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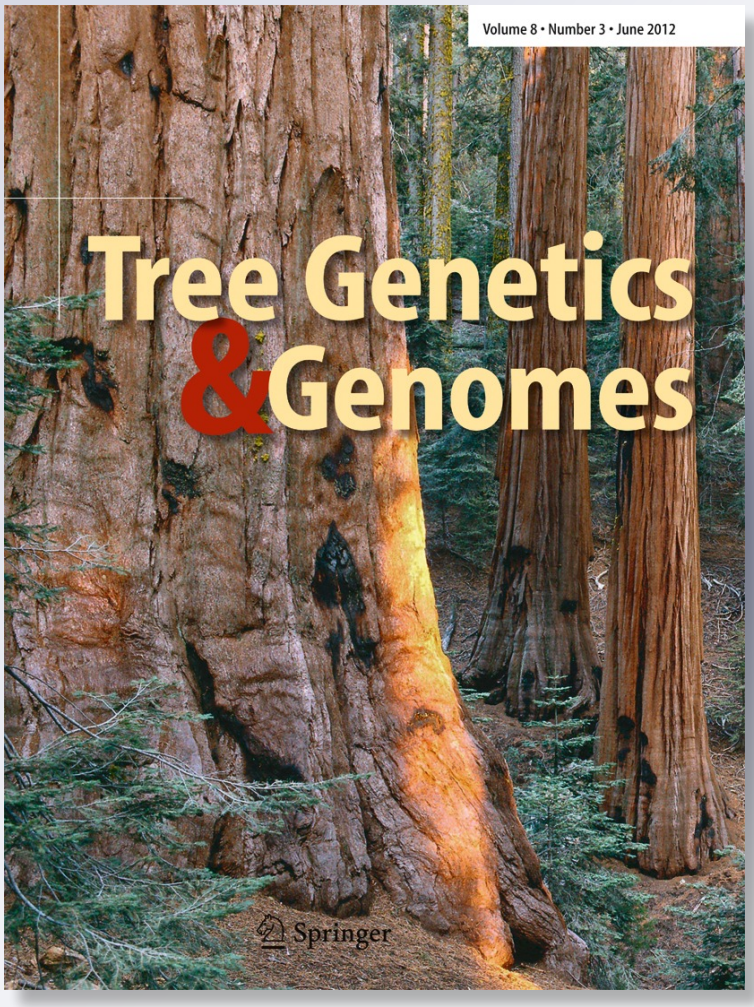


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ORIGINAL PAPER

# Phylogeography of two hybridizing southern beeches (*Nothofagus* spp.) with different adaptive abilities

**Carolina Soliani · Leonardo Gallo · Paula Marchelli**

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**Abstract** In phylogenetically related plant species, hybridization can influence their current genetic structure. Long-lasting hybridization may be related to persistence in shared glacial refugia, where the differential abilities of each species to survive could have provided adaptations to changing environmental conditions. In temperate South American forests at the Patagonia region, the pattern of Quaternary glaciations offered several opportunities for refuge. At mid-latitudes (42° to 44° S), particular topographic characteristics determined different glaciation patterns, defining the existence of a transitional zone. We studied two widespread *Nothofagus* species (*Nothofagus pumilio*, *Nothofagus antarctica*) characterized by contrasting plasticity. We screened 40 coupled populations with three cpDNA markers and found 14 different haplotypes. Both species presented significant phylogeographic structure ($N_{ST} \geq G_{ST}$, $p>0.001$), with two geographically segregated lineages (north–south). A latitudinal cline in the distribution of genetic diversity was determined, with most variable populations in the north (35°–41° S). Population diversity diminished to southern latitudes, but a particular situation occurs between 42°S and 44°S. The transition zone, a putative refuge area, presented unique haplotypes. The more plastic species, *N. antarctica*, probably persisted in more refuge areas, which could be reflected in its higher levels of diversity. In these species, sympatric distribution explains introgression ($IG>IG_e$), but the differential levels of haplotype sharing between *N. pumilio* and *N. antarctica* at population level are relevant to the understanding of phylogeographic patterns. Hybridization may have facilitated recruitment in the onset of postglacial colonization by middle to long-distance pollen dispersal. In the current scenario of climate change, the presence of hybrids with different plastic responses is of remarkable importance.

**Keywords** *Nothofagus pumilio* · *N. antarctica* · Patagonia · Genetic structure · Introgression · Chloroplast DNA markers · Shared refugia

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## Introduction

Climatic changes during the Quaternary had a great influence on the persistence and distribution of forest species. After ice advance, the remaining patches of forests withstood a great selection pressure due to adverse climatic conditions. It is already known that glaciations affected the two hemispheres in different ways, having a lower impact in the south because of its greater oceanity and smaller land masses (Markgraf et al. 1995). In southern South America, particularly in Patagonia region, glaciations occurred from the Late Miocene to the Pleistocene, the Great Patagonian Glaciation (about 1 million years before present [M years BP]) being the most important, with the maximum expansion of ice in extra-Andean Patagonia (Flint and Fidalgo 1969; Rabassa et al. 2005). By the time of the Last Glacial Maximum (LGM), about 18,000–20,000 years BP (Porter 1981), the ice covered the Patagonian Region

beyond the mountain ranges and towards the plains (Rabassa and Clapperton 1990; Glasser et al. 2008). However, several ice-free areas remained (Markgraf et al. 1995), probably even during pre-Pleistocene glaciations, constituting ancient and/or recent refugia for vegetation, some of which became the center of expansion of the biota after the ice retreated (e.g., Azpilicueta et al. 2009; Gallo et al. 2008; Marchelli and Gallo 2006; Marchelli et al. 2010).

Stratigraphic studies of the last moraine advances (<20,000 years BP) revealed different glaciation patterns between northern and southern Patagonia (Glasser et al. 2008). To the north of 40°S, outlet glaciers were more restricted in extension and formed "alpine-style" valley glaciers. Therefore, more microhabitats were potentially available as refuges for forest species. A more continuous ice cap characterized the southern region (44° to 55°S) where the minor heights of the Andes Mountains promoted the formation of extended frozen layers that covered the whole land. At mid-latitudes (42° to 44°S), a transitional zone was described with particular characteristics. In this zone, lower-height mountain chains with a north–south orientation running parallel to the Andes could have stopped the advance of the glaciers (Flint and Fidalgo 1964, 1969) and provided suitable areas for species survival. In addition, by the time of migration and recolonization of formerly glaciated areas (e.g., early Holocene 10,000–8,500 years BP), the predominance of westerlies also determined intermediate climatic conditions at those latitudes (Markgraf 1993; Markgraf et al. 2003), such as a lack of seasonality and a drier and warmer environment (Manzini et al. 2008).

Reinforcing the idea of a latitudinal trend, the evolutionary history of Patagonian vegetation can be divided into three different areas, based on a reconstruction of paleoclimates (Markgraf et al. 1996): north of 43°S, between 43° and 51°S, and south of 51°S. We can assume that each of the three zones was subjected to different selection pressures. Toward southern latitudes, the extension of the ice cap could have restricted in situ persistence, while, toward the north, other selection pressures like fire and drought could have had an impact on the populations and their genetic makeup.

Additionally, our comprehension of pre-Quaternary times is limited as well as the knowledge of past forest distribution. The former is evidenced by the lack of a extensive characterization of the pollen record for the eastern Andes Mountains (Argentina) and the impossibility of recognizing species, only "pollen types" (Heusser 1984). Notwithstanding, in southern woodlands, forest recovery from cryptic refugia was dated as early as 14,800 $^{14}$C years BP in Staten Island (55°S, 64°W) and the current continental platform could have been a refuge, since the sea level was lower than now (Rabassa et al. 2005). Traces of the southern beeches, *Nothofagus* spp., and herbaceous pollen support this hypothesis (Villagrán et al. 1995).

The geographical context of last glaciations in southern South America is well documented and dated (e.g., Rabassa et al. 2005), giving support for biogeographic and phylogeographic studies. The description of distribution patterns and population dynamics of Patagonian biota can be thought as an analogue scenario of the northern hemisphere, which gives a global interpretation of Quaternary times. Recent studies have focused on Patagonian flora and fauna, from herbaceous (e.g., Cosacov et al. 2010) to arboreal species (e.g., Marchelli and Gallo 2006), amphibian (e.g., Nuñez et al. 2011), reptilian (e.g., Breitman et al. 2011), and mammals (e.g., González-Ittig et al. 2010) taxa. Our contribution to the understanding of Patagonian history focuses on two widespread arboreal species distributed along the whole region.

## Studied species

*Nothofagus pumilio* (Poepp. et Endl.) Krasser and *Nothofagus antarctica* (Forster) Oerster are endemic species to South American Temperate Forests. In Argentina, their distribution covers around 18° of latitude, from the north of Neuquén province (36°S) to the south of Isla Grande de Tierra del Fuego (55°S). They were therefore expected to be subjected to the different glaciation patterns described for the Patagonian Region. *N. pumilio*, known as *lenga*, usually forms large masses of pure stands associated with climax forests (late-successional species), growing in well-defined environments with deep, well-drained soils and reaching the timberline, where it can tolerate low temperatures and frosts as a shrub. On the other hand, *N. antarctica*, commonly called *ñire,* is the species with the widest ecological plasticity and phenotypic variation (Ramírez et al. 1985) of their South American congeners. It occurs in cold humid valleys with heavy clay soils, in peat bogs, in rocky and xeric sites, as well as on the timberline. Due to this plastic response, it can also be found in the steppe forming monospecific masses of shrubs or small trees. Mixed stands occur in several places throughout their natural distribution, constituting an altitudinal ecological gradient, with *N. pumilio* dominating at higher elevations and *N. antarctica* at lower sites. *N. antarctica* is also a pioneer and resprouting species and has a great capacity for clonal reproduction (Premoli and Steinke 2008), while *N. pumilio* can only reproduce generatively. These species are closely related, being grouped in the same phylogenetic clade of the subgenus *Nothofagus* (Manos 1997), although recent studies suggest an ancestral position for *N. pumilio* (Acosta and Premoli 2010).

Hybridization in *Nothofagus* is a common phenomenon (e.g., Donoso 1993; Gallo et al. 1997; Premoli 1996; Stecconi et al. 2004). For the species complex, *N. pumilio*

−*N. antarctica* intermediate phenotypes were described (Donoso 2006) and can be frequently seen in the field. Hybrids have been found within *N. antarctica* forests, more than 15 km away from the nearest *N. pumilio* forests. Directional pollination of *N. pumilio* towards *N. antarctica* has been suggested (Acosta and Premoli 2010), but it is unknown if this is the only possibility. Among sympatric and phylogenetically related species, hybridization may have been more frequent during glacial times (Palmé et al. 2004; Heuertz et al. 2006), constituting an additional source of variation. The pre-condition for hybridization is the co-existence of the species in the same refuge or in neighboring areas. Potential hybridization between the *Nothofagus* species studied here should be taken into account in order to understand species recruitment and postglacial recolonization patterns.

### Aims of the study

The transitional zone may have a particular genetic structure, since different intensity and types of natural selection pressure can be assumed at central latitudes. Forests could have persisted in ice-free zones between north- and south-orientated mountain ranges, or even in stepparian refugia beyond the current eastern limits of the forests. Survival in common refugia could only have occurred when habitat conditions were suitable for both species, which is related to the intrinsic characteristics of each species (e.g., life history traits and competitive abilities). Our goal is to make inferences about the common history of two southern beeches, *N. pumilio* and *N. antarctica*, focusing on the transition zone, but taking into consideration their different life history traits.

We propose to test the following hypotheses: (a) The different types of glaciation in northern and southern Patagonia determined a latitudinal structure of genetic variation in this *Nothofagus* complex. We predict the existence of a cline in genetic variation, with the north showing more genetic diversity due to more possibilities for forests to take refuge, which diminish to the south; an intermediate situation is expected in the transition zone. (b) Due to its adaptive ability, *N. antarctica* had a greater capacity for survival in refugial conditions than *N. pumilio*. This hypothesis should be reflected in greater haplotype diversity. A broad interpretation is needed to elucidate this complex phylogeographic scenario, also taking into consideration interactions between species, such as hybridization and introgression. Hybridization might have been more frequent due to climatic constraints during Pleistocene times, which may have had an impact in the genetic composition of populations or even the species identity (the number and type of haplotypes shared by the species).

## Materials and methods

### Plant material

Forty populations of *N. pumilio* and *N. antarctica* (20 of each species) were sampled during the summer and autumn of 2007 and 2008. One stand of each species was selected at each site, thus allowing comparative analysis of genetic variation as well as common phylogeographic features. We first distinguished the *N. pumilio* population and then looked for the nearest *N. antarctica* forest, within their natural distribution in Argentina. Half the populations (20) are restricted to the transition zone, which corresponds to Chubut province, and are the focus of our study. Throughout this region, mountain ranges run parallel to the Andes Mountains, and therefore populations were sampled on each side of the ranges, distributed in three latitudinal transects. The remaining populations were collected to the north and south of the transition zone, in order to have a reference for genetic variation. Each of these regions contains ten populations (five of each species), making a total of 20. The northern group covers from 36°S to 41°S, while the southern is from 51°S to 55°S. We are therefore covering almost 20° of latitude, which corresponds to the distribution of both species in Argentina (Table 1).

Branches of about 30 cm in length, containing approximately 15 buds were collected from five to ten adult trees in each population, totalling 305 individuals. A minimum distance of 50 m was maintained between individuals in order to avoid the sampling of relatives. Branches were kept fresh until they could be frozen at −80°C and used for laboratory analysis.

### DNA extraction

Total DNA was extracted from dormant buds following the procedure described in Dumolin et al. (1995). Stock DNA was stored at −80°C, and dilutions to 5 ng/μl were prepared for restriction polymerase chain reaction-fragment length polymorphism (PCR-RFLP) analyses. A total of 151 of *N. pumilio* and 154 individuals of *N. antarctica* were analyzed ($N_t$=305). In the northern and southern groups, we evaluated five to six individuals of each species per population, while in the transition zone, we took ten individuals of each species per population in order to maximize the detection of genetic variation.

### Genetic screening

A first screening of variability was carried out in both species with a subset of 40 individuals (20 for each species) from ten different populations (two individuals per population). We tested 22 different combinations of universal

**Table 1** Geographic location and allelic richness of coupled *N. pumilio* and *N. antarctica* sampled populations ordered from north to south

| Population | Species | Latitude (S) | Longitude (W) | $N^a$ | $A_r$[b] | Stand condition[c] |
|---|---|---|---|---|---|---|
| Lagunas de Epulauquen (E) | *N. pumilio* | 36°49′39″ | 71°06′12″ | 5 | 1 | Sympatric mixed |
| | *N. antarctica* | 36°49′30″ | 71°05′51″ | 5 | 1 | |
| Caviahue (CAV) | *N. pumilio* | 37°51′18″ | 71°05′02″ | 5 | 1 | Sympatric mixed |
| | *N. antarctica* | 37°49′54,7″ | 71°01′05,1″ | 6 | 1.8 | |
| Tromen (Tr) | *N. pumilio* | 39°34′47″ | 71°27′35″ | 5 | 1 | Sympatric mixed |
| | *N. antarctica* | 39°36′ | 71°27′ | 5 | 0 | |
| Quilanlahue (Q) | *N. pumilio* | 40°07,6′59″ | 71°28,6′35″ | 5 | 2 | Sympatric non-mixed |
| | *N. antarctica* | 40°08′15″ | 71°28′1″ | 5 | 2 | |
| Challhuaco (V) | *N. pumilio* | 41°14′39″ | 71°17′09″ | 5 | 1 | Sympatric mixed |
| | *N. antarctica* | 41°14′0″ | 71°17′27″ | 5 | 1 | |
| Northern group mean | | | | 5.1 | 1.2 | |
| Cholila (ChB) | *N. pumilio* | 42°40′36,36″ | 71°29′50,81″ | 9 | 1 | Sympatric non-mixed |
| | *N. antarctica* | 42°31′32,5″ | 71°31′34,6″ | 10 | 0.5 | |
| Huemules (Hm) | *N. pumilio* | 42°50′14″ | 71°28′46″ | 10 | 0.8 | Sympatric mixed |
| | *N. antarctica* | 42°49′22″ | 71°27′53″ | 10 | 1 | |
| La Hoya (H) | *N. pumilio* | 42°50′27,3″ | 71°15′50,6″ | 10 | 0.8 | Sympatric mixed |
| | *N. antarctica* | 42°50′54″ | 71°15′33,3″ | 10 | 0.9 | |
| Nahuelpan (Np) | *N. pumilio* | 42°58′59,5″ | 71°11′23,8″ | 10 | 0.9 | Sympatric mixed |
| | *N. antarctica* | 42°59′23,8″ | 71°11′22,2″ | 10 | 0.9 | |
| Trevelin (Te) | *N. pumilio* | 43°4′0.4″ | 71°34′44.1″ | 10 | 0.8 | Sympatric mixed |
| | *N. antarctica* | 43°4′4.9″ | 71°34′28.7″ | 11 | 1.6 | |
| Lago Guacho (Ch) | *N. pumilio* | 43°48′53″ | 71°29′41″ | 10 | 0.8 | Sympatric mixed |
| | *N. antarctica* | 43°49′38″ | 71°27′01″ | 10 | 0.8 | |
| J. San Martín (JS) | *N. pumilio* | 43°49′40″ | 70°45′27″ | 10 | 1 | Sympatric mixed |
| | *N. antarctica* | 43°49′39″ | 70°45′10″ | 10 | 1 | |
| Arroyo Perdido (AP) | *N. pumilio* | 44°50′17″ | 71°41′40″ | 10 | 0.5 | Sympatric mixed |
| | *N. antarctica* | 44°50′12″ | 71°41′36″ | 10 | 1.8 | |
| Lago Fontana (Ft) | *N. pumilio* | 44°50′26″ | 71°37′58″ | 10 | 0.8 | Sympatric mixed |
| | *N. antarctica* | 44°50′35″ | 71°37′38″ | 10 | 1.9 | |
| Río Unión (U) | *N. pumilio* | 44°51′27″ | 71°39′11″ | 10 | 1 | Sympatric mixed |
| | *N. antarctica* | 44°51′30″ | 71°39′24″ | 10 | 2.8 | |
| Center group mean | | | | 10 | 1.1 | |
| Cancha Carrera (B) | *N. pumilio* | 51°13′19″ | 72°16′21″ | 5 | 0 | Sympatric non-mixed |
| | *N. antarctica* | 51°13′21″ | 72°15′34″ | 5 | 1 | |
| Mina I (Cf) | *N. pumilio* | 51°31′17″ | 72°21′02″ | 5 | 1 | Sympatric mixed |
| | *N. antarctica* | 51°31′48″ | 72°20′31″ | 5 | 0 | |
| Tierra del Fuego Norte (FuN) | *N. pumilio* | 54°04′30,00393″ | 68°31′52,40417″ | 5 | 0 | Sympatric mixed |
| | *N. antarctica* | 54°04′25,76821″ | 68°31′26,27458″ | 5 | 1 | |
| Tierra del Fuego Centro (FuC) | *N. pumilio* | 54°22′28,19493″ | 67°15′49,28046″ | 6 | 1.8 | Sympatric mixed |
| | *N. antarctica* | 54°22′14,23680″ | 67°15′34,63458″ | 6 | 1 | |
| Tierra del Fuego Este (FuE) | *N. pumilio* | 54°35′28,5966″ | 66°37′12,6020″ | 6 | 0.8 | Sympatric non-mixed |
| | *N. antarctica* | 54°34′20,9940″ | 66°38′2,4794″ | 6 | 1 | |
| Southern group mean | | | | 5.4 | 0.8 | |

$A_r$ allelic richness after rarefaction, *N* sample size, *stand condition* refers to the degree of overlapping of species in each population

[a] Total number of individuals per population

[b] Allelic richness per population (rarefaction method)

[c] Sympatric mixed refers to interespecific population's pairs where trees of different species are mixed in the field; sympatric non-mixed refers to interespecific population's pairs separated by more than 1,5 km, and consequently, trees of different species are not mixed in the field

primers that anchor cpDNA non-coding regions: *trn*K1-*trn*K2, *trn*D-*trn*T, *trn*C–*trn*D, *psa*A–*trn*S, *trn*S–*trn*T, *trn*H–*trn*K, *trn*Q–*trn*R, *trn*V–*rbc*L, *atp*H-*atp*I, *ccmp*5–*rpo*C2-r5, *rpo*C1 f5-*rpo*C1 br, *trn*G P–*trn*fM M, y*cf*3-*ccmp*6, *ccmp*6–*trn*S1 M, *trn*S–*rps*4, *trn*T–*trn*L 5′exon, *trn*L intron, *trn*L 3′ exon–*trn*F, *trn*T-*trn*F, *ccmp7–rbc*L, *trn*H–*psb*A, *rpl*20 5′-*rps*12, and *psb* B– *psb* F (see Heinze 2007). In addition, five chloroplast cpSSR regions were evaluated following

Weising and Gardner (1999): *ccmp1, ccmp2, ccmp5, ccmp6, ccmp7*. For the cpSSRs, the scoring method consisted of vertical electrophoresis of 6% denaturing polyacrylamide gels and then silver staining using the protocol by Streiff and Lefort (1997). PCR products of non-coding regions were digested with restriction enzymes, resulting in 62 different primer/enzyme combinations. Within the sub-sample, only three combinations found useful polymorphism: *trn*D-*trn*T/*Hinf*I, *trn*C-*trn*D/*Taq*I, *atp*H-*atp*I/*Hinf*I. Although we found polymorphism in two other regions (*rpo*C1 f5-*rpo*C1 br/*Taq*I and *psa*A–*trn*S/*Taq*I) and one cpSSR (*ccmp2*), these did not represent additional haplotypes and were discarded.

## PCR-RFLP analysis

*PCR conditions* The PCR was performed with 1× PCR buffer, 2 mM $MgCl_2$, 0.2 μM of each primer, 52 μg/mL of BSA, 0.2 mM of each dNTP, and 1 U of Taq DNA polymerase (Invitrogen) in a total volume of 25 μl. A general PCR programme (Heinze 2007) was used to amplify intergenic non-coding regions and chloroplast microsatellites (Weising and Gardner 1999)—3 min at 94°C, followed by ten cycles of 50 s at 94°C and 1 min at 70°C, then 35 cycles of 50 s at 94°C, 50 s at 55°C, and 2 min at 70°C, with a final extension step of 10 min at 70°C. For the amplification of *trn*D-*trn*T and *trn*C-*trn*D regions, we followed Demesure et al. (1995). The reactions were performed either in a Biometra Uno-Thermo Block or in an MJResearch PT-200 thermo cycler.

*Restriction conditions* PCR products were digested with restriction enzymes following a common restriction protocol—8 μl of PCR product, 1× buffer, and 5 U of one restriction endonuclease (BioLabs) *Hinf*I, *Taq*I, *Alu*I, *Hae*III, or *Mse*I in a total volume of 23 μl. Digestion was performed at 65°C for 3 h in the case of *Taq*I and at 37°C overnight for the other four enzymes. The reaction was stopped by adding loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol in water).

The screening of the total sample of 305 individuals was conducted using the three variable primer/enzyme combinations described above. The restricted fragments were analyzed on 8% non-denaturing polyacrylamide gels. Vertical electrophoresis, fragment staining, and gel documentation were performed as in Azpilicueta et al. (2009).

## Data analysis

### *Polymorphism, genetic diversity, and structure*

Fragments that showed useful polymorphism were sized in both species using BioDoc Analyse software version 2.0 (Biometra): *trn*D-*trn*T 1,230 bp, *trn*C-*trn*D 2,759 bp, *atp*H-*atp*I 873 bp. Polymorphism occurred both as point mutations in the restriction site and as *indels* (insertion/deletions). We labelled the fragments by decreasing order of length as visualized in polyacrylamide gels and the restriction site mutations with a number 9, as described by Demesure et al. (1996). Haplotypes were then defined according to different combinations of length variants (Tables 2 and 3).

We first obtained the average within population gene diversity ($h_s$) and the total genetic diversity ($h_t$) for both species following Pons and Petit (1995). Differentiation coefficients were calculated with PERMUT, either considering genetic similarities between haplotypes as the proportion of shared fragments ($N_{ST}$) or taking into account only the frequencies of the haplotypes ($G_{ST}$) (Pons and Petit 1995, 1996). Phylogeographic structure is evident if closely related haplotypes are found together in one population more often than expected by chance. We evaluated differences between $G_{ST}$ and $N_{ST}$ (i.e., $N_{ST} \geq G_{ST}$) with PERMUT and $N_{ST}$ vs. $N_{ST}$ permutated with SPAGeDi *v.*1.1 (Hardy and Vekemans 2002).

We calculated the allelic richness per population ($A_r$) with CONTRIB (Petit et al. 1998), which applies a rarefaction method, to avoid biases in the estimation due to differences in sample sizes. The number chosen for rarefaction was the lowest sample size.

In order to compare different geographic regions, all parameters were also estimated between populations of each group (North, Center, and South).

The geographic distribution of genetic variation was evaluated with an analysis of molecular variance (AMOVA) by testing if differences within and between a priori selected regions for both species were significantly different: North (36°S–41°S) and South (42°S–55°S) was the first tested cluster; North (36°S–41°S), Centre (42S°–44°S), and South (46°S–55°S) was the second analysis. Additionally, we run SAMOVA (spatial analysis of molecular variance) that implements an approach to define groups of populations that are geographically homogeneous and maximally differentiated from each other (Dupanloup et al. 2002) without a predefined structure.

Finally, we ran BARRIER *v.* 2.2 with the populations of the transition zone in order to identify boundaries, i.e., areas where differences between pairs of populations are greatest (Manni et al. 2004). The computational approach is based on Monmonier's maximum difference algorithm, performed on a previously drawn map where the software had already applied Voronoï tessellation and Delaunay triangulation. The first procedure finds neighboring populations and defines polygons around each sampled point (i.e., the populations, with an *X* and *Y* coordinate),

**Table 2** Description of the 14 haplotypes found in *N. pumilio* and *N. antarctica*

| *Nothofagus* spp. haplotypes | Polymorphic fragments | | | | | | | | | Total haplotype frequency | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $DT_1$ | $DT_2$ | $DT_3$ | $HI_1$ | $HI_2$ | $HI_3$ | $HI_4$ | $CD_1$ | $CD_2$ | *N. pumilio* | *N. antarctica* |
| 1 | 9 | 2 | 3 | 2 | 2 | 9 | 2 | 2 | 0 | 2 | 1 |
| 2 | 9 | 1 | 1 | 2 | 2 | 9 | 2 | 2 | 0 | 1 | 1 |
| 3 | 9 | 2 | 1 | 2 | 2 | 9 | 2 | 2 | 0 | 8 | 13 |
| 4 | 9 | 2 | 2 | 2 | 2 | 9 | 2 | 1 | 0 | 2 | 1 |
| 5 | 9 | 2 | 2 | 2 | 2 | 9 | 2 | 2 | 0 | 7 | 15 |
| **6** | **9** | **1** | **2** | **2** | **2** | **9** | **2** | **3** | **0** | – | 4 |
| 7 | 0 | 2 | 1 | 1 | 1 | 0 | 2 | 3 | 9 | 23 | 33 |
| **8** | **0** | **2** | **1** | **1** | **1** | **0** | **1** | **3** | **9** | – | 2 |
| **9** | **0** | **2** | **1** | **1** | **1** | **0** | **3** | **3** | **9** | – | 2 |
| 10 | 0 | 2 | 2 | 1 | 1 | 0 | 2 | 3 | 9 | 96 | 71 |
| **11** | **0** | **2** | **2** | **1** | **1** | **0** | **1** | **3** | **9** | – | 4 |
| **12** | **0** | **2** | **2** | **1** | **1** | **0** | **3** | **3** | **9** | – | 5 |
| 13 | 0 | 2 | 3 | 1 | 1 | 0 | 2 | 3 | 9 | 7 | 2 |
| *14* | *9* | *1* | *2* | *2* | *2* | *9* | *2* | *2* | *0* | 5 | – |

Polymorphic fragments obtained from primer/enzyme combinations are named in decreasing order of length. Number nine indicates a restriction site mutation. *N. pumilio* unique haplotypes are shown in italics. *N. antarctica* unique haplotypes are shown in bold type. Haplotype absolute frequencies are provided

which at the same time becomes the centroid of the polygon. Delaunay triangulation is a method that connects a set of points (populations) on a plane (map) by a set of triangles, and there is a unique way to make this connection, given a set of populations whose geographic locations are known. The algorithm then finds the edges associated with the highest rate of change in a given distance, namely "genetic barriers", by means of the genetic distances obtained with Biosys2 (Prevosti distances, Gregorius 1974).

**Table 3** Fragment size associated with each polymorphic fragment is expressed in base pairs

| Polymorphic fragments | Fragment size in base pairs (bp) |
|---|---|
| $DT_1$ | 328 (9), 233+103 (0) |
| $DT_2$ | 135 (1), 124 (2) |
| $DT_3$ | 46 (1), 40 (2), 32 (3) |
| $HI_1$ | 221 (1), 204(2) |
| $HI_2$ | 164 (1), 150 (2) |
| $HI_3$ | 88 (9), 57+44 (0) |
| $HI_4$ | 61 (1), 48 (2), 55 (3) |
| $CD_1$ | 964 (1), 930 (2), 904 (3) |
| $CD_2$ | 338 (9), 295+57 (0) |

Small differences in the sum of the restriction fragments in the cases of point mutations are assumed to be due to the precision of the estimation method that uses the migration of the bands and the size standard

### *Haplotype association and geographical arrangement*

To infer the relationships between haplotypes, we built haplotype networks with NETWORK software v. 4.6 (http://www.fluxus-engineering.com/sharenet.htm). We did one network per species, labelling the common haplotypes with the same number in order to facilitate comparisons. The software has two different network-building options, the *reduced median* network algorithm and the *median-joining* network algorithm. Following software guide recommendations, we run both algorithms in order to compare network outputs. To resolve ambiguous connections (loops), it is possible to use a post-processor option based on *maximum parsimony* tree building. The best haplotype arrangement can be selected from a series of possible trees generated by the software. Based on predictions from coalescent theory, three different criteria were used to finally draw the haplotype networks: (1) frequency criterion; (2) topological criterion; and (3) geographical criterion (see, Pfenninger and Posada 2002).

In addition, we represented the distribution of haplotypes on a frequency map. To test the relationship between geographic and genetic distances, we analysed an *isolation*

*by distance model* by running a Mantel test (Mantel 1967): we carried out 10,000 permutations between a geographic distance matrix constructed from latitudes and longitudes and a genetic distance matrix obtained with the pairwise genetic differentiation coefficients between populations ($G_{ST}$) using the software DISTON (http://www.pierroton.inra.fr/genetics/labo/Software/).

*Genetic introgression*

The sharing of cpDNA or mtDNA haplotypes among closely related species that hybridize and occur in sympatry is very common (Rieseberg and Soltis 1991). We calculated an introgression index (*IG*) which is a measure of the propensity of species to locally share genetic markers (Belahbib et al. 2001; Palmé et al. 2004). It ranges from zero (complete differentiation, no variants shared across species) to one (no differentiation, genetic variation is species-independent) (Belahbib et al. 2001). The index is based on "mixed" populations (i.e., population pairs where there is an overlap between species; see Table 1). We first obtained intraspecific identities for both species:

$$\widehat{J}_{1k} = \frac{\left(n_{1k}\sum_i x_{1ki}^2\right) - 1}{n_{1k} - 1}$$

(and $\hat{J}_{2k}$ respectively) where $i$ indexes the haplotype (alleles), $k$ represents the population, and $x$ the haplotype frequency. Then, we calculated interspecific identities by:

$$\widehat{J}_{12k} = \sum_i x_{1ki}x_{2ki}$$

Finally, the introgression ratio (*IG*) is defined as the mean of all pairs of interspecific identities, divided by the mean intraspecific identities:

$$IG = \frac{\sum_k \widehat{J}_{12k}}{\left(\sum_k \left(\widehat{J}_{1k} + \widehat{J}_{2k}\right)/2\right)}$$

If the species do share haplotypes but have independent geographical distribution, haplotype sharing on a local scale can be considered to occur only by chance. We computed the $IG_e$ index (expected introgression) in order to verify if there was concordant geographical organization across species, considering all populations. When *IG* is higher than $IG_e$, then sympatry could be thought of as the main cause of haplotype sharing; if, however, these ratios adopt similar values, then the current sympatric distribution of the species does not significantly influence the structure of haplotype sharing (Belahbib et al. 2001; Palmé et al. 2004).

## Results

### Polymorphism, genetic diversity, and structure

Three non-coding cpDNA regions proved variable in our study, which generated nine polymorphic fragments in both species. We found nine haplotypes in *N. pumilio* and 13 in *N. antarctica*; the two species shared eight haplotypes. We labelled the haplotypes from 1 to 14, assigning the same number to the common haplotypes (Table 2). On one hand, *N. pumilio* was less variable and showed only one unique haplotype (distributed in the northern region, in Epulauquen and Quilanlahue populations). On the other hand, the most variable region in *N. antarctica* (*atp*H-*atp*I) allowed the identification of four unique haplotypes (all localized in the transition zone). In addition, *N. antarctica* presented a fifth unique haplotype in the northern group as a consequence of a site restriction mutation in *trn*C-*trn*D (haplotype 6).

*N. pumilio* and *N. antarctica* have similar levels of average within population gene diversity ($h_s$), total genetic diversity ($h_t$), and gene differentiation across all populations ($G_{ST}$) (Table 4). Moreover, comparable amounts of differentiation were found between ordered alleles where similarities between haplotypes are counted as the proportion of shared fragments (Table 4). A remarkable result in our study is the significant phylogeographic structure in the species complex and the definition of two divergent lineages of genetic variation (evidenced by $N_{ST} \geq G_{ST}$, $p > 0.001$ and the one-tail test, $N_{ST}$ values $> N_{ST}$ permutated, $p < 0.001$) (see Figs. 1 and 2).

Allelic richness ($A_r$) was variable among populations. In the north, Quilanlahue is the population with highest diversity for both species; at mid-latitudes, Cholila, San Martín, and Río Unión for *N. pumilio,* whereas Lago Fontana and Río Unión for *N. antarctica* showed the highest allelic richness. In southern Patagonia, Tierra del Fuego Centro was the most diverse *N. pumilio* population (Table 1).

Genetic diversity in both species was higher in the northern group, with decreasing values of allelic richness (Table 1) and diversity values (Table 4) in the central and southern groups. Genetic differentiation was consequently lower in the central and the southern groups. Negative values were assumed as zero (no differentiation) (Long 1986). For *N. pumilio,* the phylogeographic structure was maintained only for the northern group, while *N. antarctica* also showed a phylogeographic structure in the central group (Table 4).

The AMOVA shows that both species have a significant, high degree of variation distributed within the populations (72% in *N. pumilio*, 70% in *N. antarctica*, $p < 0.001$). A significant proportion of variation was found inside regions for both species (47% for *N. pumilio*, 33% for *N. antarctica*

**Table 4** Average within population gene diversity (*hs*), total genetic diversity (*ht*), gene differentiation in all populations for unordered alleles ($G_{ST}$), and ordered alleles ($N_{ST}$) for all the populations and for each geographic group

| Geographic group | | *N. pumilio* | *N. antarctica* |
|---|---|---|---|
| Total | *hs* (SD) | 0.424 (0.0460) | 0.488 (0.0506) |
| | *ht* (SD) | 0.645 (0.0865) | 0.761 (0.0536) |
| | *G*ST (SD) | 0.344 (0.0589) | 0.359 (0.0786) |
| | *N*ST (SD) | 0.885 (0.0217)[a] | 0.841 (0.0318)[a] |
| North | *hs* (SD) | 0.600 (0.632) | 0.447 (0.1323) |
| | *ht* (SD) | 0.836 (0.0560) | 0.745 (0.1321) |
| | *G*ST (SD) | 0.282 (0.1162) | 0.401 (0.1725) |
| | *N*ST (SD) | 0.467 (0.1291)[a] | 0.599 (0.0922)[a] |
| Center | *hs* (SD) | 0.391 (0.0302) | 0.545 (0.0597) |
| | *ht* (SD) | 0.385 (0.0335) | 0.680 (0.0636) |
| | *G*ST (SD) | −0.013 (0.0162) | 0.197 (0.1246) |
| | *N*ST (SD) | −0.013 (0.0162) NS | 0.615 (0.1449)[a] |
| South | *hs* (SD) | 0.293 (0.1376) | 0.413 (0.1083) |
| | *ht* (SD) | 0.316 (0.1366) | 0.434 (0.0810) |
| | *G*ST (SD) | 0.070 (NC) | 0.047 (0.1635) |
| | *N*ST (SD) | 0.070 (NC) NS | 0.047 (0.1635) NS |

*SD* standard deviation, *NS* no evidence of phylogeographic structure

[a] Phylogeographic structure

for north–south division; 33% for *N. pumilio*, 21% for *N. antarctica*, for North–Centre–South division). When SAMOVA algorithm was performed, two groups of populations ($K$=2) were significantly resolved, with slight differences among species. Northern populations (Epulauquen, Caviahue, Tromen, Quilanlahue, Chalhuaco) were clearly separated from the rest for *N. pumilio* structure ($F_{CT}$=0.9206; $p$<0.001). In the case of *N. antarctica* Cholila (42°31′ S), instead, was grouped within the northern cluster ($F_{CT}$=0.8601; $p$<0.001). For increasing values of $K$ ($K$>2), the additional clusters consisted of a unique population, which has no meaning to the purpose of the analysis (both species presented the same picture; data not shown).

We performed a set of analysis in a bounded geographic area, and we found different genetic barriers between populations for *N. pumilio* with respect to *N. antarctica* (transition zone). In Fig. 3, we show the first six barriers for both species that correspond to distances ($d_0$) between 0.15 and 0.9. The easternmost population (San Martín) differs from the rest for *N. pumilio* whereas, for *N. antarctica*, it is clearly separated from the southern and Lago Guacho populations. This barrier may have a correspondence with a geographic isolation because of the existence of an arid region not suitable for these taxa. On the other hand, there is an important barrier ($d_0$=0.9) separating *N. antarctica* Cholila population from the others, and a similar scenario was found for *N. pumilio* Nahuelpan population (see Fig. 3 for details).

## Geographical arrangement of haplotype variation

We found a significant correlation between geographical and genetic pairwise distances in both species (Mantel tests, $p$>0.01). The distribution pattern of genetic variation was similar in both species, the north (36–41°S) being distinctly different from the rest of the distribution area (Fig. 1). Some rare haplotypes remained concentrated in a single population or in a single region. In northern populations, *N. pumilio* presented five different haplotypes not found in the other two groups (below 41°S). Haplotype 3 was the most frequent (5.3%) followed by number 5 (4.6%), and one unique haplotype was detected at low frequencies (number 14, 3.3%). The most diverse population was Quilanlahue. In the south, the most widely distributed haplotypes were number 10 (63.6%) and number 7 (15.2%). The Tierra del Fuego Centro population presented three different haplotypes (7, 10, and 13) with a high allelic richness (Table 1).

In the case of *N. antarctica*, we found six distinct haplotypes in the northern group. Number 5 was the most frequent (9.7%) followed by number 3 (8.4%). Caviahue and Quilanlahue were the most variable populations (three haplotypes in each) also with high allelic richness values. In addition, haplotype 6 was unique for this species and for the northern group (2.6%). In the transition zone, Cholila was the only population with a mixture of southern and northern haplotypes (5 and 10). Among the southern populations, there are seven distinct haplotypes, two of which are widely distributed: number 7 (21.4%) and number 10 (46.1%). Moreover, southern Chubut populations presented unique haplotypes (between 43° and 44°S) in low frequencies: numbers 8 (1.3%), 9 (1.3%), 11 (2.6%), and 12 (3.2%). The Río Unión population, in the transition zone, presented the maximum value of $A_r$.

Haplotype networks showed a relevant outcome: in both species two haplotype groups were identified separated by six mutations (three of which affected restriction sites). According to the coalescent theory (Posada and Crandall 2001), the most frequent haplotypes are considered to be the most ancient ones, which is the case of number 10 (south) for both species. However, if a topological criterion is prioritized, internal haplotypes become the most ancient. Then, for *N. pumilio*, internal haplotypes are 5 (north) and 10 (south) whereas for *N. antarctica* 3 and 10 are the oldest (Fig. 2). These intra-network differences between species may be related with different evolutionary histories typical of each of them. In both networks, unique variants are tip haplotypes (derived haplotypes with low frequencies), and in the case of *N. antarctica*, a missing haplotype is proposed (not sampled) that connects the two main groups.

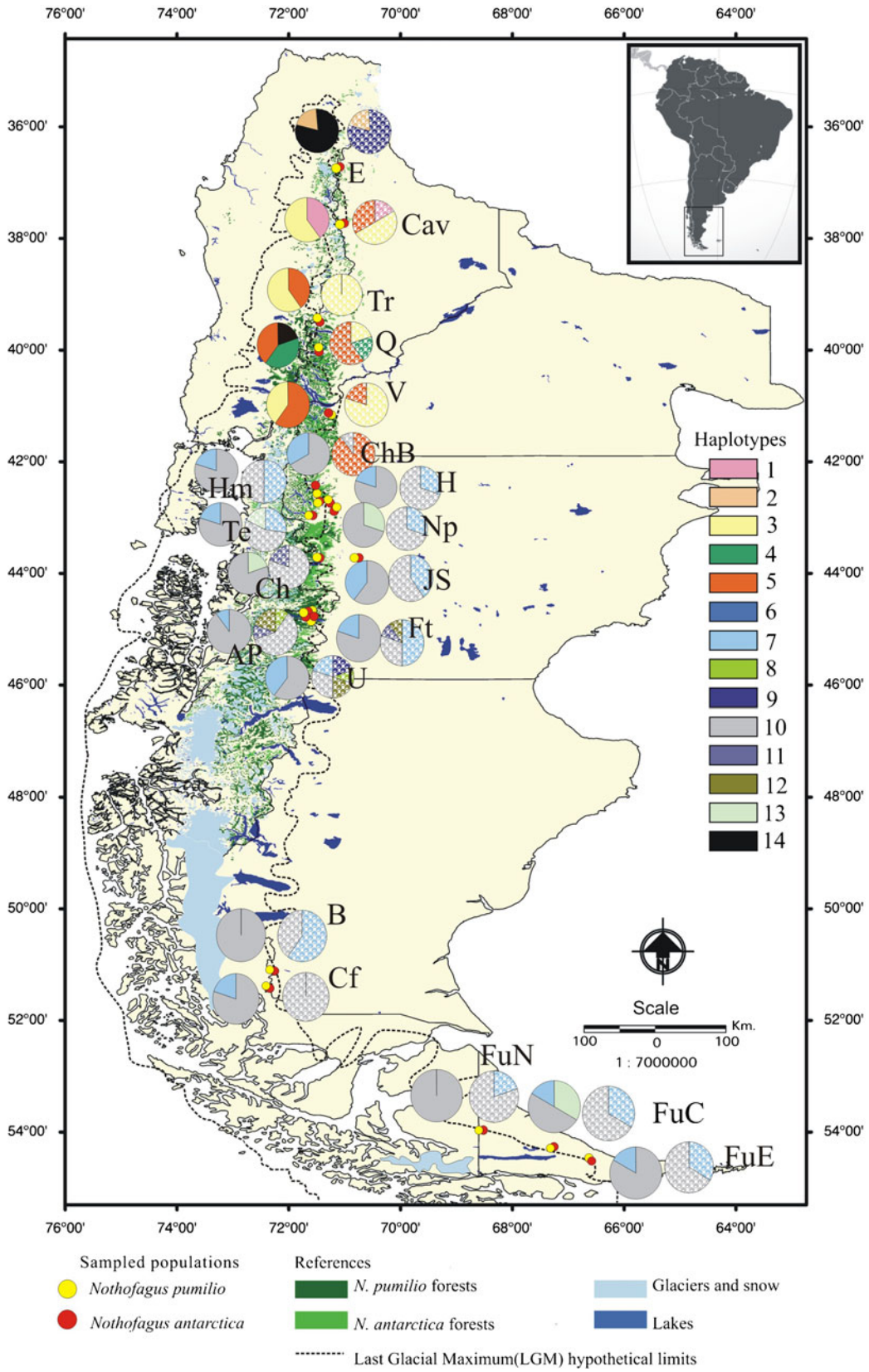


**Fig. 1** Geographic distribution of sampled populations of *Nothofagus* species. Haplotype frequency diagrams per population are shown for each species, *plain-color diagrams* correspond to *N. pumilio* whereas texture color diagrams correspond to *N. antarctica*. For population codes, see Table 1. LGM is based on Hollin and Shilling (1981)

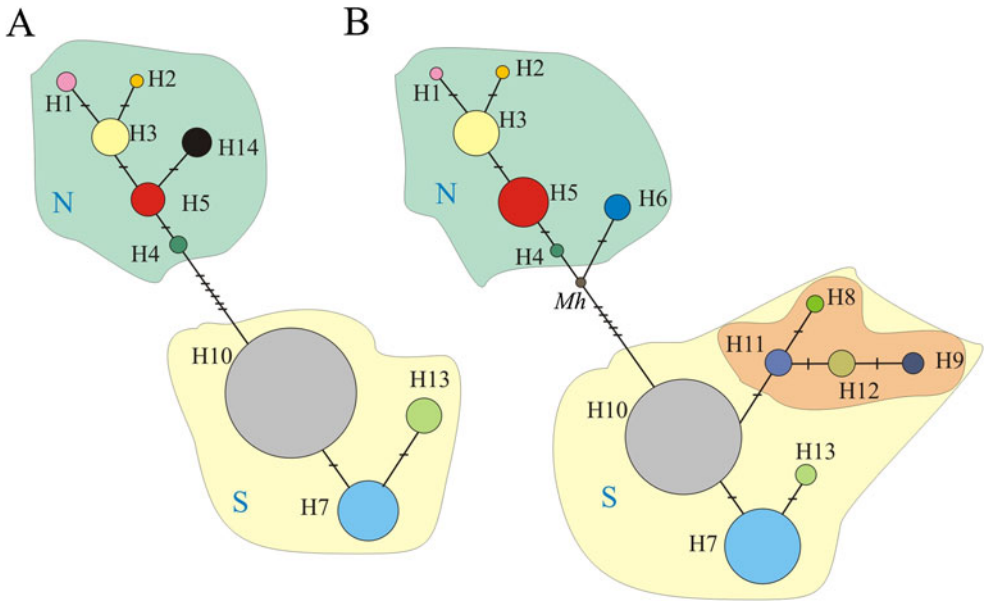


**Fig. 2** Haplotype networks for the nine haplotypes identified in *N. pumilio* populations (**a**), and for the 13 haplotypes identified in *N. antarctica* populations (**b**). Haplotypes are represented according to their frequencies; two main groups are recognizable which correspond to the north–south division at about 42°S of latitude (N and S, respectively). Common haplotypes are labelled with the same *number* and *color* in both species. Mutational steps between haplotypes are represented. *Mh*: missing haplotype

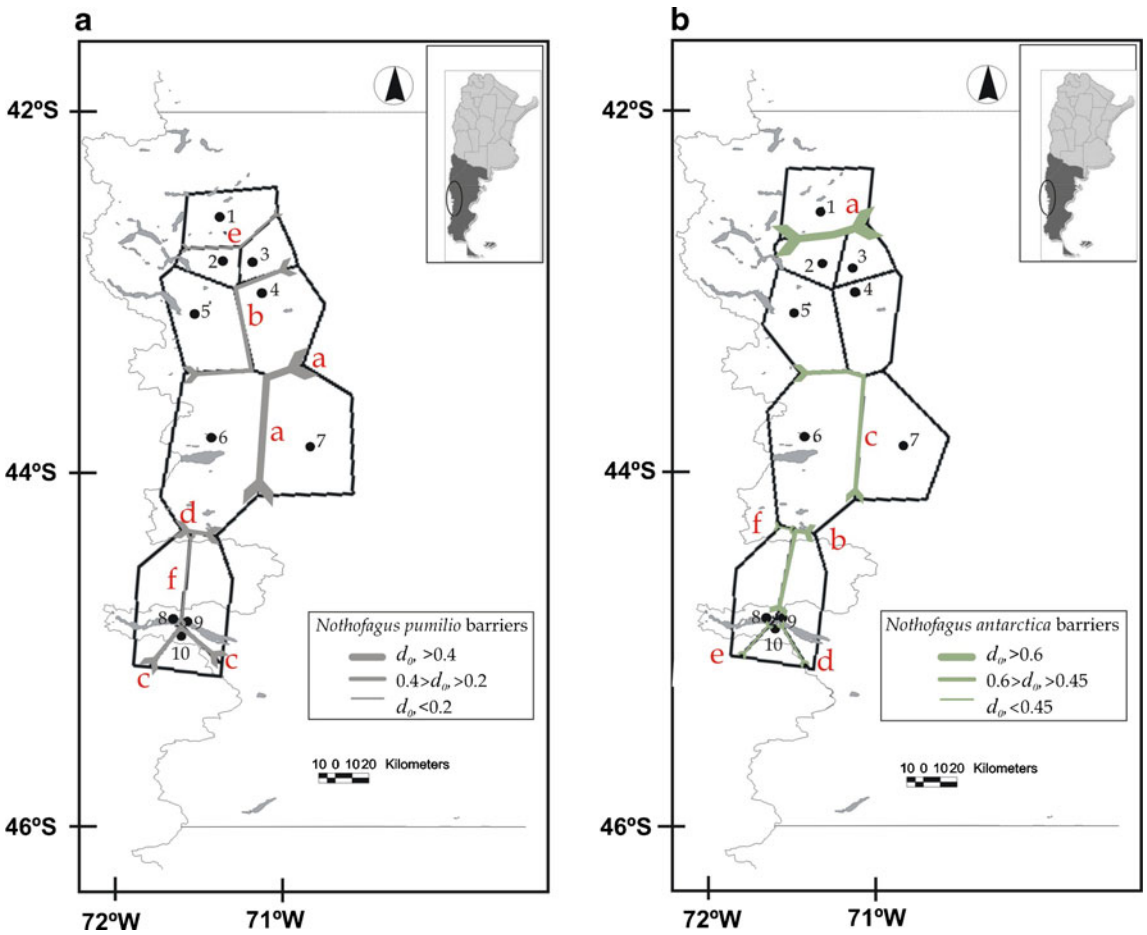


**Fig. 3** Genetic barriers between Chubut populations. **a** *N. pumilio* barriers. **b** *N. antarctica* barriers. **a**–**f**: Genetic barriers associated with corresponding genetic distances between populations based on haplotype frequencies ($d_0$, Gregorius 1974). *1–10*: sampled populations. *1*: Cholila, *2*: Huemules, *3*: La Hoya, *4*: Nahuelpan, *5*: Trevelin, *6*: Lago Guacho, *7*: J.San Martín, *8*: A.Perdido, *9*: L. Fontana, *10*: Río Unión

### Genetic introgression

A high introgression ratio was calculated ($IG$=0.90) indicating that genetic variation is species-independent in this *Nothofagus* species-complex. The expected introgression index considering the complete natural range of the species was lower ($IG_e$=0.437). Haplotype sharing between sympatric population pairs is a common observation in this species complex, but differences at population level are important (see for example Epulauquen, Cholila, Lago Guacho, and Lago Fontana on the map, Fig. 1).

## Discussion

### Phylogeographic patterns among southern beeches

In the present study, we compared the genetic structure of two related, sympatric species making inferences about their phylogeographical patterns. Our results were congruent and showed that the strong latitudinal structure of cpDNA haplotypes ($N_{ST} \geq G_{ST}$, $p$>0.001) shared by sympatric populations of *N. pumilio* and *N. antarctica* together with the differential levels of haplotype-sharing at population scale are relevant outcomes.

We found a north–south division in the haplotype distribution at about 42° 30′ S, reflecting the presence of two distinct, geographically segregated lineages. This might be consistent with the action of the two different types of glaciations in Patagonia, supporting our first tested hypothesis. A latitudinal trend in genetic variation was first described for the geographically restricted species *Nothofagus nervosa* with a transition near Lanín volcano (about 39° 30′S) (Marchelli and Gallo 2006), also confirmed in its congener *N. obliqua* (Azpilicueta et al. 2009). This tendency was also found in three important conifer species of southern forests, *Austrocedrus chilensis* (Pastorino and Gallo 2002), *Pilgerodendron uviferum* (Premoli et al. 2002), and *Araucaria araucana* (Bekessy et al. 2002) for nuclear DNA variation (isozymes and RAPDs) and in our preliminary work with a subset of the northern populations of *N. antarctica* with maternal and isozyme markers (Pastorino et al. 2009). A similar trend was recently reported for *N. pumilio* (Mathiasen and Premoli 2010).

In addition to latitudinal geographical distribution, we found that *N. pumilio* and *N. antarctica* do share the most frequent haplotypes, but each species also has unique haplotypes. Our results provide detailed information on intrapopulation polymorphisms. Intrapopulation variation is not always characterized by the same haplotypes in each species. Haplotype sharing among different *Nothofagus* species (including the two species studied here) has already been reported, showing a fixed variant in each population (Acosta and Premoli 2010). As mentioned before, we did find intrapopulation variation and unique haplotypes, which could be related to differences in the sampling design of our study and that carried out by Acosta and Premoli (2010).

### Glacial history

Seed dispersal in *Nothofagus* occurs by gravity and wind (Veblen et al. 1996), reaching distances of no more than 50 m from the mother tree, the majority remaining beneath it (Rusch 1993). This poor capacity for long-distance seed dispersal together with landscape fragmentation produced by several glaciations may be the cause of the significant phylogeographic structure found in our study. The strong north–south division around 42°S indicates a different evolutionary history for these groups of populations. Each group must have survived in different glacial refugia, supporting the evidence of multiple refugia in Patagonia (e.g., Marchelli and Gallo 2006; Pastorino and Gallo 2002; Premoli et al. 2000, 2002). In addition, however, since six mutations separate the two groups (three being point mutations) and considering that the rate of nucleotide substitutions of the chloroplast genome is of $1.1 \times 10^{-9}$ substitutions per site per year (Curtis and Clegg 1984), the hypothesis of pre-Quaternary fragmentation and isolation is supported (Azpilicueta et al. 2009; Gallo et al. 2008; Marchelli and Gallo 2006; Marchelli et al. 2010; Mathiasen and Premoli 2010).

Allelic richness ($A_r$) is a proper parameter for the identification of historical processes like bottlenecks or population admixture because of its independence from population size (Widmer and Lexer 2001). Refuge location or meeting points of colonization routes can be inferred with this parameter, which is also very meaningful for conservation purposes (Petit et al. 1998). In northern Patagonia, where glacier setting provided more possibilities for refuge, populations have a greater amount of variation. Quilanlahue (in the Lácar Lake region) presented a high value of $A_r$ for both *Nothofagus* species suggesting a putative relictual zone, in agreement with recent studies on plants and animals (Cosacov et al. 2010; Breitman et al. 2011). Although it is difficult to assert a specific temporal scale, previous works have postulated that this zone is reflecting the conditions at pre-Pleistocene times, as evidenced in other *Nothofagus* species (Azpilicueta et al. 2009; Marchelli and Gallo 2006). Palynological evidence supports this assumption with records from 40° to 41° S, which show that this pollen became dominant 15,000 years BP (Markgraf et al. 2002). The establishment of present-day vegetation (6,000 years BP) at the same latitudes is suggested by the abundance of Cupressaceae pollen, most likely *A. chilensis*, together with *dombeyii*-type pollen

(Markgraf et al. 2002; Whitlock et al. 2006), which is associated with both species studied here.

On Tierra del Fuego Island, we found the second most diverse *N. pumilio* population (Tierra del Fuego Centro). Pollen records from the late Pleistocene (Lago Fagnano; 54°57′ S, 67°62′ W) (Borromei and Quatrocchio 2008 and references therein) support our results. By the Holocene, the more abundant pollen sequences available (reviewed by Borromei and Quatrocchio 2008) in Tierra del Fuego revealed vegetation changes and probably the expansion of the forests till their current configuration. This *N. pumilio* population was more variable than the sympatric *N. antarctica* population. This case, an exception to our second tested hypothesis, probably reflects a pre-Quaternary scenario, in which repeated ancient glaciation events did not substantially affect *N. pumilio* variation (ancient refugia in Staten Island with lower sea levels) since regeneration settlement was successful many times over the generations. The case of Tromen (in the North), the other exception, is similar, where volcanism (Lanín volcano activity) could probably have affected the gene pool composition in a different way, favoring *N. pumilio* (higher haplotypic diversity). This species is apparently capable of renewing their stands in an explosive way after disturbances related to volcanism (Veblen et al. 1996), which could have favored its persistence in situ in the area. Supporting evidence for our second tested hypothesis is the high variability of *N. antarctica* populations, especially in the transition zone, which, having superior adaptive ability and plastic responses, found more microhabitats in which to persist (Río Unión, L. Fontana, A. Perdido, Trevelin, Lago Guacho). Environmental conditions and species responses therefore promoted different haplotype composition.

A latitudinal trend is also observed among populations in the transition zone. This variation might be associated with the effect of glaciers on a particular landscape configuration (north–south-orientated mountain chains at 42–43°S, west–east orientated watershed at 44°S; see genetic barriers on Fig. 3). Irregular masses of ice sheets between mountain chains (Flint and Fidalgo 1969) could allow the endurance of the species in ice-free areas such as piedmonts. Furthermore, within a mountain system, different ecological niches would be available, *nunatak*, *peripheral*, and/or *lowland* refugia (Holderegger and Thiel-Egenter 2009) providing a suitable habitat for these species.

To the north of the transition zone (Cholila population), there is evidence of admixture of colonization routes connecting the two main groups of populations (Pastorino et al. 2009). Pollen records reveal the development of open *Nothofagus* forest from the late-glacial to the early Holocene (11,400–6,000 cal years BP) (Whitlock et al. 2006). The south of the transition zone is characterized by unique haplotypes at low frequencies for *N. antarctica*. A cryptic refuge area could be proposed supported by the great amount of $A_r$ among these populations, but unfortunately no pollen records are available to confirm this prediction. Nonetheless, the capacity of cold-tolerant species to survive at high latitudes could not be discarded (Palmé et al. 2004; Fussi et al. 2010).

Haplotype networks add valuable information to the comprehension of evolutionary processes. Networks of both species showed little ambiguities about the haplotypes that can be considered "ancient". Clearly, haplotype 10 in the southern lineage is ancient in both species, either following the topological or the frequency criterion. Differences arise in the northern lineage where either haplotype 3 or 5 could be the oldest. Both of them are internal haplotypes, with no significant differences in their frequencies within the species. Besides, these haplotypes are broadly distributed in the northern region, which stress their putative ancestral condition. Populations where both ancestral (frequent and internal) and derivate (rare and tip) variants are present, which also hold a great amount of allelic richness, might have been a glacial refuge (Widmer and Lexer 2001). This is evident in the northern region (i.e., Quilanlahue), as well as in the central and southern regions (i.e., Fontana Lake Region, Tierra del Fuego Centro). Besides, differences between specific networks may be reflecting distinct evolutionary histories, a fact that is stressed by the presence of unique variants geographically restricted in one species (*N. antarctica*).

However, it is not possible to reach a definitive argument since hybridization between the species could be compounding the haplotype arrangements.

Regardless of which hypothesis is the most plausible, we assume that at least one, but probably more, refugia at high latitudes existed in Patagonia for our cold-tolerant species (south of 42°S), as in other parts of the world (e.g., Magri et al. 2006; Palmé et al. 2004). Even though conditions in the south were more extreme (extended ice cap and climate), pollen in small amounts and beetle assemblages associated with *Nothofagus* woodlands on the west side of the Andes (Chile) indicate that the massive growth of ice sheets during full-glacial times did not completely eliminate this biota (Ashworth et al. 1991). In the eastern foothills (Argentina), it is highly probable that a similar situation could have occurred before entering the arid conditions of the Patagonian steppe, since the ice sheet was much more fragmented (Glasser et al. 2008); however, traces of pollen have not yet been detected.

## Hybridization and genetic introgression

Hybridization is a widespread phenomenon in many plant taxa, and one of its consequences is cytoplasmic introgression (Rieseberg and Soltis 1991). Among the three main

causes of haplotype sharing (convergence, ancestral polymorphism, and hybridization/introgression), interspecific gene flow and backcrossing offspring, causing introgression, are the most suitable explanations in our species complex. A similar geographical pattern for haplotype distribution in both species, not only in the more frequent but also the less abundant variants, supports the idea of recent or at least postglacial hybridization. If the similarities were only the consequence of ancestral polymorphism, those patterns should be totally independent of each other. In this case, sympatric distribution significantly influences the haplotype composition of a given population more than species identity ($IG > IG_e$).

Ecological features also support hybridization in our species-complex: Flowering phenology and pollen release overlap (Donoso et al. 2006; González et al. 2006), although other pre- and even post-zygotic barriers can also occur. Directional pollination towards *N. antarctica* (acting as the mother tree) may be favored by the predominance of westerlies in the region and the altitudinal ecological gradient formed by both species. Based on our results, it is possible that species interactions like hybridization and introgression may contribute to persistence and recolonization processes during glaciations. It is possible that, under stressful climatic conditions, the pioneer *N. antarctica* could not rely only on seed movement to spread, but used its sprouting ability to enhance its persistence and dispersion capacity. At the same time, inter-specific gene flow with incoming *N. pumilio* pollen from neighboring or relatively nearer populations (middle- to long-distance dispersal) could in part counterbalance the minor capacity of *N. antarctica* to fertilize intraspecific flowers. The adaptive mechanism of *N. antarctica* to extend its forests and persist could be the proper combination of seed movement and clonal reproduction. *N. pumilio,* the late-successional species, would also overcome the lack of natural regeneration in severe environmental conditions (e.g., eastern marginal populations) through interspecific gene flow with its congener. These *Nothofagus* species do not develop persistent seed banks (Veblen et al. 1996), and its germination capacity varies a lot depending on local habitat circumstances. In this way, common or neighboring refugia would have played an important role during glacial times since interactions between species could facilitate their persistence (e.g., Fussi et al. 2010).

Notwithstanding, to elucidate fine details of the hybridization/introgression process between these *Nothofagus* species, a more restricted spatial-scale study would be necessary, also taking into consideration other age classes, like seedlings, as well as hybrid trees. A new experimental design with a smaller scale of analysis would allow the testing of well-known hypotheses concerning hybridization mechanisms in trees (e.g., pollen swamping hypothesis) (Potts and Reid 1988; Petit et al. 1997; Belahbib et al. 2001).

## Final conclusions

We provide evidence from maternal lineages that genetic variation was modelled by glaciations in Patagonia following a north–south latitudinal gradient. A latitudinal cline in the distribution of genetic diversity was determined, with a great amount of variation that diminish from north to south and with unique variants at the transition zone (42–44°S). This trend agrees with information in many different taxa from Patagonia, including both plants and animals, suggesting a relevant impact of Pleistocene climatic changes. Moreover, hybridization and introgression between these *Nothofagus* species are the main causes of haplotype sharing. If this was a long-lasting process that occurred in shared refugia, it might have facilitated *Nothofagus* persistence and expansion in a complex geographical scenario. *N. antarctica* introgressed individuals possessing some intermediate traits of *N. pumilio* phenotypes would deserve special protection status. The presence of hybrids (sensu *lato*) could be a clear sign that the introgression process took place, with probably a different behavior of its descendants (increased plasticity). In the current scenario of climate change, this is of remarkable importance.

Future studies including also the nuclear genome will contribute to the understanding of current patterns of gene flow and the thorough detection of the introgression process. Combined information from different types of molecular markers would provide tools to ultimately define conservation units. A balance between conservation and the use of the genetic resources of Andean Patagonia is our desired goal.

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