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Evidence of chloroplast capture in South American *Nothofagus* (subgenus *Nothofagus*, Nothofagaceae)

M. Cristina Acosta *, Andrea C. Premoli

Centro Regional Universitario Bariloche-Universidad Nacional del Comahue, INIBIOMA-CONICET, Quintral 1250, 8400 Bariloche, Argentina

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ABSTRACT

Subgenus *Nothofagus*, although geographically restricted at present to temperate areas of South America, has captured much attention in discussions of plant biogeography due to its widespread distribution through Gondwanan continents during the Tertiary. However, phylogenetic relationships within the subgenus *Nothofagus* have not yet been resolved. We examined geographic patterns of intraspecific and interspecific genetic variation to detect whether incongruences in nuclear or plastid DNA phylogenies occur, in order to better understand the evolutionary history of the subgenus *Nothofagus*. We conducted spatially-explicit sampling at 10 distinct locations throughout the range of austral South American forests and sampled all present *Nothofagus* species. We used ITS and chloroplast DNA sequences to estimate phylogenetic relationships. A phylogeny constructed from nuclear genes resolved the subgenus *Nothofagus* as monophyletic. We found that *N. antarctica* was a sister to a clade of evergreen species (*N. betuloides*, *N. dombeyi*, and *N. nitida*), while *N. pumilio* likely diverged earlier. Nine cpDNA haplotypes were distinguished in the subgenus *Nothofagus* which were associated to geographic locations rather than to taxonomic relationships. This species-independent cpDNA phylogeographic structures within the subgenus *Nothofagus* may be related to repeated chloroplast capture events over geological time in Patagonia.

1. Introduction

Nothofagus Blume (Nothofagaceae) is the most characteristic genus within austral forests of southeast Australia including Tasmania, New Caledonia, New Guinea, New Zealand, and southwestern South America. This ancient genus has been an important focus for discussions of biogeographical history because of its distribution in Gondwana, the fact that its nuts are apparently limited in dispersal movement, and the existence of a phylogenetically relevant fossil record of distinctive pollen types (Hill and Read, 1991). The phylogeny of *Nothofagus* has been investigated by comparing extant and fossil morphologies as well as DNA sequences from chloroplast (cpDNA) and nuclear (nDNA) genomes (Hill and Read, 1991; Hill and Jordan, 1993; Martin and Dowd, 1993; Setoguchi et al., 1997; Manos, 1997). These previous phylogenetic analyses have confirmed the monophyly of the four extant subgenera and their relationships [*Lophozonia* (Turcz.) Krasser [*Fuscospora* R. S. Hill and J. Read [*Nothofagus*, *Brassospora* W. R. Philipson and M. N. Philipson]]].

Here, we have chosen to focus on the subgenus *Nothofagus* because the interspecific relationships within this subgenus remain controversial. *Nothofagus* is endemic to South America and includes the deciduous *N. antarctica* (G. Forst.) Oerst. and *N. pumilio* (Poepp. & Endl.) Krasser as well as three evergreen species [*N. betuloides* (Mirb.) Oerst., *N. dombeyi* (Mirb.) Oerst., and *N. nitida* (Phil.) Krasser; Veblen et al., 1996]. Although the subgenus *Nothofagus* is geographically restricted to temperate areas of South America at present, it was widespread among Gondwanan continents in the Tertiary (Dettmann et al., 1990). Most species within *Nothofagus*, although ecologically and genetically distinct, are similarly widespread and encompass the entire distributional range of temperate forests in southern South America. For example, while the evergreen *N. betuloides* and *N. dombeyi* are usually associated with humid climates, the deciduous *N. pumilio* is the dominant, almost only, tree found in high-elevation environments of Patagonia. The deciduous *N. antarctica* displays the greatest morphological variation of the five members of the subgenus, inhabiting low to high-elevation environments, valley bottoms with cold air drainage, and wetlands (Veblen et al., 1996).

Previous studies have yielded conflicting topologies for the phylogenetic tree within subgenus *Nothofagus*. A tree based on rbcL cpDNA sequences (Martin and Dowd, 1993) showed that *N. dombeyi* diverged first, but could not resolve the relationships between *N. nitida*, *N. betuloides*, and *N. pumilio*–*N. antarctica*, suggesting a close relationship between the deciduous taxa. Using the atpB-rbcL cpDNA intergenic spacer, Setoguchi et al. (1997) found an

* Corresponding author. Fax: +54 2944 422111.
E-mail addresses: mcacosta@crub.uncoma.edu.ar (M. Cristina Acosta), apremoli@crub.uncoma.edu.ar (A.C. Premoli).

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unresolved polytomy of the five species within the subgenus. In Manos (1997), one of two most parsimonious trees based on the ITS nuclear region showed *N. antarctica* diverging first from two clades, *N. nitida–N. betuloides* and *N. pumilio–N. dombeyi*, indicating that deciduous species are not sister taxa. In addition, a strict consensus tree including the morphological characters defined by Hill and Jordan (1993) showed *N. pumilio* diverging first, followed by *N. antarctica* and subsequently *N. betuloides* (*N. dombeyi–N. nitida*). Moreover, combined analyses of ITS, cpDNA rbcL sequences (from Martin and Dowd, 1993), and morphological characters yielded (*N. dombeyi* (*N. nitida–N. betuloides*) (*N. antarctica–N. pumilio*)) clades in the strict consensus tree. The predicted phylogenetic relationships within the subgenus are inconsistent across the three data sets, and a resolution based on combined data is unsatisfactory.

Incongruence between phylogenies based on nuclear and chloroplast markers are generally caused by convergent evolution, lineage sorting, or hybridization and introgression (McKinnon et al., 2001). Hybridization is a common phenomenon between sympatric species belonging to the same pollen group in *Nothofagus* (Donoso, 1996; Veblen et al., 1996). Notably, putative hybrids have been recognized between certain combinations of South American species within subgenus *Nothofagus* (Premoli, 1996a,b; Stecconi et al., 2004; Quiroga et al., 2005). Previous studies that used chloroplast DNA markers to examine genetic variation in Fagales suggested that cpDNA haplotypes are shared among closely related species, and that there is considerable intraspecific polymorphism. Consistently, cpDNA haplotypes were found to correlate with geographically circumscribed regions rather than with species *per se* (Whittemore and Schaal, 1991; Petit et al., 1993; Thórsson et al., 2001). Rieseberg and Soltis (1991) have suggested that nuclear and chloroplast DNA-based studies with comprehensive sampling methods are needed in order to avoid erroneous phylogenetic conclusions. The objective of this study is to examine the geographic patterns of intraspecific and interspecific genetic variation at chloroplast and nuclear markers to understand the evolutionary history within subgenus *Nothofagus*, and to determine whether reticulate evolution occurs in this subgenus.

2. Materials and methods

2.1. Taxon sampling

Leaf material was collected from a total of 32 populations of the five species (*N. antarctica*, *N. betuloides*, *N. dombeyi*, *N. nitida*, and *N. pumilio*) of subgenus *Nothofagus* (Table 1). Sampling followed a spatially-explicit design in which samples were collected for all *Nothofagus* species present at each of 10 distinct locations throughout the geographic range of temperate forests in southern South America. In addition, the outgroups consisted of samples of one individual of each *N. fusca* (Hook. fil.) Oerst. and *N. solandri* (Hook. fil.) Oerst. belonging to the subgenus *Fuscospora*. Also we included one individual of *N. cunninghamii* (Hook. fil.) Oerst. and *N. menziesii* (Hook. fil.) Oerst. together with two individuals of *N. obliqua* (Mirb.) Oerst. and *N. nervosa* (Phil.) Dim. & Mil. [= *N. alpina* (Poepp. & Endl.) Oerst.] from the subgenus *Lophozonia*. For the nuclear ITS region, we tested the effect of subgenus *Brassospora* as outgroup since *N. grandis* is available in GenBank (Accession No. DQ499088). The voucher specimens were deposited in the herbarium of Centro Regional Universitario Bariloche, Argentina (BCRU).

2.2. DNA extraction, amplification, and sequencing

Fresh tissue was kept in a portable cooler until it arrived at the laboratory at Universidad Nacional del Comahue. Total DNA was extracted with a DNeasy plant mini kit (Qiagen, Hilden, Germany). A reaction of 1–2 μl of DNA extract (10 ng) and 4–6 μl of GeneReleaser® (BioVentures Inc., Murfreesboro, TN, USA) was performed for 15 min at 85 °C (followed by a hold at 32 °C) prior to PCR to facilitate DNA release. The complete internal transcribed spacer (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 population due to highly conserved DNA sequences of such nuclear region for any given species. The PCR mix contained 1 μl of template DNA (10 ng), 0.625U GoTaq DNA polymerase (Promega, Madison, WI, USA), 5 μl 5× Green GoTaq® reaction buffer (Promega), 0.25 mM of each dNTP, and 0.3 μM of each primer in a total volume of 25 μl. The PCR cycling scheme 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.

Three non-coding regions of the chloroplast genome were amplified from two individuals of each species collected at any one location using primer pairs *psb*B–*psb*H (Hamilton, 1999), *trn*L–*trn*F (Taberlet et al., 1991), and *trn*H–*psb*A (Hamilton, 1999). The PCR mix was the same as for ITS except that 2 μl of template DNA was used. An initial denaturation step at 95 °C for 4 min was followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C, 54 °C, or 56 °C (depending on the primer pair used) for 1 min, extension at 72 °C for 1:30 min; a final extension at 72 °C for 7 min and a final hold at 15 °C. All reactions were carried out on an Applied Biosystems 2720 thermocycler (Applied Biosystems, Foster City, CA, USA). Amplification products were separated by electrophoresis on a 1% agarose gel, stained with Sybr Safe (Invitrogen, Eugene, OR, USA), and visualized with a UV transilluminator. PCR products were cleaned with Exonuclease I (Fermentas, Ontario, Canada) and Shrimp Alkaline Phosphatase (USB, Cleveland, OH, USA). Cycle sequencing was performed using Big Dye terminator chemistry (Applied Biosystems, Foster City, CA, USA). Automated sequencing using both forward and reverse amplification primers was conducted on an ABI PRISM 3100 AVANT at the sequencing facility of the Universidad Nacional del Comahue.

2.3. Phylogenetic analysis

Sequences were aligned using MEGA 3.1 (Kumar et al., 2004) with manual adjustments as needed. One sequence of each population and species were used in the phylogenetic analyses except for antLLIC1 and antLLIC2 that were slightly different and therefore were both included. The effect on the stability of ingroup topology and on branch support of each subgenus that were used as outgroups or a combination of them was tested. Phylogeny reconstruction under parsimony was conducted separately on nuclear and chloroplast datasets using PAUP* v. 4.0b10 (Swofford, 2003). Heuristic searches included 1000 random addition replicates, TBR branch swapping, and gaps were coded following the "simple indel coding" method (Simmons and Ochoterena, 2000). Support for monophyly was determined by non-parametric bootstrapping (Felsenstein, 1985) on 1000 bootstrap replicates using the same criteria as did the regular parsimony searches. Bayesian analyses of the separate ITS and cpDNA data sets were conducted using MrBayes v. 3.1.2 (Huelsenbeck and Ronquist, 2001) with a model of sequence evolution generated by MrModeltest v. 2.2 (Nylander, 2004) that implemented the AIC criteria. Evolution models that best fit ITS and chloroplast data sets were GTR + G and GTR + I, respectively (Rodríguez et al., 1990). The analysis consisted of 3×10^6 generations with four chains (three heated and one cold) and trees were saved every 1000 generations in each run. Approximately 10% of the trees (corresponding to the burn-in period) were discarded and a 50% majority rule consensus tree was constructed from the remaining trees. Finally, the haplotype median-joining network was constructed using Network v. 4.1.1.2 (Bandelt et al., 1999).

Table 1
Collection sites, code, voucher and GenBank accession numbers of sampled populations of *Nothofagus*.

Location	Latitude (°S)	Longitude (°W)	Species, code, and voucher number	GenBank Accession No.	
				ITS1-5.8S-ITS2	*psb*B–*psb*H *trn*L–*trn*F *trn*H–*psb*A
Argentine, Prov. Neuquen, Dept. Huiliches, Tromen Lake	39° 34′	71° 25′	*N. antarctica*, antNLTR, *MCA & EK 137*	GQ863229	GQ863269 GQ863297 GQ863325
			N. dombeyi, domNLTR, *MCA & EK 135*	GQ863230	GQ863270 GQ863298 GQ863326
			N. pumilio, pumNLTR, *PM 17*	GQ863231	GQ863397 GQ863379 GQ863388
			N. nervosa, nerNLTR, *MCA & EK 136*	GQ863232	GQ863271 GQ863299 GQ863327
Argentine, Prov. Neuquen, Dept. Lácar, San Martín de Los Andes	40° 07′	71° 26′	*N. antarctica*, antNSMA, *MCA & EK 87*	GQ863233	GQ863272 GQ863300 GQ863328
			N. dombeyi, domNSMA, *MCA & EK 142*	GQ863234	GQ863273 GQ863301 GQ863329
			N. pumilio, pumNSMA, *PM 18*	GQ863235	GQ863398 GQ863380 GQ863389
			N. nervosa, nerNSMA, *MCA & EK 85*	GQ863236	GQ863274 GQ863302 GQ863330
			N. obliqua, oblNSMA, *MCA & EK 140*	GQ863237	GQ863275 GQ863303 GQ863331
Chile, Región de Los Lagos, Antillanca Valley	40° 47′	72° 11′	*N. betuloides*, betLLAN, *MCA & ACP 50*	GQ863238	GQ863276 GQ863304 GQ863332
			N. dombeyi, domLLAN, *MCA & ACP 111*	GQ863239	GQ863277 GQ863305 GQ863333
			N. nitida, nitLLAN, *MCA & ACP 52*	GQ863240	GQ863278 GQ863306 GQ863334
			N. pumilio, pumLLAN, *PM 20*	GQ863241	GQ863399 GQ863381 GQ863390
			N. obliqua, oblLLAN, *MCA & ACP 109*	GQ863242	GQ863279 GQ863307 GQ863335
Chile, Región de Los Lagos, Osorno Volcano	41° 07′	72° 31′	*N. antarctica*, antLLVO, *MCA 133*	GQ863243	GQ863280 GQ863308 GQ863336
			N. betuloides, betLLVO, *MCA 132*	GQ863244	GQ863281 GQ863309 GQ863337
			N. dombeyi, domLLVO, *MCA 131*	GQ863245	GQ863282 GQ863310 GQ863338
			N. pumilio, pumLLVO, *PM 22*	GQ863246	GQ863400 GQ863382 GQ863391
Argentine, Prov. Río Negro, Dept. Bariloche, Otto Hill	41° 08′	71° 19′	*N. antarctica*, antRNBA, *MCA & EK 49*	GQ863247	GQ863283 GQ863311 GQ863339
			N. dombeyi, domRNBA, *MCA & EK 47*	GQ863248	GQ863284 GQ863312 GQ863340
			N. pumilio, pumRNBA, *MCA & EK 40*	GQ863249	GQ863401 GQ863383 GQ863392
Chile, Región de Los Lagos, Chiloé Island, Huillinco	42° 42′	73° 53′	*N. antarctica*, antLLIC, *MCA & ACP 95*	GQ863250	GQ863285/6 GQ863313/4 GQ863341/2
			N. betuloides, betLLIC, *MCA & ACP 99*	GQ863251	GQ863287 GQ863315 GQ863343
			N. dombeyi, domLLIC, *MCA & ACP 96*	GQ863252	GQ863288 GQ863316 GQ863344
			N. nitida, nitLLIC, *MCA & ACP 94*	GQ863253	GQ863289 GQ863317 GQ863345
Argentine, Prov. Chubut, Dept. Futaleufu, Sierra Colorada	43° 10′	71° 23′	*N. antarctica*, antCHSC, *MCA et al. 116*	GQ863254	GQ863290 GQ863318 GQ863346
			N. dombeyi, domCHSC, *MCA et al. 118*	GQ863255	GQ863291 GQ863319 GQ863347
			N. pumilio, pumCHSC, *PM et al. 30*	GQ863256	GQ863402 GQ863384 GQ863393
Argentine, Prov. Santa Cruz, Dept. Lago Argentino, El Chaltén	49° 17′	72° 54′	*N. antarctica*, antSCCH, *MCA & LG 77*	GQ863257	GQ863292 GQ863320 GQ863348
			N. pumilio, pumSCCH, *PM 48*	GQ863258	GQ863403 GQ863385 GQ863394
Argentine, Prov. Santa Cruz, Dept. Lago Argentino, Los Glaciares National Park	50° 25′	72° 45′	*N. antarctica*, antSCPG, *MCA & LG 73*	GQ863259	GQ863293 GQ863321 GQ863349
			N. betuloides, betSCPG, *MCA & LG 72*	GQ863260	GQ863294 GQ863322 GQ863350
			N. pumilio, pumSCPG, *PM 50*	GQ863261	GQ863404 GQ863386 GQ863395
Argentine, Prov. Tierra del Fuego, Dept. Usuahia, Estancia María Luisa	54° 27′	66° 30′	*N. antarctica*, antTDF, *MCA & LG 57*	GQ863262	GQ863295 GQ863323 GQ863351
			N. betuloides, betTDF, *MCA & LG 66*	GQ863263	GQ863296 GQ863324 GQ863352
			N. pumilio, pumTDF, *PM 61*	GQ863264	GQ863405 GQ863387 GQ863396
Australia, Tasmania, cultivated	–	–	*N. fusca*, fusCV, *MCA 183*	GQ863265	GQ863367 GQ863371 GQ863375
			N. solandri, solCV, *MCA 184*	GQ863266	GQ863368 GQ863372 GQ863376
			N. cunninghamii, cunCV, *MCA 185*	GQ863267	GQ863369 GQ863373 GQ863377
			N. menziesii, menCV, *MCA 186*	GQ863268	GQ863370 GQ863374 GQ863378

Collector's names: MCA, M. C. Acosta; LG, L. Garibaldi; EK, E. Kowaljow; PM, P. Mathiasen; ACP, A. C. Premoli.

3. Results

3.1. Nuclear DNA

The length of the ITS1-5.8S-ITS2 region within subgenus *Nothofagus* varied slightly from 354–164–288 bp in *N. antarctica* and *N. nitida* to 356–164–289 bp in *N. betuloides* and *N. dombeyi*, and 354–164–293 bp in *N. pumilio*. While in *Fuscospora* the sequence length was constant at 354–164–273 bp in *Lophozonia* ranged from 348–164–278 bp in *N. nervosa* and *N. obliqua* to 353–164–294 bp in *N. cunninghamii* and *N. menziesii*. The sequence of the 5.8S rDNA was invariable in all species within subgenus *Nothofagus*. Alignment of the final matrix required the occurrence of 14 1 bp indels, two 2 bp indel, and the introduction of five larger gaps of 5–18-bp. The highest number of autapomorphies (six) was observed in *N. pumilio* and included one duplication of 5 bp. Intraspecific sequence variation was not observed (Fig. 1A), except in *N. dombeyi* individuals from the RNBA location (Ribotype 3, $R = 3$) that had one transversion. The remaining *N. dombeyi* and *N. betuloides* samples had identical ITS sequences (Ribotype 2, $R = 2$). No evidence of multiple rDNA repeat types was found in any of the analyzed taxa by means of superimposed or smaller peaks that would provide evidence of introgression in nuclear genes.

3.2. Chloroplast DNA

Nine distinct cpDNA haplotypes were found in the subgenus *Nothofagus* and are shared among all species found at any one location (Fig. 1B). The size of the *psb*B–*psb*H intergenic spacer was

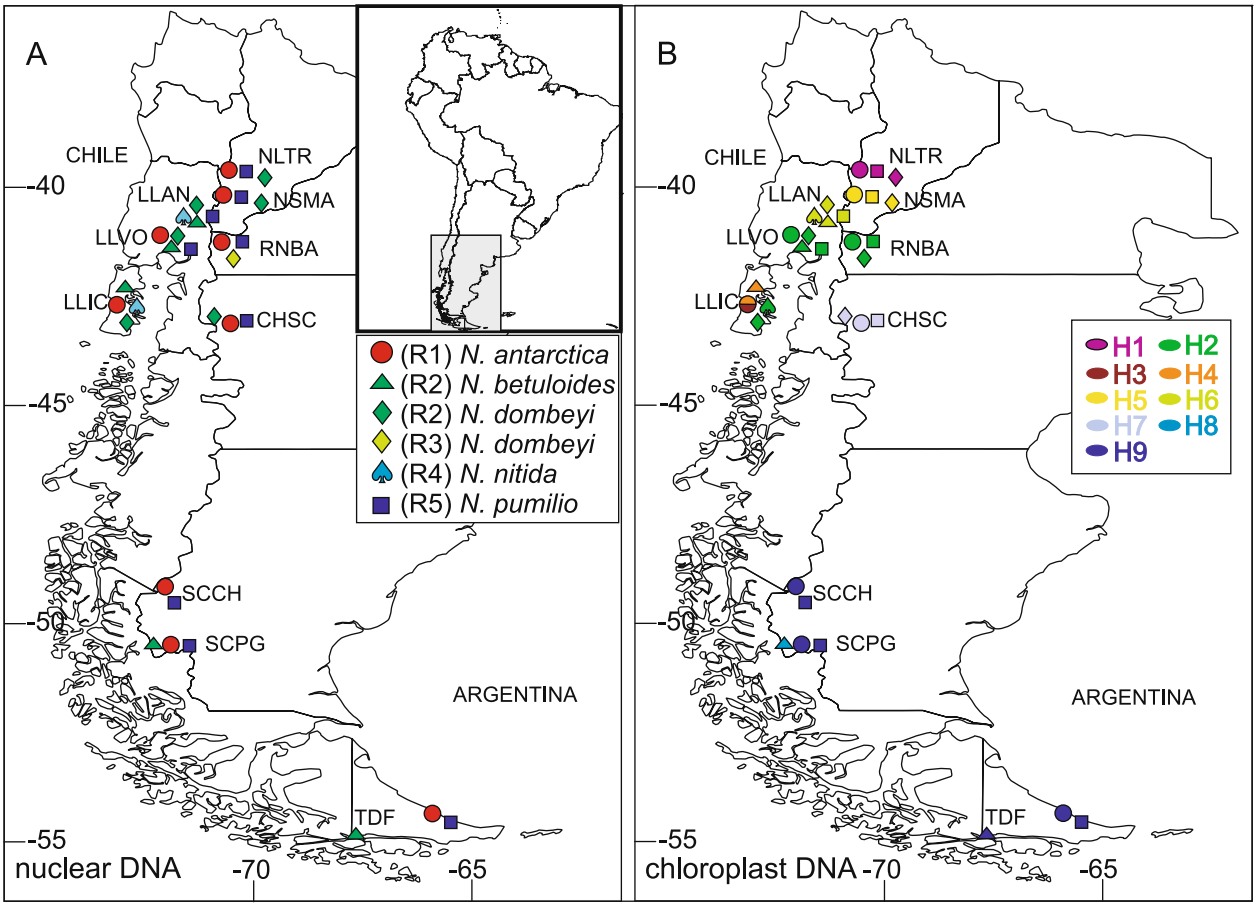

Fig. 1. Map of southern South America showing the distribution of the 32 sampled *Nothofagus* populations, their ribotypes (R) and haplotypes (H). Codes of populations are as in Table 1. (A) ITS data. (B) Combined cpDNA data. Symbols indicate different species. Colors correspond to the ITS ribotypes and chloroplast haplotypes shown in A and B, respectively. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

791 bp in haplotypes H1–H6 and 776 bp in haplotypes H7–H9. The lengths of *trn*L–*trn*F varied from 417 in haplotype H1 to 437 bp in haplotype H7, while the intergenic spacer *trn*H–*psb*A was more variable than the others and ranged between 420 (H8) and 445 bp (H4, H5, and H6). Within subgenus *Nothofagus*, eight gaps were introduced, ranging from 1 to 19 bp in length. Haplotypes H1–H6 have one insertion of 15 bp in *psb*B–*psb*H, haplotype H1 has one deletion of 17 bp in the *trn*L–*trn*F spacer, and haplotypes H2–H6 have one insertion of 19 bp in *trn*H–*psb*A. In addition, cpDNA haplotypes within the subgenus have single-base deletions in a poly-T region at *trn*L–*trn*F and *trn*H–*psb*A, as well as single-base deletions in a poly A-region in the latter. Within subgenus *Lophozonia* the lengths and sequences of cpDNA regions were highly conserved among the sampled populations of the two South American species (*N. nervosa* and *N. obliqua*, that comprised *psb*B–*psb*H 768 bp, *trn*L–*trn*F 437 bp, and *trn*H–*psb*A 411 bp) which differed to those in species from Australia (*N. cunninghamii* 768–436–425 bp) and New Zealand (*N. menziesii* 768–434–419 bp). The only difference in cpDNA between species from subgenus *Fuscospora* was a unique indel in the *trn*H–*psb*A intergenic spacer (*N. solandri* 776–433–402 bp and *N. fusca* 776–433–403 bp).

3.3. Phylogenetic analysis

Parsimony analysis and Bayesian inference yielded trees with same ingroup topology but different branch support when different outgroups were used. In the ITS analyses, bootstrap values and bayesian posterior probabilities are markedly lower when we used *Brassospora* as outgroup, possibly due to its closer sister relation with the subgenus *Nothofagus* (Manos, 1997). Using only *Lophozonia* as outgroup, which in turn is considered the most phylogenetically distinct to subgenus *Nothofagus*, yielded relatively lower branch support values in the ITS tree than those obtained using a combination of *Fuscospora* + *Lophozonia*. However, the inclusion of *Fuscospora* reduced the branch support value for monophyly of subgenus *Nothofagus* in the cpDNA tree probably due to the phylogenetically closer relationship between these later two subgenera. In spite of this, both subgenera *Fuscospora* and *Lophozonia* were used as outgroups (Fig. 2).

Parsimony analyses of the aligned ITS data alone returned four most parsimonious (MP) trees, while eight MP trees were produced by cpDNA analyses (Table 2). Nuclear DNA analyses showed the subgenus *Nothofagus* to be monophyletic [BS (bootstrap support) = 99%, BPP (Bayesian posterior probability) = 1.00, Fig. 2A]. Also, *N. antarctica* and *N. pumilio* were resolved as monophyletic (BS = 97%, BPP = 1.00; BS = 96%, BPP = 1.00, respectively). Noteworthy, *N. betuloides* and *N. dombeyi* shared the same ribotype (BS = 99%, BPP = 1.00) and are grouped in a unique clade. This phylogenetic analysis provides strong support for the evergreen species as monophyletic (*N. nitida*, *N. betuloides*, and *N. dombeyi*, BS = 90%, BPP = 1.00). Among the deciduous forms, *N. antarctica* was resolved as a sister to a clade of evergreen species (BS = 98%, BPP = 1.00). Thus, *N. pumilio* probably diverged first from the remaining species in the subgenus. The consensus tree from phylogenetic analyses of cpDNA within *Nothofagus* shows two major divergent haplotypes with high support values, a northern group (H1–H6; BS = 89%, BPP = 0.94, Fig. 2B) and a southern group (H7–H9; BS = 94%, BPP = 0.96). Within the northern group, there are also

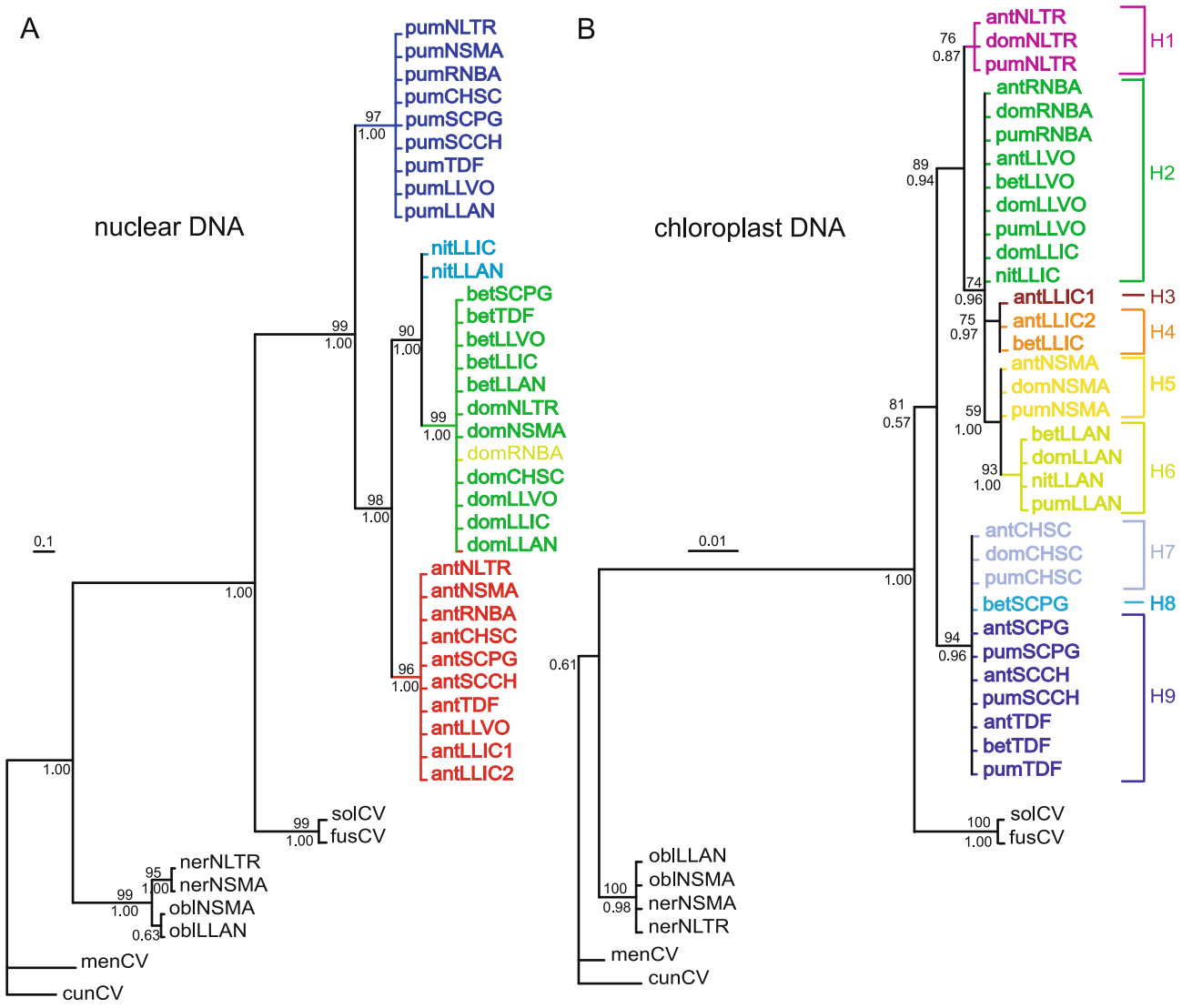

Fig. 2. Phylogenetic trees showing relationships between *Nothofagus* species from analyses of nuclear and chloroplast data sets. Numbers above and below branches indicate bootstrap values for maximum parsimony and posterior probabilities from Bayesian inference analyses, respectively. (A) ITS data. (B) Combined chloroplast data. Different colors indicate the different ribotypes and haplotypes (H) observed. Taxa names are given in Table 1. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

two clades, one consisting of the haplotype shared by the northernmost analyzed population (H1; BS = 76%, BPP = 0.87) and the other formed by the remaining northern haplotypes (H2–H6; BS = 74%, BPP = 0.96). The latter clade is further split into two subclades, one with two haplotypes found on Chiloé Island (H3 and H4, BS = 75%, BPP = 0.97) and the other composed of haplotypes from San Martín de los Andes and Antillanca (H5 and H6; BS = 59%, BPP = 1.00), both of which are derived from the most common northern haplotype, H2. As a result, three haplotypes were observed in Chiloé Island (H2, H3 and H4). In the southern group, there is a unique unresolved clade containing three different haplotypes (H7–H9). The topology of the haplotype network connects all haplotypes into a single network without loops (Fig. 3). In general, the configuration of the haplotype network is consistent with the clades predicted by the cpDNA phylogenetic tree.

Table 2
Summary statistics for the molecular data sets analyzed.

Region	Aligned length (base pairs)	No. parsimony informative characters		Pairwise divergence		No. most parsimonious trees	Length of tree	CI/RI (excluding uninformative characters)	Best fitting model of evolution
		Within *Nothofagus*	Entire data set	Within *Nothofagus*	Entire data set				
ITS	828	22	85	0–2.4%	0–7.7%	4	119	0.857/0.966	GTR + G
*psb*B–*psb*H *trn*L–*trn*F *trn*H–*psb*B	1700	21	76	0–1%	0–4.2%	8	101	0.931/0.984	GTR + I

Abbreviations: CI, consistency index; RI, retention index.

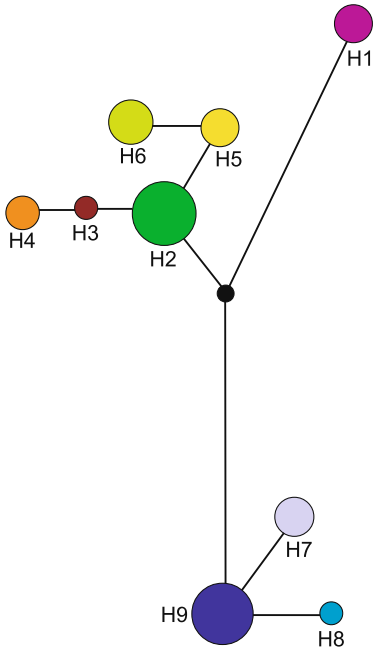

Fig. 3. A median-joining network showing the relationships among chloroplast DNA haplotypes (H) found in 32 analyzed populations within subgenus *Nothofagus*. The black circle indicates a hypothetical mutation that is required to connect existing haplotypes.

4. Discussion

Incongruent nuclear and plastid DNA phylogenetic analyses were observed within subgenus *Nothofagus*. While the distribution of the ITS ribotypes illustrate relationships among the delimited species within subgenus *Nothofagus*, the cpDNA phylogeny is geographically structured. Species-independent geographic distribution of cpDNA haplotypes has been reported in other Fagales (*Alnus* L.: King and Ferris, 2000; *Betula* L.: Thórsson et al., 2001; *Corylus* L.: Palmé and Vendramin, 2002; *Juglans* L.: Potter et al., 2002; *Lithocarpus* Blume: Cannon and Manos, 2003; *Fagus* L.: Okaura and Harada, 2002; *Quercus* L.: Whittemore and Schaal, 1991; Petit et al., 1993; Belahbib et al., 2001; Jiménez et al., 2004).

4.1. Chloroplast capture

Processes that might explain discordances between nuclear and chloroplast phylogenies include convergent evolution, lineage sorting, and reticulate evolution. Sequence convergence would be highly unlikely. The probability that nine haplotypes had occurred simultaneously and independently in 10 distinct areas separated by more than 2000 km, and within two to four different species, is extremely small. Furthermore, many of the observed mutations arose within non-coding regions; it is very unlikely that these would reflect the influence of selection. Also, lineage sorting requires the ancestor of subgenus *Nothofagus* to be polymorphic for all haplotypes, and this stochastic process would not be expected to show the strong geographical partitioning observed here for all chloroplast haplotypes. Chloroplast capture, i.e., where the cytoplasm of one species is replaced by that of another species through hybridization/introgression, is the most likely explanation for the pattern of haplotype sharing observed here (Fehrer et al., 2007). This is mainly due to the high potential for interspecific gene flow in plants (Rieseberg and Soltis, 1991). Chloroplast capture could occur frequently in species with sympatric distribution and reproductive compatibility. In *Nothofagus*, hybridization seems to occur only between species with the same pollen type and is thought to be due to weak reproductive barriers between species (Veblen et al., 1996). Interestingly, it has been suggested that interspecific crosses as well as backcrosses between parents and progeny may be favored when outcrossed pollen is scarce and interspecific flowers outnumber intraspecific flowers (Premoli, 1996a). Natural hybridization among species within the subgenus *Nothofagus* has been recorded between the deciduous species *N. antarctica* and *N. pumilio* (Quiroga et al., 2005), among the evergreen species *N. betuloides*, *N. dombeyi*, and *N. nitida* (Premoli, 1996a,b), and between the deciduous *N. antarctica* and evergreen *N. dombeyi* (Stecconi et al., 2004).

Interspecific pollen competition is one mechanism that plays an important role in controlling the formation of hybrids in several plant groups (Rieseberg, 1995). Hybridization is likely to take place in populations where individuals of one species are in the minority and receive foreign pollen belonging to related taxa. In fact, the minority species will almost inevitably be the female parent of the hybrid. According to this pollen competition scenario, catastrophic geological events (e.g., vulcanism or glaciations) that have affected the geography of Patagonia (Ortiz-Jaureguizar and Cladera, 2006) may have resulted in the survival of a few remaining individuals of a single *Nothofagus* species, which will be the source for postdisturbance colonization (Poole et al., 2001). Some species within the subgenus *Nothofagus* regenerate profusely after large-scale disturbances; this may have distinct genetic consequences depending on whether they establish by seed, as does *N. dombeyi* (Premoli and Kitzberger, 2005) or resprouting, as does *N. antarctica* (Premoli and Steinke, 2008). The latter species is a possible candidate for female parent status because of its widespread distribution, the broad variety of habitat types that it occupies, and its capacity to resprout after disturbance events. If *N. antarctica* individuals receive pollen from distant parental species with which it can hybridize (*N. pumilio* or an evergreen species), such progeny could become hybrid founder populations (Fig. 4, adapted from Rieseberg et al., 1995). Continual introgressions would quickly lead to a majority of individuals containing exclusively *N. antarctica* cpDNAs but mostly *N. pumilio* nuclear genes. As a result, individuals from the hybrid founder population could subsequently colonize a complete region and potentially expand the geographic distribution of the species. Thus, hybridization may facilitate long-distance pollen dispersal (Potts and Reid, 1988) that, in combination with a selective regime favoring each ecologically-distinct *Nothofagus* species, may lead to differential patterns of cytoplasmic and nuclear introgression observed here.

If chloroplast capture occurs among species within the subgenus *Nothofagus*, it would likely involve the entire plastid genome. As a consequence, previous phylogenetic relationships generated from cpDNA data may only reflect geographic relationships (Martin and Dowd, 1993; Setoguchi et al., 1997). In addition, one of the two most parsimonious trees previously published for *Nothofagus* based on ITS nrDNA sequences differs from the tree shown here (Manos, 1997). This is probably due to the fact that the only one sequence for *N. dombeyi* included in the phylogenetic analysis performed by Manos was apparently closer to *N. pumilio*, which in turn was interpreted as evidence of hybridization between these species. We found no ribotype polymorphism for *N. dombeyi*, nor hybrids between *N. dombeyi* and *N. pumilio* have been described so far under natural conditions. Thus, extensive population sampling is needed to avoid erroneous conclusions. We further suggest that nuclear DNA should be used in phylogenetic studies of related species, particularly if chloroplast capture occurs.

4.2. Phylogeny

Our nuclear phylogenetic analysis shows subgenus *Nothofagus* to be a well-supported clade. The monophyletic nature of *Nothofagus* is

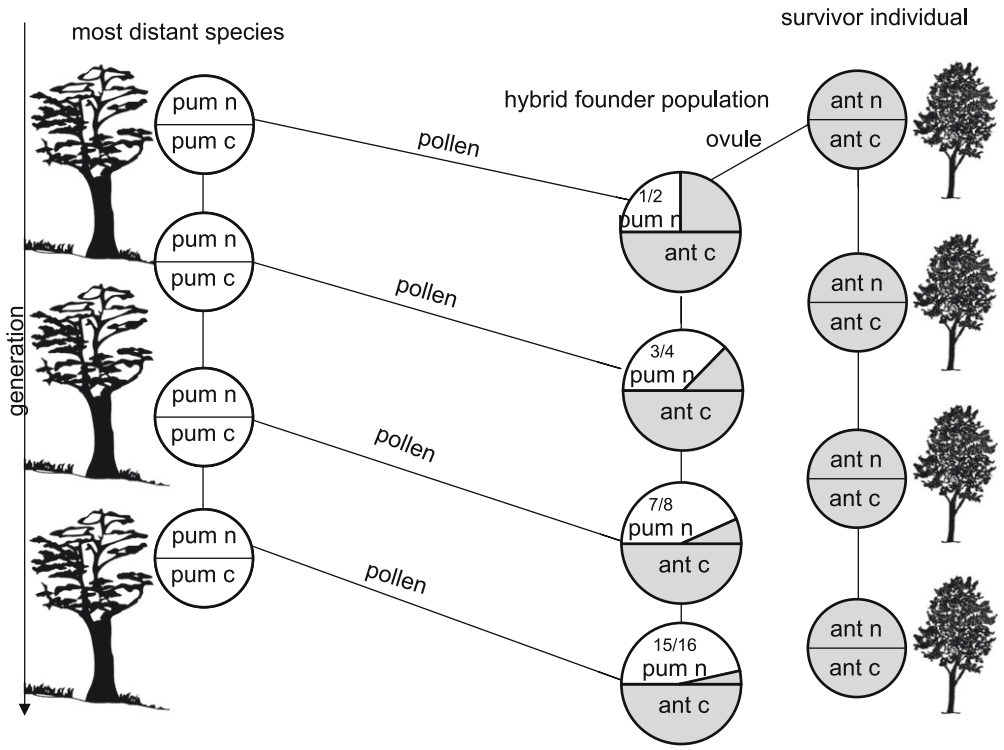

Fig. 4. A hypothetical scenario for cytoplasmic introgression in a population of *Nothofagus* sp. (adapted from Rieseberg et al., 1995). Abbreviations: pum, *N. pumilio*; ant, *N. antarctica*; n, nuclear DNA; c, cytoplasmic DNA.

also supported by shared morphological features, such as cupules with non-glandular lamellae, a tubular male perianth with fewer than 20 stamens, fusca 1 type pollen, leaves with a single unicellular trichome type C, T-pieces of cutin at stomatal poles, and stomata oriented in parallel to the long axis of the leaf. Within subgenus *Nothofagus*, *N. pumilio* diverged first, followed by *N. antarctica*, and then evergreen species *N. betuloides*, *N. dombeyi*, and *N nitida*. This is similar to the result obtained by the strict consensus tree based on the reanalysis of morphology from Hill and Jordan (1993) in Manos (1997), except for the topology of the interspecific relationships among evergreens. Thus, evergreen species are supported as sister taxa, whereas the two deciduous species *N. pumilio* and *N. antarctica* are not. *Nothofagus pumilio*, although widespread, is mostly restricted in habitat and dominates subalpine communities in which prostrate individuals characterize the upper tree line (Premoli, 2003). Hill and Read (1991) divided *Nothofagus* into two groups, with the monotypic section *Pumiliae* containing *N. pumilio* and the section *Nothofagus* containing the rest of the species. *Nothofagus pumilio* has many morphological and molecular autapomorphies that support this separation. The typical arrangement of cupules and flowers found in most cool-temperate *Nothofagus* is a symmetrical, four-valved cupule that encloses a dichasium of three flowers, two lateral tricarpellate and one central bicarpellate. In contrast, *N. pumilio* has two-valved asymmetric cupules bearing a single tricarpellate flower, which signals the loss of the corresponding opposing pair of cupule valves. It is thus unique in having a single trimerous fruit, suggesting that one lateral fruit has survived evolutionary reduction. Furthermore, *N. pumilio* is a distinctive species on the basis of its cuticular morphology, containing two unique trichome types and no glandular trichomes in common with other species (Hill and Jordan, 1993). Finally, based on leaf architectural features, while *N. pumilio* has crenate margins the rest of the species within the subgenus have dentate margins (Gandolfo and Romero, 1992). Even though *N. pumilio* and *N. antarctica* share a deciduous habit, the female reproductive structure, fruit, leaf morphology, and cuticle characters of *N. antarctica* are more closely related to *N. betuloides* and *N. dombeyi* than to *N. pumilio*. This relationship is reflected in our phylogenetic tree. The sister-group relationship between evergreen species is also supported by detailed protein electrophoresis and multivariate analyses of leaf architecture that suggested a close relationship between *N. betuloides* and *N. dombeyi* (Premoli, 1996b). *Nothofagus nitida* may have become restricted to the western slopes of the Andes sometime in the past, and is considered to have a narrow ecological tolerance, growing in Valdivian rainforests where mean annual precipitation is high (Premoli, 1997). The observation of the same ribotype in *N. betuloides* and *N. dombeyi* suggests that either these species have differentiated recently, in response to climatic changes during the Pliocene and Pleistocene (Romero, 1986), or that they are older taxa that have been hybridizing and exchanging chloroplast and nuclear genes for a long period of time (Premoli, 1996b).

Striking species-independent patterns of phylogeographic structure were found within the subgenus *Nothofagus* in southern South America. This can be explained by a long-lasting concordant distribution of most species of the subgenus associated with their spread within South America from austral latitudes (Premoli et al., unpubl.). Local processes of hybridization/introgression/chloroplast capture may have favored their persistence and hence the development of a significant spatial structure throughout their evolutionary history.

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