocene; Mast, Maastrichtian; P, Pleistocene; Pl, Pliocene; Q, Quaternary; S, Santonian.

distributed in the south. Haplotypes found at any one location were shared among all present species (Fig. 3). Two well-supported clades were identified, one including northern haplotypes (bootstrap support (BS) = 97%; Bayesian posterior probability (BPP) = 1.00) and the other composed of southern haplotypes (BS = 96%; BPP = 1.00) (Fig. 4). The phylogenetic analysis revealed two subclades within the northern clade, whereas no divergence was detected within the southern clade encompassing *c.* 15° of latitude. It was noteworthy that the northern-most lineage clustered with the southern lineage (Fig. 3, network inside top right panel).

The distribution of the ITS ribotypes illustrates the relationships among the delimited species within subgenus *Nothofagus*, whereas the cpDNA phylogeny supports the geographic location. The phylogeny constructed from nuclear genes showed that *N. antarctica* was sister to a clade of evergreen species (*N. betuloides*, *N. dombeyi* and *N. nitida*) (BS = 79%; BPP = 0.98), whereas *N. pumilio* probably diverged earlier (BS = 86%; BPP = 0.99) (Fig. 5).

The trees recovered by ML and BI methods for both chloroplast and nuclear markers yielded the same topology, consistent with the phylogenetic relationships among the subgenera. All subgenera were monophyletic (BS = 61–100%; BPP = 0.71–1.00; Figs 4, 5). A closer relationship was found for cpDNA haplotypes between subgenera sharing *fusca*-type pollen (BS = 99%; BPP = 1.00). These are subgenera *Fuscospora* with *fusca*-type (a) and *Nothofagus* with *fusca*-type (b) pollen. The only member of subgenus *Fuscospora* in South America, the deciduous *N. alessandri*, had a similarly basal position in both phylogenetic trees.

Molecular dating

Divergence times among populations and species within *Nothofagus* were estimated separately using the three combined cpDNA

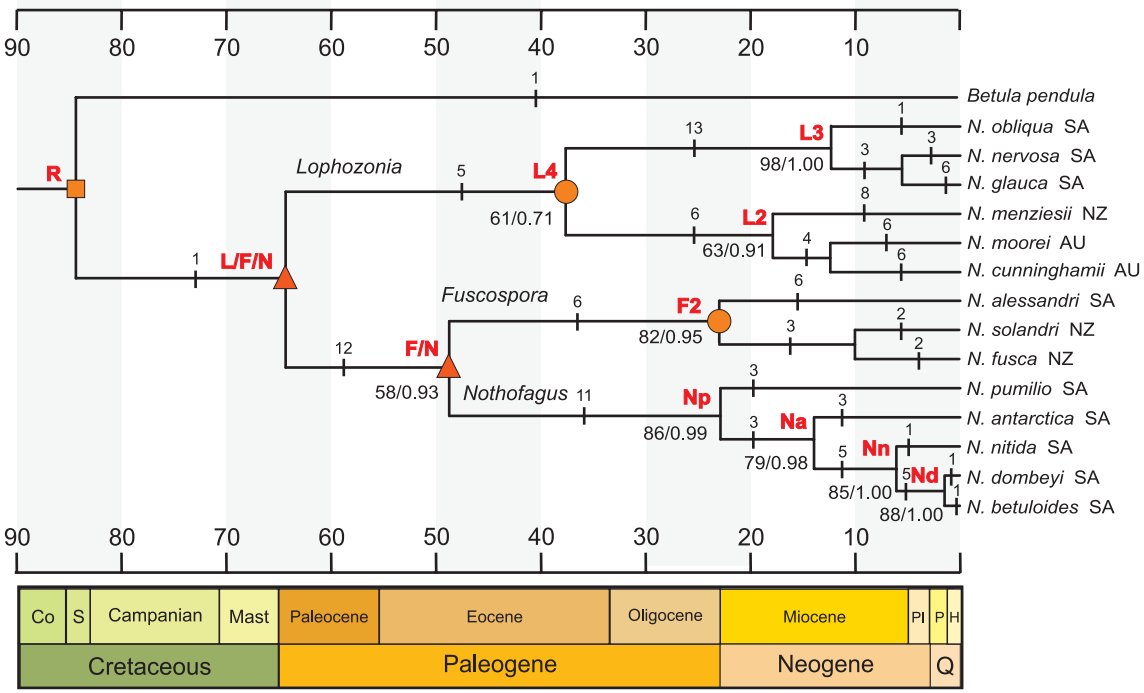

Fig. 5 Chronogram indicating the evolutionary relationships among *Nothofagus* species by means of internal transcribed spacers (ITSs). The number of changes is indicated by numbers above the lines along each branch. Numbers below the branches indicate bootstrap and posterior probability values for maximum likelihood and Bayesian inference analyses, respectively. Red identifiers correspond to the estimated divergence age for nodes as in Table 4. The root node was fixed and is indicated by an orange square. Additional time constraints are indicated on the nodes by orange triangles (microfossils) and orange circles (macrofossils). The location of each species is indicated: AU, Australia; NZ, New Zealand; SA, South America. Co, Coniacian; H, Holocene; Mast, Maastrichtian; P, Pleistocene; Pl, Pliocene; Q, Quaternary; S, Santonian.

regions and ITS dataset implemented by MULTIDIVTIME and BEAST (Figs 4, 5). Both methods yielded considerable deviations of average values by means of 95% confidence intervals. The use of BEAST provided relatively more recent mean age estimates compared with those obtained by MULTIDIVTIME. Given that no major differences were obtained in BEAST by the use of different calibration point combinations for both cpDNA and ITS datasets, these were averaged and considered as minimum ages, and were used in further discussions and to construct the chronograms (Tables 3, 4 and Figs 4, 5). Divergence times (in Myr) between cpDNA northern and southern clades (N3) containing populations of all species within subgenus *Nothofagus* occurred on average at 32 Myr, whereas age estimates of distinct subclades within the northern clade were N2 = 18 and N1 = 13 (Table 3). Age estimates yielded a divergence time of subgenus *Lophozonia* (L4 = 33) that was within the range of previous estimates (L4 = 34/25; Knapp *et al.*, 2005), whereas the estimated divergence of *fusca*-type pollen (F/N = 48) and subgenus *Fuscospora* (F2 = 27) yielded ages at the lower end of those reported previously (F/N1 = 72/70 and F2 = 61/42; Knapp *et al.*, 2005). However, only one

Table 3 Bayesian relaxed molecular clock age estimates (million yr (Myr)) for different chloroplast DNA haplotype clades within subgenus *Nothofagus* (N) using MULTIDIVTIME and BEAST

	MULTIDIVTIME			BEAST		
Node	Microfossils	Macrofossils	Micro + Macro	Microfossils	Macrofossils	Micro + Macro
R	84.00 (79.00–89.00)	84.00 (79.00–89.00)	84.00 (79.00–89.00)	85.70 (70.92–101.58)	81.46 (63.21–100.80)	82.62 (65.87–99.38)
N1	19.57 (4.52–41.74)	16.33 (3.54–38.70)	16.48 (3.51–37.99)	15.38 (5.00–29.32)	13.86 (4.46–25.22)	11.11 (4.25–20.40)
N2	36.27 (13.92–63.47)	30.15 (9.84–57.19)	30.43 (10.11–57.56)	21.75 (8.65–37.73)	16.14 (6.08–27.68)	16.67 (6.81–28.41)
N3	62.20 (37.77–85.15)	51.17 (25.84–79.75)	51.60 (26.45–80.29)	38.17 (20.03–56.26)	28.68 (12.73–45.67)	29.37 (14.16–45.34)
F2	55.61 (30.80–79.33)	47.39 (32.17–73.74)	47.94 (32.20–74.80)	28.72 (8.42–50.80)	25.13 (11.21–38.76)	25.83 (11.03–39.23)
F/N	80.84 (73.25–97.68)	66.05 (43.51–92.67)	66.52 (44.04–92.79)	58.98 (44.38–73.08)	42.21 (24.40–62.07)	43.63 (26.48–62.26)
L2	15.03 (4.61–35.30)	22.86 (9.64–42.54)	22.76 (9.29–42.72)	20.42 (6.55–37.16)	16.66 (4.82–28.86)	16.68 (6.37–28.86)
L4	26.61 (11.37–52.37)	39.98 (31.73–60.08)	39.96 (31.72–60.88)	37.29 (18.54–59.73)	30.66 (16.47–45.04)	31.59 (17.53–46.42)

Node names for subgenera *Lophozonia* (L) and *Fuscospora* (F) are according to Knapp *et al.* (2005); 95% confidence and highest posterior density intervals are given in parentheses for MULTIDIVTIME and BEAST, respectively.

Table 4 Bayesian relaxed molecular clock age estimates (million yr (Myr)) for different nuclear DNA ribotype clades within subgenus *Nothofagus* (N) using MULTIDIVTIME and BEAST

	MULTIDIVTIME			BEAST			
Node	Microfossils	Macrofossils	Micro + Macro	Fixed rate	Microfossils	Macrofossils	Micro + Macro
R	84.00 (79.00–89.00)	84.00 (79.00–89.00)	84.00 (79.00–89.00)	232.80 (196.43–264.54)	85.32 (68.37–101.06)	81.66 (62.49–101.05)	84.31 (68.42–103.67)
Np	43.95 (21.05–71.18)	34.88 (14.72–62.73)	35.00 (14.58–63.04)	18.63 (11.71–25.87)	31.61 (8.11–56.57)	19.34 (4.67–40.79)	21.67 (2.83–41.56)
Na	31.62 (11.88–58.06)	25.17 (8.63–50.52)	25.05 (8.57–51.13)	11.32 (6.17–17.11)	18.85 (3.36–39.19)	12.11 (2.61–28.26)	12.69 (2.08–27.87)
Nn	18.67 (3.44–42.94)	14.98 (2.66–36.63)	14.75 (2.43–36.56)	5.09 (1.73–8.85)	8.02 (0.60–20.42)	5.15 (0.55–13.68)	5.52 (0.44–13.74)
Nd	4.99 (0.14–18.60)	3.96 (0.11–15.86)	3.94 (0.11–15.42)	0.58 (0.0005–1.77)	1.73 (0.0003–6.47)	0.94 (0.00006–3.44)	1.01 (0.001–3.59)
F2	49.46 (23.09–75.27)	43.62 (31.90–68.23)	43.99 (31.89–70.00)	17.71 (9.69–26.67)	25.36 (2.77–59.20)	20.73 (3.78–36.33)	24.38 (6.57–40.09)
F/N	81.55 (73.28–99.04)	65.86 (43.74–92.89)	66.47 (43.56–93.41)	43.01 (32.01–55.23)	66.61 (50.21–83.24)	38.93 (12.05–70.69)	48.68 (22.38–77.81)
L2	25.30 (8.04–55.50)	29.48 (11.06–60.42)	30.06 (11.34–61.66)	23.31 (14.62–32.32)	17.27 (2.98–38.17)	13.84 (2.81–28.57)	15.88 (2.83–31.38)
L3	13.98 (2.45–39.18)	16.47 (3.24–43.26)	16.84 (3.25–44.24)	9.98 (4.75–15.82)	15.38 (2.17–33.86)	10.76 (1.34–23.84)	12.64 (1.57–27.21)
L4	47.68 (20.58–84.60)	54.68 (32.82–88.43)	55.62 (33.03–89.71)	50.39 (36.76–64.80)	36.52 (11.25–64.87)	30.17 (14.11–46.38)	33.11 (16.68–48.20)

Node names for subgenera *Lophozonia* (L) and *Fuscospora* (F) are according to Knapp *et al.* (2005); 95% confidence and highest posterior density intervals are given in parentheses for MULTIDIVTIME and BEAST, respectively.

species, and therefore one haplotype, of subgenus *Nothofagus* was included in the analysis by Knapp *et al.* (2005), which may have biased the molecular dating estimations.

Within subgenus *Nothofagus*, ITS age estimates (in Myr) yielded more recent species' divergences (Nd = 1 – Np = 23) than that found between the oldest split dated by cpDNA (N3 = 32) (Table 4; Fig. 5). Divergence between subgenera *Fuscospora* and *Nothofagus* by ITS (F/N = 49) yielded a similar estimate to that obtained by cpDNA data (F/N = 48). We found similar estimates for common nodes, such as F2 = 22 and 27, L2 = 18 and 18, and L4 = 38 and 33, from ITS and cpDNA trees, respectively.

Discussion

Ancient lineage divergence

We found a major latitudinal divergence among chloroplast lineages within subgenus *Nothofagus* that dates back to the early Oligocene and at least no younger than the middle Miocene (Table 3, Fig. 4). Reciprocal monophyly between north and south, *c.* 42–43°S, and the absence of common as well as intermediate haplotypes along the > 2000 km range strongly suggest long-lasting isolation between latitudinally divergent lineages. In addition, more recent disjunctions that occurred during the Miocene exist further north. To the best of our knowledge, no study has reported such ancient and geographically concordant differentiation using molecular dating methods. Yet, the confidence intervals of age estimates were wide. 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). Uncertainties in calibrations may also be caused by taxon sampling effects and tree topology (Ho *et al.*, 2005; Renner, 2005). In order to reduce these sources of error, we used distinct relaxed clock Bayesian models, a thorough sampling schedule and a combination of multiple available fossil data (Near & Sanderson, 2004) and molecular markers, which, in turn, yielded similar age estimates for most comparable nodes. Nevertheless, methodological limitations related to the amount of sequence variation may also explain the wide confidence intervals (Brown & Yang, 2010). Nonetheless, the calibration of molecular phylogenies may be difficult in *Nothofagus* which is now well past its maximum diversity and distribution (Hill, 2001). This is particularly the case for subgenus *Nothofagus* which, although currently restricted to southern South America, was widely distributed across South America, Antarctica, Australia and New Zealand by the mid–late Eocene (Dettmann *et al.*, 1990). Molecular evidence of such former widespread distribution is provided by the internal positions of latitudinally extreme cpDNA haplotypes in the network. Thus, cpDNA structure probably reflects the ancestral distribution of subgenus *Nothofagus* that predates the divergence of extant species.

Phylogeographic patterns are usually interpreted in concert with the glacial history of most plant taxa, including tree species. However, independent pieces of evidence, including pollen fossil

data, suggest that cold-tolerant species within subgenus *Nothofagus* may have survived throughout glacial cycles of the Neogene in ice-free areas along the southern Andes (Markgraf *et al.*, 1995). Ecological niche modeling of Last Glacial Maximum scenarios in Patagonia indicates that numerous areas remained suitable for the survival of the cold-tolerant *N. pumilio* as far south, that is, cold, as Tierra del Fuego at 54°S (Premoli *et al.*, 2010). This is in agreement with nuclear markers, which show no large-scale range shifts of *N. pumilio* towards the north, that is, warm, but local movements with ice sheets (Mathiasen & Premoli, 2010). A recent phylogeographic study conducted beyond forest areas in the Patagonian steppe, combining ecological niche modeling and molecular data of *Hordeum* species, also provided evidence for *in situ* survival within their extant distribution ranges (Jakob *et al.*, 2009). Thus, the genetic structure of cold-hardy *Nothofagus* mirrors an ancient biogeographic history that was not reset by Quaternary glaciations. Yet, the analysis of merely the north-eastern range of *N. antarctica* (subgenus *Nothofagus*) by means of one restriction fragment length polymorphism (RFLP) cpDNA marker yielded two haplotypes north and south of 42°30′S which were explained as distinct refugia (Pastorino *et al.*, 2009). Although long-lasting persistence throughout ice ages may reinforce ancestral genetic signatures (Premoli *et al.*, 2010), no parsimonious explanation based on glacial history exists for the absence of shared, that is, widespread, haplotypes between north and south gene pools of all species within subgenus *Nothofagus*, including *N. antarctica* (M. C. Acosta, unpublished). Similarly, deep chloroplast divergence was also found within subgenus *Lophozonia* in south-eastern Australia. Interestingly, the single cpDNA haplotype of the sister species *N. moorei* was nested among the haplotypes of the geographically disjunct *N. cunninghamii*. This was interpreted as ancient hybridization and chloroplast capture either with *N. moorei* or the use of an extinct species as a bridge (Worth *et al.*, 2009). Therefore, phylogeographic evidence within subgenera *Nothofagus* and *Lophozonia* calls for ancient distribution patterns of *Nothofagus* inhabiting complex landscapes of southern land masses.

Concordant phylogeographic patterns

In this analysis, cpDNA haplotypes were shared by all present species within a given area. Our results reinforce a recent analysis of all species of subgenus *Nothofagus* at 10 locations in Patagonia, where all extant species were screened for both chloroplast and nuclear DNA sequences. It was demonstrated that, although ITS sequences resolved the phylogeny within the subgenus, cpDNA polymorphisms were associated with geography (Acosta & Premoli, 2010). Region-specific cpDNA was thus explained by local processes of long-term hybridization and chloroplast capture events that favored the development of a concordant spatial genetic structure (Acosta & Premoli, 2010). Species within subgenus *Nothofagus* are self-incompatible (Riveros *et al.*, 1995). They share *fusca*-type (b) pollen, and hybrids between most species' pairs have been described in nature (Premoli, 1996; Stecconi *et al.*, 2004; Quiroga *et al.*, 2005). Although mainly outcrossed, species in mixed stands yield reduced outcrossing rates, suggesting that the mating system may be influenced by the physical and biotic environment or selection against hybrids (Premoli, 1996). Multilocus estimates from progeny arrays suggest that the variable composition of pollen loads may foster interspecific hybridization, particularly among taxa with weak reproductive barriers, as within subgenus *Nothofagus*. This may be favored during post-disturbance colonization or range expansion, where the minority species may consist of a few individuals within small populations that act as recipients (maternal parent and thus retain cpDNA) of pollen produced by long-distance dispersal from the majority species consisting of large populations (paternal parent and therefore pollen donor). Unequal population sizes result in pollen competition, and backcrosses may occur towards the foreign species. The resulting population from introgression will mainly consist of chloroplasts of the recipient species and nuclear genes of the donor (Fig. 4 in Acosta & Premoli, 2010). Predominant westerly winds will accentuate pollen dispersal, and thus interspecific gene flow within a given region over long time periods. Yet limited seed dispersal intensifies the spatial signal of the chloroplast which strengthens latitudinal divergence. Although chloroplast capture has been documented in many plant species (e.g. Rieseberg & Soltis, 1991; Chan & Levin, 2005), to the best of our knowledge this is the first report of such a spatially and temporally extended hybridization–introgression phenomenon.

Most surprisingly, the data presented here show that phylogenetic discontinuities were also concordant among taxa. These 'genetic breaks' *sensu* Avise *et al.* (1987) were found in intraspecific phylogenies of all five species. For example, northern and southern clades within *N. pumilio* differ by 11 mutational steps (Mathiasen & Premoli, 2010). The most likely explanation for major genetic discontinuities that display geographic orientation involves long-term, extrinsic barriers to gene flow (Avise *et al.*, 1987). However, the major and oldest divergence within *Nothofagus* reported here occurs in areas in which extant species are nowadays found as continuously distributed populations. Such genetic discontinuity was also recorded by means of nuclear markers in the cold-tolerant tree *Podocarpus nubigena* (Podocarpaceae) (Quiroga & Premoli, 2010). Similarly, a north–south divergence at *c.* 43°S was found in the cold-hardy *Embothrium coccineum* (Proteaceae), although a shallow structure by cpDNA sequences resulted from small population sizes and greater effects of drift (Vidal-Russell *et al.*, 2011). Concordant divergence within phylogenetically independent lineages strengthens the vicariance hypothesis.

Biogeographic reconstruction

Allopatric divergence between populations of all species developed as a result of geographic barriers encountered by cold-tolerant *Nothofagus* during mid–late Paleogene land-based expansion. Although abundant stratigraphic and tectonic information exists about southern South America, few attempts have been made to cross-link paleogeographic features of the landscape with patterns of divergence of southern lineages (Romero, 1986b). The physical settings of Eocene–Miocene Patagonia consisted of two different realms (Ramos, 2005; Lisker *et al.*, 2006) characterized

by mostly segmented territory (Fig. 2). The fossil record confirms the presence, probably with a restricted range and as low-abundance populations, of subgenus *Nothofagus* in Eocene Patagonia (Romero, 1986b); *fusca*-type (b) pollen was recovered towards the south from the Ligorio Márquez Formation (*c.* 47°S, 72°W) (Okuda *et al.*, 2006) with a K–Ar date of 47.6 ± 0.78 Myr (Yabe *et al.*, 2006), and also further north at Río Pichileufú (*c.* 41°S, 71°W) (W. Volkheimer, pers. comm.) with a $^{40}Ar/^{39}Ar$ depositional age of 47.46 ± 0.05 Myr (Wilf *et al.*, 2005). Furthermore, major changes in climate from an Eocene glasshouse to an Oligocene icehouse (Huber & Nof, 2006) resulted in the development of rather cooler trends. As a consequence, range expansion of south Antarctic cold-hardy elements followed.

The spread towards the north occurred in warmer areas where, until most of the Eocene climatic optimum, shade-intolerant *Nothofagus* was probably outcompeted by shade-tolerant warm-loving taxa. Given that a significant uplift of the southern Andes occurred no earlier than the Miocene (Blisniuk *et al.*, 2005), available land for plants was probably segmented. This was particularly critical during the Oligocene at northern latitudes between 36 and 41°S where, under higher sea level, fluvial systems were drowned, producing numerous partially detached lacustrine basins throughout almost 700 km (Fig. 2; Ramos, 1982). In addition, during the colonization process to the north, the aperture of an additional open-ocean late Oligocene–Miocene embayment at *c.* 40°S latitude isolated northernmost populations (leRoux & Elgueta, 2000). This probably explains the minor genetic discontinuity found in the northern clade. Suitable habitat and corridors for plant survival and migration in warmer latitudes were probably provided by the Coastal Range in southwestern continental Chile (Duhart & Adriasola, 2008; Glodny *et al.*, 2008) and numerous elevated terrains of the northern Patagonian Batholith close to the Patagonian steppe in northern Patagonia, Argentina (Ramos, 1982, 2005; Pankhurst *et al.*, 1999; Giacosa & Heredia, 2004). As a result of a fragmented paleogeography, populations and species within subgenus *Nothofagus* in northern latitudes suffered from isolation and drift that resulted in greater cpDNA haplotype diversity (Mathiasen & Premoli, 2010). By contrast, vast marine ingressions from the Atlantic occurred from the Oligocene until 15 Myr BP and covered southern-most Patagonia (Austral Basin; Fig. 2). Extensive Paleogene emerged continental deposits of the southern segment (Ramos, 2005) allowed the long-lasting persistence of cold-loving elements. As a result, taxa of subgenus *Nothofagus* developed the ability to withstand cooling and became adapted to harsher conditions. The proof of long-term *in situ* persistence is evident in the south without major lineage divergence. Similar cpDNA homogeneity across large areas in the south was obtained for the widespread *Embothrium coccineum* (Vidal-Russell *et al.*, 2011). This is congruent with low extinction and evolutionary rates, reflected in the morphological stasis observed in the fossil record of woody lineages from Patagonia (Markgraf *et al.*, 1995).

Once paleobasins were drained, probably as a result of the uplift of the main Andean Cordillera during the Miocene, deep-rooted genetically distinct lineages came into contact at *c.* 40 and 43°S (Fig. 3). Hence, such genetically diverse and segmented zones are secondary contact areas between populations with different biogeographic histories significantly controlled by geology. Our results highlight the need for the revision of phylogeographic ideas, particularly on taxa and geographic areas where climatic oscillations of the Neogene have not reset ancient historical signals. In these cases, cpDNA polymorphisms can be mistakenly interpreted as a result of glacial history. Therefore, deep chloroplast divergence within subgenus *Nothofagus* is most likely explained by Paleogene events. These include divergence caused by paleobasins and the synergistic effects of climate driving the expansion of cold-hardy taxa.

Divergence in dynamic landscapes

Extinct and extant taxa within subgenus *Nothofagus* have a shared evolutionary history (Acosta & Premoli, 2010). Extant species have distinct life history traits, inhabit different environments and the majority are regionally widespread and locally sympatric. The three evergreen species *N. betuloides*, *N. dombeyi* and *N. nitida* often occur in relatively mesic environments. They are morphologically similar and produce hybrids in mixed populations (Premoli, 1996). This was interpreted as recent differentiation (Romero, 1986a), which is confirmed by molecular dating of the ITS phylogeny. The relatively basal position of *N. nitida* within the evergreen clade supports previous isozyme data which suggested an early diversification (Premoli, 1996). This probably resulted from mountain building since the upper Miocene (14 Myr BP) when *N. nitida* developed adaptations to most humid climates. It is the only species of the subgenus restricted to the western slopes of the Andes. The sister species to the evergreen clade is the deciduous *N. antarctica* which occurs in the most extensive range of habitat types. Divergence, as well as the development of significant phenotypic variation, as a result of both plasticity and genotypic effects (Steinke *et al.*, 2008), most probably occurred in response to the cooler and drier trends of the early to middle Miocene (Blisniuk *et al.*, 2005). Similarly widespread is the deciduous *N. pumilio*, although restricted to high-elevation and high-latitude environments. The basal placement of *N. pumilio* within subgenus *Nothofagus* mirrors that of *N. alessandri*, the only South American member of subgenus *Fuscospora*. Although the divergence and range expansion of *N. pumilio* took place in response to cooler trends since the Oligocene, warm-loving lineages, such as *N. alessandri*, became restricted to the warmer climates of northwestern Patagonia.

Extant species within subgenus *Nothofagus* are shade intolerant and have, in common, the fact that they are affected by natural disturbances. These range from large-scale events, such as volcanic ash deposition, landslides, fire and windstorms, which devastate entire stands, to small-scale events in the form of individual tree falls (Veblen & Donoso, 1987). Nonequilibrium forest stands recurrently affected by natural disturbances at distinct spatial scales maintain complex genetic structures (Premoli & Kitzberger, 2005; Premoli & Steinke, 2008; Mathiasen, 2010). Adaptations, such as sprouting and short-distance seed dispersal, seem to have been selected for local persistence in *Nothofagus*. When vacant niches become available, such as after disturbance,

frequency-dependent survival and long-distance dispersal via pollen into areas dominated by other species may favor hybridization–introgression (Chan & Levin, 2005). Nonetheless, *Nothofagus* species maintain their identity through ecological divergence in contrasting environments.

Conclusions

We have shown that detailed phylogeographic analyses and molecular dating by independent cpDNA and ITS markers bring together fossil evidence and paleogeographic features of the landscape. Age estimates by cpDNA and ITS show that the divergence of subgenus *Nothofagus* dates back to the mid-Eocene (48 Myr), which is consistent with the presence of *fusca*-type (b) fossil pollen at 41 and 47°S latitude in Patagonia. In addition, the occurrence of geographically structured cpDNA haplotypes confirms an ancient widespread distribution of subgenus *Nothofagus*. Spatially assorted distribution of cpDNA lineages was found at mid-latitudes of Patagonia in an area in which extant species occur as continuous populations. An age estimate of such major phylogeographic divergence was dated as upper Eocene–lower Oligocene (32 Myr). Other minor lineage disjunctions occurred further north during the Miocene (13 and 18 Myr). We show that paleobasins prevented transoceanic dispersal during land-based spread at the onset of cooler trends of the Oligocene. Thus, our results provide evidence of geological barriers impeding the dispersal of ancestral lineages within subgenus *Nothofagus*, which reconciles vicariance with land dispersal mechanisms. Gene pools of cold-hardy *Nothofagus* lineages, which, in turn, were not reset by the climatic oscillations of the Neogene, mirror paleogeographic features consisting of an archipelago-like landscape. Concordant cpDNA phylogeographic patterns reflect cycles of hybridization–introgression among extant and ancestral taxa, whereas the ITS phylogeny reveals that species' identity is probably maintained by adaptive divergence in the absence of complete reproductive barriers in *Nothofagus*.

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