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Management of Nothofagus genetic resources: Definition of genetic zones based on a combination of nuclear and chloroplast marker data

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ABSTRACT

Development of appropriate forest conservation and management measures for a given tree species includes the identification of distinct genetically homogeneous units across its overall distribution range, which depends on the availability of knowledge on genetic, morphological and adaptive variation. This has important implications for germplasm transfer within and between areas, for example in reforestation or restoration activities. Genetic zones are defined as genetically more or less homogeneous regions within which propagation material can be transferred with relative certainty not to cause changes at the genetic structure level. The main goal of this study was to define genetic zones for *Nothofagus nervosa* and *Nothofagus obliqua* within their Argentinean natural distribution range. A total of 823 individuals belonging to 24 populations (14 of *N. nervosa* and 10 of *N. obliqua*, $\mu = 34 \pm 5$) were genotyped by means of seven nuclear microsatellite markers. The geographic clustering encountered through the application of a Bayesian approach was analyzed in combination with additional information on diversity parameters, chloroplast DNA and isozymes. For assembling the maps showing the distribution of clusters, we averaged cluster membership values of trees in each grid cell. To visualize geographical patterns in nSRR richness and the distribution of different clusters of both species, we carried out grid-based spatial analyses using 30 s grid cells (~1 km at the equator) as the unit of analyses. We distinguished five genetic zones for *N. nervosa* and three for *N. obliqua,* consistent with the management history of the species, and displaying a certain level of geographic congruence. Higher allelic richness values were found in the surroundings of areas identified as potential glacial refugia, which suggest that the distribution pattern of allelic richness is closely associated with the glacial history of the species within the region. We expect this information to constitute a valuable tool for the identification of seed transfer zones, and to guide recruitment and plantation activities in the context of domestication programs of both species in Argentina. For assisted migration programs under progressive climate change the identification of these zones together with knowledge concerning hotspots at genetic diversity level should constitute an input for planning the activities.

1. Introduction

1.1. Management units: definition and considerations

Development of appropriate forest conservation and management measures for a given tree species includes the identification of distinct genetically homogeneous zones across its overall distribution range on the basis of available information on molecular and phenotypic variation within and between populations. Such management units are pivotal for steering the decision-making process concerning germplasm transfer within and between areas, for example in reforestation or restoration activities (Newton et al., 1999). Over the last years, many concepts have been put forward to define management units (e.g. Fraser and Bernatchez, 2001; Sáenz-Romero et al., 2003; de Guia and Saitoh, 2007; Lefèvre et al., 2012). The first definition was coined by Ryder (1986) who described evolutionary significant units (ESUs) as 'populations possessing genetic attributes significant for present and future generations of the species in question'. Later, Moritz (1994) defined management units as 'populations with significant divergence of allele frequencies at nuclear or mitochondrial loci – regardless of their phylogenetic distinctiveness', which are therefore, the most appropriate units for population monitoring and demographic studies.

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Meanwhile, Crandall et al. (2000) highlighted the importance to preserve adaptive traits associated with their ecological implications. A similar concept commonly used among forest practitioners is 'seed zones'. Seed zones are restricted geographic areas delineated to allow for the selection of adequate planting stock which is best matched to particular target sites such that it minimizes the loss of productivity and forest health due to maladaptation (Hamman et al., 2011).

Although all the previous concepts can be useful in a variety of contexts, here we prefer to use the notion of genetic zones for their relatively simple and probably less controversial nature. Genetic zones are defined as genetically homogeneous regions within which propagation material can be transferred, minimizing the occurrence of changes at the level of genetic structure (e.g. Honjo et al., 2009; Pastorino and Gallo, 2009). Identification of genetic zones can be highly informative for definition of management units, as they reflect a population of interbreeding trees adapted to geographically restricted areas.

Genetic analyses based on molecular markers and adaptive traits are useful tools for identifying and assessing genetic zones, and hence also management units (e.g. Hogbin and Peakall, 1999; Honjo et al., 2009; Pastorino and Gallo, 2009). Neutral molecular markers have been used successfully to define management units and/or genetic zones for several species (e.g. Honjo et al., 2009; Bucci and Vendramin, 2000; Derory et al., 2002). However, it is increasingly being recognized that better definitions can generally be obtained from the combined use of different genetic markers (Bucci et al., 2007; Cavers et al., 2003). In addition, it is advisable to further complement molecular information with other types of data (Hogbin and Peakall, 1999). For example, Pastorino and Gallo (2009) combined information concerning the geographical setting of populations and historical processes like glaciations with genetic data, to identify genetic management units for the South American conifer species *Austrocedrus chilensis*. Based on divergences found at organellar and nuclear DNA, Cavers et al. (2003) uncovered two evolutionary significant units (ESUs) in Costa Rican populations of *Cedrela odorata*, which corresponded to two morphologically, physiologically and molecularly distinct ecotypes defined previously (xeric and humid). González-Martínez et al. (2004) argued for the need to base the original identification of management units (MUs) proposed by Newton et al. (1999) for *Pinus pinaster* on the conjunction of molecular and adaptive traits.

1.2. The species of interest

Nothofagus nervosa (Phil) Dim. et Mil. and *Nothofagus obliqua* (Mirb.) Oerst are wind-pollinated, outcrossing, anemochorous, deciduous forest trees, representing two of the most ecologically and economically important species of the genus in the southern South American temperate forests. Natural hybridization between *N. obliqua* and *N. nervosa* is known to occur (Donoso et al., 2004), especially within sympatric altitudinal areas where both species co-occur (Gallo, 2004). Even more, the role of hybridization in the long-term maintenance of intra-specific variation has been highlighted (Gallo, 2004). In isozyme analyses, species-specific alleles have been described as diagnostic markers of hybridization (Gallo et al., 1997; Marchelli and Gallo, 2000).

Both species occur naturally between 33° and 40°S along the Pacific coastal mountains and the longitudinal valleys in Chile, and on both sides of the Andes Mountains, in Chile and Argentina, respectively. Both species produce valuable wood for furniture and structural uses, which has resulted in over-exploitation in the past and drastically reduced the remaining populations, particularly in Chile (Donoso and Lara, 1995). In Argentina, where the species occur between 36° and 40° S, protected areas have been established on the basis of ecological factors, and currently most of the natural distribution of both species is located within Lanin National Park Administration area (Sabatier et al., 2011), where different protection levels exist. Political decisions about the protection status of some areas have recently been taken, based on the results of genetic variation studies (Gallo et al., 2009). In addition, genetic considerations have recently been integrated in a 'best practices' manual for forest stand management of northern Patagonian forests (Chauchard et al., 2012).

Previous studies based on cpDNA markers and palinological and paleo-geographic information have provided evidence about the regional glacial history of both species (Marchelli et al., 1998; Marchelli and Gallo, 2006; Azpilicueta et al., 2009). The detection of a clear latitudinal pattern in the haplotypic diversity allowed the identification of at least two different glacial refugia for each of the species. In addition, along the continuous forests growing at the south (Lácar watershed), a colonization route coming from the west was inferred for *N. nervosa*, while an eastern refugia area was proposed for *N. obliqua*. The highest level of genetic diversity was observed at the northern distribution range in the case of *N. obliqua*, and at the western populations for *N. nervosa*. These results were also confirmed with isozyme markers (Marchelli and Gallo, 2000, 2001, 2004; Azpilicueta and Gallo, 2009).

As is the case in most countries from the southern hemisphere, commercial forestry in Patagonian Argentina involves primarily exotic conifer species (mainly *Pinus ponderosa* (Dougl.) Laws and *Pseudotsuga menziesii* (Mirb.) Franco), in spite of the fact that native species have higher quality wood. Recently, a domestication and breeding program has been initiated in Argentina to reduce the impact of logging on native forests through the introduction of native species into commercial plantation forestry, including *N. nervosa* and *N. obliqua*. For this purpose, it is imperative to define genetic zones within which safe transfer of seeds/seedlings is guaranteed. Plantation and restoration activities with native species are currently being highly promoted in Argentina through a new legislation[1] which aims at the conservation, restoration and sustainable management of native forests.

Vergara (2000) identified and characterized 14 provenances for the entire natural range in Chile and Argentina of both *N. nervosa* and *N. obliqua* taking together. This identification was predominantly based on climatic and geographic information, including the natural range of the species considering their phytogeographic limits, and only to minor extent on genecological variation (described by Donoso, 1979). The 11 provenances identified for the Chilean forests of *N. nervosa* and *N. obliqua*, which occupy an extensive latitudinal gradient (Vergara, 2000) have a clear practical utility for management and conservation programs (Gutiérrez, 2003). By contrast, the three main provenance regions identified for the corresponding Argentinean forests are less satisfactory, considering the high fragmentation and spatial heterogeneity of these forests. Only relatively few practical outcomes can be derived from these provenances for Argentinean forest management.

Our main goal here is to define, for the first time, genetic zones for *N. nervosa* and *N. obliqua* within their Argentinean natural distribution range. To this end, we carried out a molecular marker study based on nuclear microsatellites and combined and compared the results with previous findings based on isozymes (Marchelli and Gallo, 2000, 2001, 2004; Azpilicueta and Gallo, 2009) and chloroplast DNA (cpDNA) (Marchelli et al., 1998; Marchelli and Gallo, 2006; Azpilicueta et al., 2009). We expected that the high level of polymorphism of microsatellite markers would allow for an unambiguous discrimination between genetic zones, with a higher level of detail as compared to cpDNA and isozymes.

[1] http://www.ambiente.gov.ar/archivos/web/SUBordenamiento/file/ley_26331_presupuestos_minimos_bosques_nativos.pdf.

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Information concerning genetic zones will constitute a valuable tool to adequately plan management interventions in ways that preserve the genetic structure of Argentinean *N. nervosa* and *N. obliqua* populations, especially within national parks, and therefore, to maintain their adaptive variation. Genetically homogenous management units will additionally be valuable for the certification of seedlots and breeding activities (Bucci and Vendramin, 2000), considering that nowadays seed production areas exist for each of these species at Lácar watershed within Lanin National Park (Azpilicueta et al., 2010). Our research is also expected to generate useful information for designing *in situ* gene conservation units.

2. Materials and methods

2.1. Plant material and DNA analysis

We sampled a total of 749 adult trees and 74 nursery seedlings belonging to 24 populations (14 of *N. nervosa* with 486 individuals and 10 of *N. obliqua* with 337 individuals; 34.3 ± 5.0 individuals per population on average) across the species natural range in Argentina (Table 1). To prevent collecting closely related individuals, a minimum distance of 50 m between sampled trees was maintained. Total DNA was extracted from leaf or bud tissue following the procedure described in Dumolin et al. (1995). Seven nuclear microsatellite (nSSR) loci, developed for the genus (Azpilicueta et al., 2004; Marchelli et al., 2008; Soliani et al., 2010), were selected based on their high polymorphism, and amplified using labeled primers (NnBIO11, NgBIO14, NnBIO111, and NnBIO37) or M13 methodology (Schuelke, 2000) (Npum9, Npum14 and Oak64). The polymerase chain reaction (PCR) was carried out in a total volume of 15 μl, using 1X Green Buffer and Go Taq for all primers, except for primer NgBIO11 which was amplified with Taq Platinum (Invitrogen). In all cases we used ~10 ng of template DNA per reaction and PCR conditions as described in Azpilicueta et al. (2004), Marchelli et al. (2008) and Soliani et al. (2010), with minor modifications. For amplifications we used either a BIORAD or a BIOMETRA Thermal Cycler with the following program: 4 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at the annealing temperature and 1 min at 72 °C, followed by a final elongation of 30 min at 72 °C. PCR products were visualized on 2% agarose gel stained with Sybr Safe (Invitrogen). Individual genotyping was done using an ABIPRISM 3700 capillary sequencer and allele assignment with *GeneMapper* v.3.7 (Applied Biosystem).

2.2. Data analysis

On the basis of our nSSR analysis, genetic characterization at species and population level was done using the number of alleles (*NAs*), genetic diversity *v* (Gregorius, 1978), observed and expected heterozygosities (H_O and H_E respectively) (Nei, 1973). Genetic differentiation was quantified by means of both fixation indexes at population level F_{ST} (Wright, 1951) and R_{ST} (Slatkin, 1995) assuming a step-wise mutation model and considering the limitations of F_{ST} (Gregorius and Roberds, 1986), especially when working with highly polymorphic markers (Jost, 2008). Given that genetic parameters of allele numbers are more adequate for conservation purposes (El Mousadik and Petit, 1996), we calculated allelic richness corrected by rarefaction (Hurlbert, 1971). Number of exclusive alleles are also provided. Calculations were done with FSTAT v.2.9.3 (Goudet, 2001) and GenAlEx (Peakall and Smouse, 2006). Fixation (*F*) was additionally estimated with *GDA_NT* (Degen, unpublished) using a permutation test (1000 permutations) for estimating probability values.

The presence of null alleles at population level and at each analyzed *locus* was tested with Micro Checker (van Oosterhout et al., 2004). The predicted values for allele frequencies were estimated using *FreeNA* (Chapuis and Estoup, 2007). As F_{ST} may be overestimated in the case of null alleles (Chapuis and Estoup, 2007), its potential effect on genetic differentiation was evaluated by calculating F_{ST} values using the 'excluding null allele' (ENA) method by Chapuis and Estoup (2007) implemented in *FreeNA*.

To detect population structure and infer genetic clusters of individuals, we used two alternative methods implemented in STRUCTURE (Pritchard et al., 2000) and BAPS (Corander et al., 2003). Both methods are based on Bayesian statistics with differences in the inclusion or not of geographic information. STRUCTURE assigns individuals probabilistically (data set of multi-locus genotypes) to K assumed populations/clusters, characterized by a set of allele frequencies at each locus – with no prior knowledge of the geographical locations (Pritchard et al., 2000). By contrast, BAPS (Bayesian Analysis of Genetic Population Structure) is conditioned on the geographical location of pre-assigned groups of individuals (Corander et al., 2003), as those given in Table 1 in this paper. Because STRUCTURE failed to detect a clear clustering within both species, here we only present the results obtained with BAPS. We complemented the clustering with chloroplast DNA (Marchelli et al., 1998; Marchelli and Gallo, 2006; Azpilicueta et al., 2009) and isozyme information (Marchelli and Gallo, 2001, 2004; Azpilicueta and Gallo, 2009) obtained from the same populations. Additionally, information about historical management practices within National Parks, such as logging activities, was taken into account for identifying the clusters. Genetic differentiation among the identified clusters was further tested with a nested AMOVA using GenAlEx (Peakall and Smouse, 2006). Each zone was then characterized on the basis of *NA* (total number of alleles), r_g (allelic richness) and H_E (Nei diversity) parameters, including number of *EA* (exclusive alleles) detected. The chloroplast maternal lineage identified in previous studies complemented the information provided at cluster level.

As nursery material and adults collected in the field have different selection histories, pooling both together may lead to erroneous interpretations of observations resulting from genetic analysis. In order to avoid this we checked the existence of a genetic bottleneck within the nursery material running BOTTLENECK (Cornuet and Luikart, 1996) assuming the stepwise mutation model.

To visualize geographical patterns in nSRR richness and the distribution of different clusters of both species, we carried out grid-based spatial analyses at a resolution of 30 s (~1 km at the equator). To obtain sufficient and more evenly distributed data points for constructing high resolution maps, we constructed circular neighborhoods of 5 min diameter (~10 km at the equator) around the locations of all trees sampled, following van Zonneveld et al. (2012) and Thomas et al. (2012). With this we assume that each sampled tree is representative for a circular area with a diameter of 5 min around it. Consequently, each tree was replicated in all 30 s grid cells contained in a circular area of 5 min diameter around its location. This diameter was chosen to allow comparison of the genetic profile of adjacent populations and hence detect spatial patterns at landscape level across the species distributions. Maps of allelic richness were corrected by rarefaction to a minimum sample size of 14 and 19 trees per grid cell for *N. nervosa* and *N. obliqua*, respectively. For assembling the maps showing the distribution of clusters, we averaged cluster membership values of trees in each grid cell. Only mean membership values of at least 0.5 are displayed on the maps to improve visibility. Circular neighborhoods were constructed in R statistical package version 2.14 (R Development Core Team, 2011) with Raster package (Hijmans and van Etter, 2012), and rarefaction was performed using HP-rare (Kalinowski, 2005). All maps were edited in ArcMap 10.

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Table 1
List of the analyzed populations: geographic location and genetic parameters at population level are presented.

Species	Watershed	Population	ID	Latitude	Longitude	Altitude (m asl)	N	*NA*	*v*	r_g	H_O	H_E	*F*	*EA*	*BAPS cluster*	*PML*
Nothofagus nervosa	Espejo	Espejo chico	E1	40° 34′ 48″	71° 43′ 12″	1000	30	29	1.893	27.86	0.422	0.472	0.153*	2 (1)	5	1
	Lácar	Bandurrias	L1	40° 09′ 00″	71° 21′ 00″	980	35	23	1.713	20.79	0.319	0.416	0.194*	0	2	1
		Yuco	L2	40° 09′ 07″	71° 30′ 39″	930	31	32	1.940	27.61	0.497	0.485	0.056	2 (2)	4	1
		Quilanlahue	L3	40° 08′ 18″	71° 28′ 04″	913	31	31	1.900	27.13	0.379	0.474	0.246*	0	4	1
		Quechuquina	L4	40° 10′ 12″	71° 40′ 12″	900	25	33	1.897	29.13	0.417	0.473	0.095	0	4	–
		Cerro Malo	L5	40° 08′ 17″	71° 37′ 36″	1000	44	36	1.861	27.93	0.403	0.463	0.105	0	4	–
		Hua Hum	L6	40° 07′ 55″	71° 40′ 02″	940	50	36	1.880	28.53	0.450	0.468	0.053	1	4	1
		Quila Quina	L7	40° 10′ 40″	71° 26′ 37″	980	35	29	1.804	25.96	0.372	0.446	0.183*	0	4	–
	Lolog	Puerto Arturo	Lo1	40° 01′ 12″	71° 22′ 48″	850	34	31	1.878	26.93	0.372	0.467	0.202*	0	1	1
		Boquete	Lo2	40° 01′ 12″	71° 35′ 24″	720	36	34	1.801	27.24	0.384	0.445	0.095	1	4	1
	Curruhue–Huechulafquen	Curruhue	C1	39° 51′ 00″	71° 29′ 26″	970	35	31	1.780	26.56	0.382	0.438	0.110	1	4	1
		Lanin	C2	39° 42′ 16″	71° 34′ 15″	970	36	28	1.892	25.44	0.418	0.471	0.117	0	1	2
		Paimún	C3	39° 45′ 00″	71° 37′ 48″	970	29	27	1.666	–	0.399	0.400	–	0	1	1
	Tromen	Tromen	T1	39° 36′ 00″	71° 19′ 48″	1100	35	33	1.681	28.84	0.395	0.405	0.043	2 (1)	3	2
Nothofagus obliqua	Lácar	Bandurrias	L1	40° 09′ 00″	71° 21′ 00″	850	35	32	1.879	29.33	0.281	0.468	0.250*	4 (4)	2	1
		Yuco[a]	L2	40° 09′ 07″	71° 30′ 39″	930	36	23	1.770	21.70	0.290	0.435	0.233*	0	3	1
		Quilanlahue	L3	40° 08′ 18″	71° 28′ 04″	913	30	30	1.838	29.11	0.267	0.456	0.355*	1 (1)	4	–
		Nonthué[a]	L4	40° 08′ 46″	71° 37′ 03″	680	38	31	1.856	28.32	0.299	0.461	0.199*	2 (2)	4	1
		Hua Hum	L5	40° 07′ 55″	71° 40′ 02″	670	35	30	1.714	25.86	0.246	0.417	0.240*	0	4	1
		Quila Quina	L6	40° 10′ 40″	71° 26′ 37″	983	34	26	1.710	24.06	0.226	0.415	0.315*	0	4	1
	Quillén	Corral Bueyes	Q1	39° 22′ 16″	71° 17′ 31″	1140	34	31	1.857	29.61	0.320	0.462	0.174	1 (1)	1	2
	Ñorquinco	Seccional	Ñ1	39° 09′ 11″	71° 15′ 03″	1071	35	32	1.847	29.70	0.276	0.459	0.229*	2 (1)	1	2
	Aluminé	Pilolil	P1	39° 30′ 05″	70° 57′ 44″	836	31	29	1.860	27.04	0.267	0.462	0.286*	0	1	2
	Epulauquen	Epulauquen	E1	36° 49′ 09″	71° 04′ 07″	1000	29	34	2.088	32.33	0.380	0.521	0.183*	5 (2)	1	2

N = sample size (number of diploid individuals).
NA = total number of alleles.
v = gene allelic diversity (Gregorius, 1978).
r_g = allelic richness (El Mousadik and Petit, 1996 from Hurlbert, 1971) (g = 28 for *N. nervosa* excluding Paimún population based on its proportion of missing values and g = 38 for *N. obliqua*).
H_O = observed heterozygosity.
H_E = expected heterozygosity or Nei diversity (Nei, 1973).
F = fixation index
EA = number of exclusive alleles at population level within the species, shared alleles with the other species is presented between brackets.
BAPS clusters: each number indicates the cluster to which each population belongs to, based on the Bayesian Analysis of Genetic Population Structure (BAPS program).
PML: principal maternal lineage based on main cpDNA haplotype found.
[a] Individuals analyzed from tissue belonging to seedlings at the nursery coming from seed pools harvested in the population from a theoretical number of 40–50 mother trees.
* Statistical significant differences (α = 0.05).

3. Results and discussion

3.1. Genetic variation at species and cluster level

Our set of microsatellites detected a total number of 55 and 57 alleles for *N. nervosa* and *N. obliqua*, respectively, with 46 alleles shared between both species. Only 9 and 11 alleles were found exclusively in respectively *N. nervosa* and *N. obliqua*; *N. nervosa* showed values of 0.401 and 0.452 for H_O and H_E, respectively whereas *N. obliqua* exhibited values of 0.285 and 0.455. The differentiation at species level was explained by an F_{ST} value of 0.061 and 0.049 for *N. nervosa* and *N. obliqua*, respectively. Differentiation measured by R_{ST} showed lower values ($R_{STnervosa}$ = 0.023 and $R_{STobliqua}$ = 0.026) (Table 1). For means of comparison, microsatellite analyses in two other American *Nothofagus* species showed higher R_{ST} values (0.112 and 0.062 in *N. pumilio* and *N. antarctica*, respectively), which is probably related with their higher distributional range (Soliani, 2012).

No evidence of a potential genetic bottleneck was found in the nursery samples. Null alleles were detected at four loci (NnBIO11; NnBIO37; NnBIO111 and Npum9) at low frequencies (except for locus NnBIO11 in both species). Notwithstanding, the F_{ST} value (0.061 and 0.049, for *N. nervosa* and *N. obliqua*, respectively) was not significantly different from the corrected-F_{ST} (0.056 and 0.047 for *N. nervosa* and *N. obliqua*, respectively) indicating that null alleles were not strongly biasing the differentiation index.

Bayesian cluster analysis carried out with BAPS identified five and four distinct groups in *N. nervosa* and *N. obliqua*, respectively (Table 1). Analysis of molecular variance revealed 9% and 4% ($p < 0.001$ in both cases) of between group variation and 4% and 3% within group variation for *N. nervosa* and *N. obliqua*, respectively (Table 2). In the following we validate and discuss the clusters identified in light of available information on maternal lineages (cpDNA), isozyme markers and management history of both species to achieve a more comprehensive description of their genetic zones.

3.1.1. N. nervosa genetic zones

Three of the five clusters identified for *N. nervosa* are represented by a unique peripheral population (cluster 2 easternmost population, cluster 3 northernmost population and cluster 5 southernmost population; Fig. 1). Cluster 1 is represented by marginal populations towards the east and northwest. By contrast, cluster 4 has a more continuous distribution in the central area of the species distribution range in Argentina, where the most genetically diverse populations are located, based both on previous (Marchelli et al., 1998; Marchelli and Gallo, 2000, 2001, 2004, 2006) and present analyses (Table 1, Fig. 2). The local diversity hotspot at Hua Hum population (L6) reinforces its recently changed conservation status, from management unit to protected area (Gallo et al., 2009). Current patterns in genetic diversity of *N. nervosa* southern forests grouped in cluster 4 are probably a footprint of the species'

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Table 2
Nested AMOVA for *N. nervosa* (upper part) and *N. obliqua* (lower part) groups for the clustering level detected by BAPS.

Source	df	SS	MS	Est. Var.	%	Stat.	Value	Prob
Among clusters	4	147.576	36.894	0.375	9	Φ_{RT}	0.089	0.001
Among Pop/Clusters	9	89.683	9.965	0.180	4	Φ_{PR}	0.047	0.001
Indiv/Within Pop	472	1720.018	3.644	3.644	87	Φ_{PT}	0.132	0.001
Total	485	1957.278		4.200	100			
Among clusters	3	102.704	34.235	0.290	4	Φ_{RT}	0.044	0.001
Among Pop/Clusters	6	73.662	12.277	0.187	3	Φ_{PR}	0.030	0.001
Indiv/Within Pop	327	1989.139	6.083	6.083	93	Φ_{PT}	0.073	0.001
Total	336	2165.504		6.559	100			

df: degrees of freedom; SS: sum of squares; MS: mean square; Est. Var.: estimated variance; %: percentage of the estimated variance; Φ_{RT}: differentiation among defined clusters; Φ_{PR}: differentiation among populations within clusters; and Φ_{PT}: differentiation among individuals within populations.

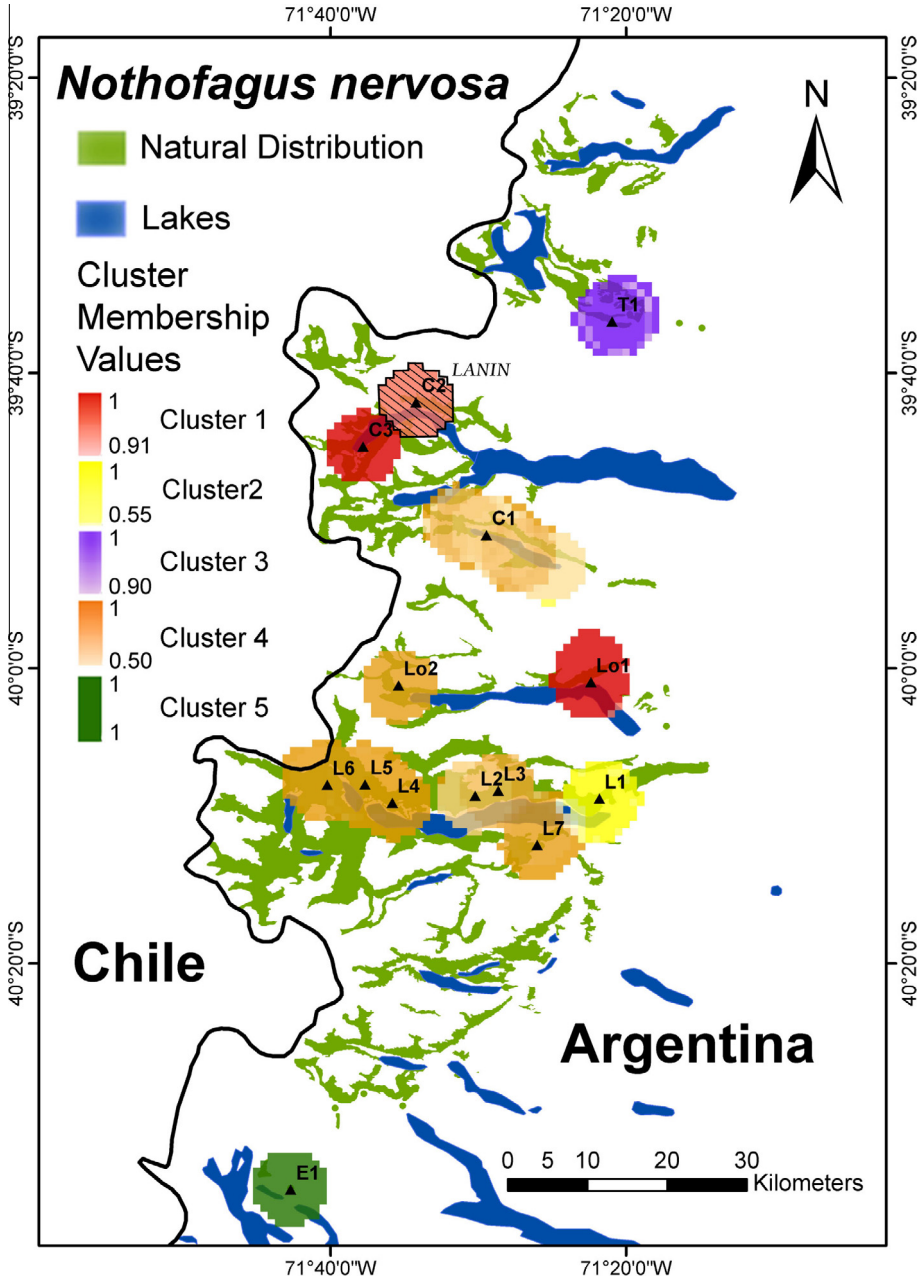

Fig. 1. Distribution of clusters of *Nothofagus nervosa*, based on nuclear microsatellite markers. The population of Lanin (hatched) is considered to be a sub-cluster of cluster 1, based on the findings of a previous study of chloroplast DNA (Marchelli and Gallo, 2006).

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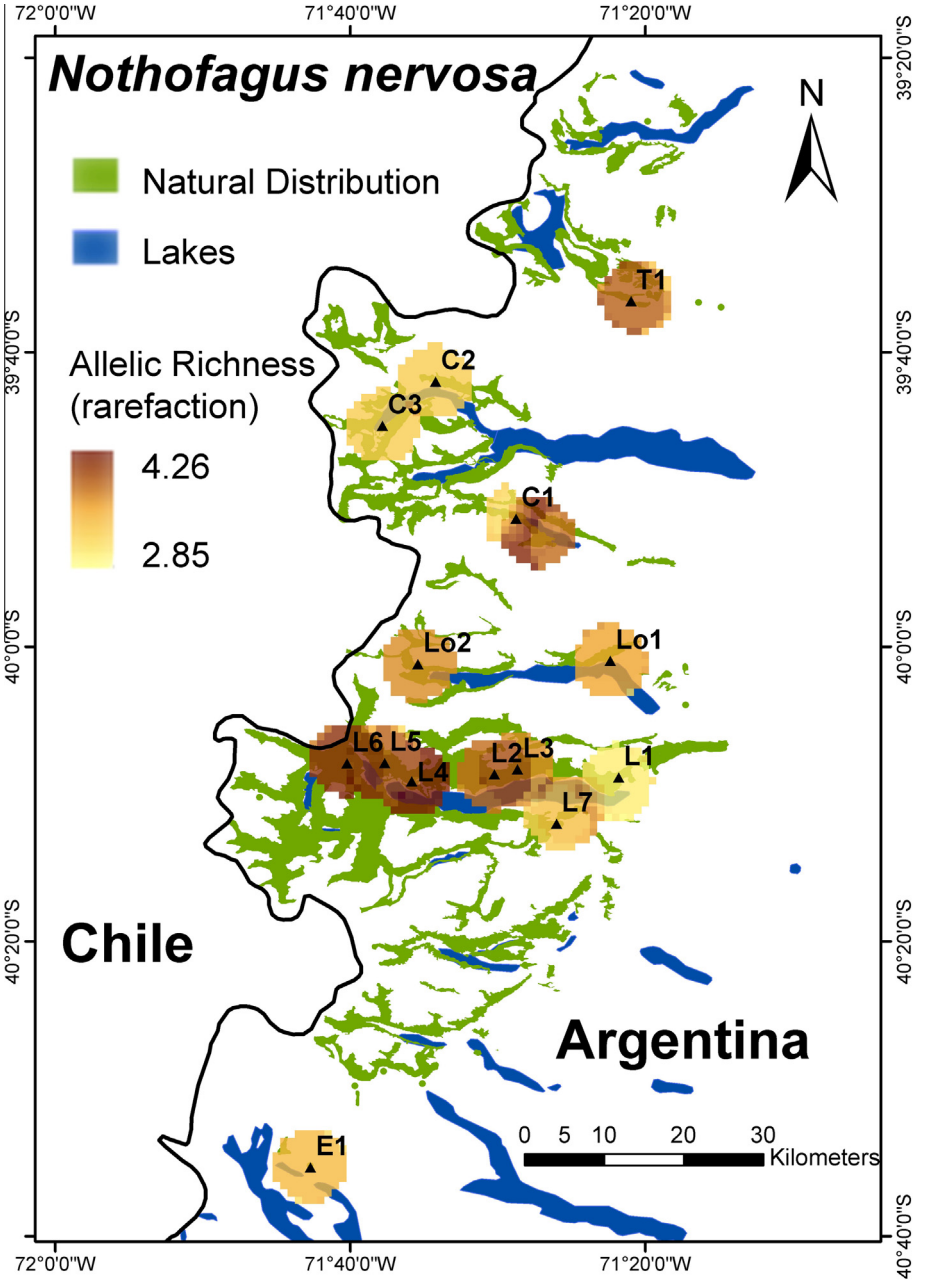

Fig. 2. Spatial variation of nSSR allelic richness of *Nothofagus nervosa*, corrected by rarefaction ($g = 28$) and represented at a resolution of 30 s grid cells and a circular neighborhood of 5 min.

post-glacial re-colonization (Marchelli and Gallo, 2006), whereby western relict populations acted as source material for eastward range expansion after ice-retraction. Because half of the exclusive alleles detected in *N. nervosa* were also found in *N. obliqua*, a high rate of hybridization may be taking place in these watersheds where both species co-occur.

The eastern-most population, located at Lacar watershed, was separated in a single group (cluster 2), probably based on its low diversity and the occurrence of hybridization with *N. obliqua* (Marchelli and Gallo, 2001, 2004). Finally, the populations at the northern and southern edges of the natural distribution range in Argentina (Tromen T1 and Espejo E1, respectively) clustered in geographically discrete groups, representing each the northern and southern haplotype of *N. nervosa*, respectively (Marchelli and Gallo, 2006) (Fig. 1; Table 3). Tromen population (T1), localized at the northern extreme of the species distribution is highly differentiated, and may represent a rear edge population (Hampe and Petit, 2005; Eckert et al., 2008) of the northern maternal lineage (PML = 2; Table 3). Rear edge populations are considered 'long-term stores of the species genetic diversity' (Hampe and Petit, 2005) and therefore, highly important for understanding past and ongoing effects over species survival. Meanwhile, Espejo (E1) could represent a rear edge population of a previous extended distribution at higher latitudes (Auer, 1958). On the other hand, it could also be part of a leading edge of a southern expansion from lower latitudes refuges. We do not have enough evidence to discern between one or the other hypothesis.

The distribution of the five clusters of *N. nervosa* (Fig. 1, Table 3) does not entirely correspond with the lineages obtained with chloroplast DNA, which divide the populations in two main groups

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Table 3
Genetic characterization of the genetic zones defined at each species and principal maternal lineage (PML) assigned by the occurrence of cpDNA haplotype.

Species	Genetic zone	Pop/Cluster	*NA*	r_g	*EA*	H_E	*PML*
N. nervosa	1	**C2**-C3-Lo1	38	28.61	0	0.477	1–2
	2	L1	23	21.07	0	0.416	1
	3	T1	33	28.49	2 (1)	0.405	2
	4	L2-L3-L4-L5-L6-L7-Lo2-C1	49	29.21	8 (4)	0.477	1
	5	E1	29	26.49	2 (1)	0.472	1
N. obliqua	1	**E1**-Q1-Ñ1-P1	46	34.85	12 (6)	0.497	2
	2	L1	32	30.33	4 (4)	0.478	1
	3	L2-L3-L4-L5-L6	41	30.03	4 (3)	0.462	1

Cluster: indicates the cluster each population belongs to, based on the Bayesian Analysis of Genetic Population Structure (BAPS program).
NA = total number of alleles.
r_g = Allelic richness (El Mousadik and Petit, 1996 from Hurlbert, 1971) (g = 30 for *N. nervosa* and g = 46 for *N. obliqua*).
EA = number of exclusive alleles at population level within the species, shared alleles with the other species is presented between brackets.
H_E = expected heterozygosity or Nei diversity (Nei, 1973).
PML = principal maternal lineage based on cpDNA (Marchelli et al., 1998; Marchelli and Gallo, 2006; Azpilicueta et al., 2009).
Highlighted with bold type, the populations defined as sub-groups within their own cluster based on their special characteristics.

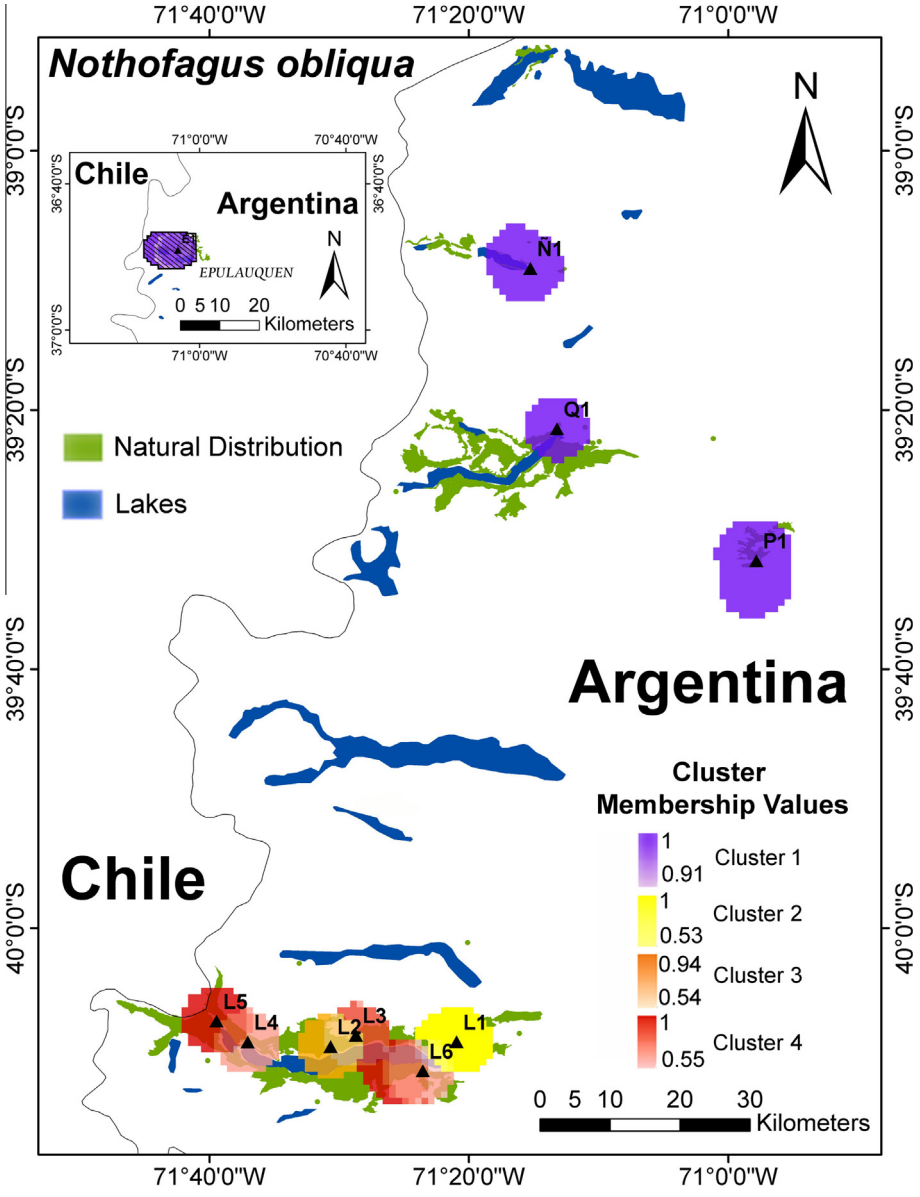

Fig. 3. Distribution of clusters of *Nothofagus obliqua*, based on nuclear microsatellite markers. The northernmost population of Epulauquen (hatched) is considered to be a sub-cluster of cluster 1, based on the findings of a previous study of chloroplast DNA (Azpilicueta et al., 2009).

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(northern and southern lineages) presented as principal maternal lineage PML in Table 1 (Marchelli and Gallo, 2006). Four nSSR clusters are composed exclusively of populations of one maternal lineage (clusters 2, 4 and 5 PML = 1 and cluster 3 PML = 2; Table 1), whereas cluster 1 displays a mixed composition (PML 1 and 2). The two populations in this cluster that are totally differentiated at chloroplast – but not at nuclear level (C2 and C3) (Milleron et al., 2008) are geographically close, although lava sediments lay between them which most likely acted as a physical barrier during historical colonization processes. This could mean that the maternal lineages were partially erased by historically recent (about 2700 BP, Lara et al., 2004) and contemporary pollen flow. Hence, based on its glacial history, we consider the *N. nervosa* population at Lanin (C2) should be treated separately, as a sub-group within its cluster (hatched area in Fig. 1; Table 3).

Finally, five distinct genetic zones were found within the Argentinean distribution range of *N. nervosa*, including one with a nested sub-zone (Table 3). Therefore, the present study allowed the identification of distinct zones within the unique provenance region defined previously by Vergara (2000). It is expected that this new zonation will allow a more appropriate conservation and management of the species.

3.1.2. N. obliqua genetic zones

The populations of *N. obliqua* were grouped in four clusters (Fig. 3). The four northern watersheds (Epulauquen, Ñorquinco, Quillén and Aluminé) clustered in one unique group (cluster 1), while the forests at Lácar lake, the southernmost watershed of the Argentinean distribution of *N. obliqua*, were sub-divided in three different clusters, two of them being composed of a single population. The northern populations included in cluster 1 share haplotype 2, whereas haplotype 1 is common to all populations of the southern clusters (2, 3 and 4; Table 1). The variation found between these two main chloroplast groups explains 89% of the total variation (Azpilicueta et al., 2009), coinciding also with two of the provenance regions defined by Vergara (2000). Allelic richness of populations belonging to the northern group is generally higher than for the southern populations of the Lacar watershed, but com-

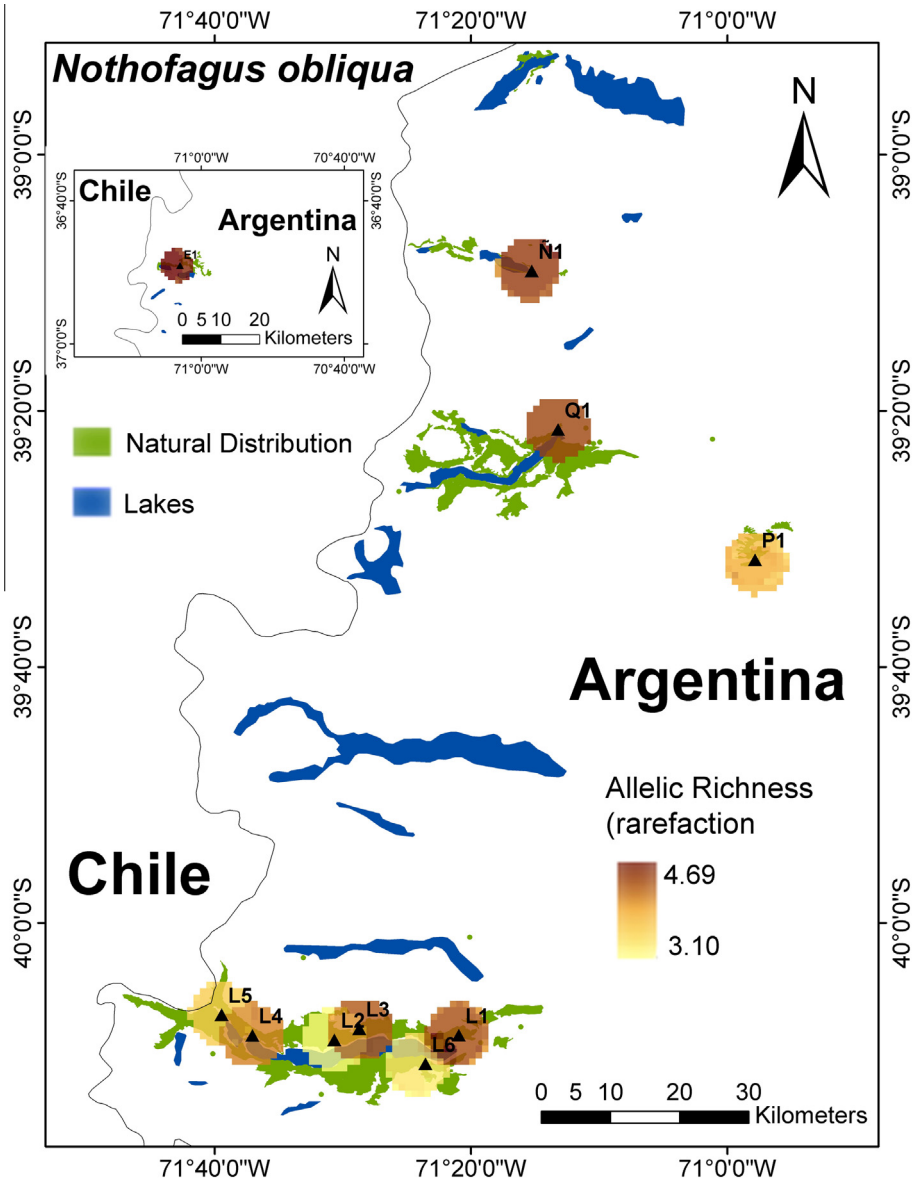

Fig. 4. Spatial variation of nSSR allelic richness of *Nothofagus obliqua*, corrected by rarefaction ($g = 38$) and represented at a resolution of 30 s grid cells and a circular neighborhood of 5 min.

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parably high values were registered for the Bandurrias (L1) population (up to 4.44 alleles per locus and per grid cell; Fig. 4) at the east side of the southern distribution area.

These observations are furthermore corroborated by the results of a previous study based on isozyme markers (Azpilicueta and Gallo, 2009), which yielded a similar percentage of differentiation as obtained for the microsatellites markers used in the present study. In conformity with Ryder's (1986) definition of ESUs, these two distinct groups could represent ESUs for the species. Restriction of their respective source populations in northern and southern refugia during the last glacial period may be at the origin of their distinct evolutionary histories (Azpilicueta et al., 2009).

Within the southern part of the species'distribution range, we identified three clusters. The Bandurrias population (L1) is located east of Lácar lake, and can be distinguished as a single cluster (cluster 2), exhibiting a high number of exclusive alleles within the species, all of which are shared with *N. nervosa* (Table 1), suggesting, a high level of inter-specific hybridization between both species. Elevation gradients in this area are reduced (González Peñalba, pers. com), probably softening the genetic barrier between both species which is known to be conditioned by a pronounced altitudinal gradient and reflected in altitudinal differences in reproductive phenology (Gallo, 2002). The probable occurrence of mostly unidirectional pollen flow could be the reason why this pattern of introgression was not observed in the *N. nervosa* Bandurrias population. The Yuco population (L2), located at the center of Lácar watershed, was identified as a distinct group (cluster 3). However, of all the *N. obliqua* populations it had the lowest observed allelic richness (Table 1, Fig. 4) with no exclusive alleles which is probably related to the long history of over-exploitation of this population – mainly during the XIXth century within Lanín National Park (González Peñalba et al., 2008). In their review on the impact of fragmentation on the genetic diversity of woody plants, Vranckx et al. (2012) advocated that a decrease in allelic richness as a consequence of habitat fragmentation is generally detected in areas where the time of fragmentation exceeds the lifespan of the trees being sampled. Therefore we did not consider this population as a separate genetic zone but rather as part of a bigger zone together with the adjacent populations from cluster 4 (Fig. 3). The remaining populations at cluster 4 share the same fixed haplotype, with two extra haplotypes in Quila Quina (L6) (Azpilicueta et al., 2009) which can be related to a historical southward range expansion of the species based on the occurrence of its fossil pollen type within that region (Auer, 1958).

Although the northern group of *N. obliqua* populations was identified as a more homogeneous region, some considerations are in place. The northernmost population (Epulauquen E1, Argentina; hatched area in Fig. 3) not only exhibited the highest levels of allelic richness and exclusive alleles (two of them being shared with *N. nervosa*) in the total species range in Argentina, but also showed polymorphism at the cpDNA level (Azpilicueta et al., 2009). Following Hampe and Petit (2005), Epulauquen (E1) can therefore be considered a rear edge population, growing within a region which was only partially affected by the last glaciation and nowadays represents a particular habitat with a high level of isolation from the rest of the species populations. Furthermore, distinction of this population based on morphological descriptors such as leaf and bud shape, seed size, and architectural features (data not published) highlights the need to consider it as a special sub-group within its cluster, until more information becomes available to elucidate its correct taxonomic status. Therefore, we propose to define three distinct genetic zones including one with a nested sub-zone for Argentinean *N. obliqua* forests on the basis of molecular and morphological data, together with information concerning management history of these forests (Table 3).

4. Conclusions

The cluster analysis conducted in this study on the basis of microsatellite marker data corresponded with the general patterns identified previously by means of isozymes and cpDNA. On the basis of this combined information, we identified five and three genetic zones for *N. nervosa* and *N. obliqua*, respectively. While in *N. nervosa* the number of genetic zones coincides with the number of clusters, in *N. obliqua* two pre-defined clusters were combined into a single genetic zone based on their high allelic affinity and geographical proximity. Interpretation of existing chloroplast and morphological data at sub-cluster level led to the identification of two additional sub-zones, one for each species. Our definition of genetic zones with the help of the nSSRs outperformed the previous classification by Vergara (2000), identifying more regions. For *N. nervosa* the unique region defined previous was sub-divided in five distinct zones, whereas for *N. obliqua*, a third zone is proposed in addition to the two zones described previously. The overall set of genetic units is intended to guide the establishment of seed transfer zones for restoration and management purposes.

In spite of the value of genetic studies for guiding the development of species management and conservation policies, they provide baseline information about the distribution of neutral genetic diversity (Bucci et al., 2007). Ideally, this information needs to be combined with information about ecogeographic distribution (climate, soil, barriers, etc.), adaptive traits and management regimes to allow for the definition of practical conservation and breeding zones. In the end, conservation units should explicitly be defined based on (adaptive) traits that enhance the potential of species survival (Crandall et al., 2000). The neutral genetic variation described in this paper is not necessarily linked to genetic variation and population differentiation at quantitative, adaptive traits (Holderegger et al., 2006). Analysis of provenance trials of *N. nervosa* and *N. obliqua* installed within the region over the past 15 years (Gallo et al., 2008) are expected to complement the results obtained in the present study. This will allow defining adequate units for *N. nervosa* and *N. obliqua* in Argentina, based on both neutral and adaptive trait data. In combination with ecogeographic studies and distribution modeling, new suitable geographic areas can be identified where genetically matched germplasm from specific conservation and management units can be planted. This is for example relevant for assisted migration of provenances to higher elevations to keep abreast with progressive climate change (Saénz-Romero et al., 2006). At the same time, it is important to promote local adaptation of seed sources by maintaining sufficiently large population sizes. Furthermore, studies are underway on seed harvesting, nursery seedling production and silvicultural practices for both species and results will additionally be used to fine-tune the design of the eventual management units.

We recommend future transfer of planting material in light of restoration activities and assisted natural regeneration in Argentinean *N. nervosa* and *N. obliqua* forests to be limited to the same genetic zone from where it is collected, at least until a more elaborate definition of adequate Management Units will become available. Moreover, for assisted migration programs under progressive climate change the identification of these zones together with knowledge about hotspots of genetic diversity should constitute an input for planning the activities. This information could also be used as a guiding tool for identifying the origin of seed or other plant material in the context of certification. Further studies and ideas about how to make best use of the information obtained from the present study are recommended, because after all, the development of seed transfer guidelines is 'as much as an art as a science' (Ying and Yanchuk, 2006; Hamman et al., 2011).

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