

REVIEW

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Robles in Lagunas de Epulauquen, Argentina: previous and recent evidence of their distinctive character

María M Azpilicueta^{1*}, Mario J Pastorino^{1,2}, Javier Puntieri^{2,3}, Fernando Barbero^{1,2}, Alejandro Martinez-Meier¹, Paula Marchelli^{1,2} and Leonardo A Gallo¹

Abstract

The northernmost 'robles' of Argentina, located in Lagunas de Epulauquen (northwest Patagonia), are considered a population of *Nothofagus obliqua*, even though they differ in some respects from individuals of other populations of the species. In order to analyse a possible genetic basis of their distinctive character, we revised previous information and added new evidence based on biochemical (isozyme) and molecular (chloroplast DNA and nuclear microsatellite) markers, as well as quantitative trait variation (seed traits, leaf morphology, plant architecture and field performance). The comparison of Lagunas de Epulauquen specimens with specimens from other areas in Argentina demonstrated their genetic distinction. Glacial history, in conjunction with hybridisation processes, provides support for the main hypothesis to explain these results. Future research lines are proposed, aimed at identifying the taxonomic status of the Lagunas de Epulauquen population. The conservation value of these forests is highlighted.

Keywords: *Nothofagus obliqua*; Taxonomy; Patagonia; Glacial history; Interspecific hybridisation

Resumen

Los robles más septentrionales de la Argentina, ubicados en Lagunas de Epulauquen (noroeste de Patagonia) son considerados una población de la especie *Nothofagus obliqua*, pero presentan características diferenciales respecto al resto de las poblaciones argentinas de esa especie. El presente trabajo reúne información generada previamente y otra original que demuestra el carácter genéticamente distintivo de esa población. Se presentan los resultados logrados a través de análisis genéticos con marcadores bioquímicos (isoenzimas) y moleculares (de ADN de cloroplasto y marcadores de microsatélites nucleares), con caracteres cuantitativos seminales, de morfología foliar, de arquitectura de las plantas y de aptitud a campo. La historia glacial de la región, en conjunción con procesos de hibridación pasados, se postula como la causa de esta diferenciación. Se propone la realización de nuevas líneas de investigación que permitan confirmar o re-definir el estatus taxonómico de los árboles de esta población. Adicionalmente se presenta una breve consideración sobre el valor de conservación de los bosques en Lagunas de Epulauquen, en relación a los resultados aquí presentados.

Palabras clave: *Nothofagus obliqua*; Taxonomía; Patagonia; Historia glacial; Hibridación interespecífica

* Correspondence: azpilicueta.maria@inta.gob.ar

¹INTA (Instituto Nacional de Tecnología Agropecuaria), EEA Bariloche, Modesta Victoria 4450, 8400 Bariloche, Argentina

Full list of author information is available at the end of the article

Review

Nothofagus obliqua characteristics

Nothofagus obliqua (Mirb.) Oerst. (Nothofagaceae, Hill and Jordan 1993), known as 'roble', 'pellín', 'roble pellín' or 'hualle', is a tree species endemic to South American temperate forests. It belongs to the *Lophozonia* sub-genus [recently upgraded to genus in the classification by Heenan and Smissen (2013)] along with other six species (Hill and Read 1991; Martin and Dowd 1993; Heenan and Smissen 2013), three of which are also South American: *Nothofagus alpina* (= *N. nervosa*) (Poepp. & Endl.) Oerst., *Nothofagus glauca* (Phil.) Krasser (Veblen et al. 1996) and *Nothofagus macrocarpa* (A. DC.) F. M. Vázquez and R. Rodr. (Vázquez and Rodríguez 1999).

N. obliqua is a wind-pollinated, outcrossing, anemochorous and deciduous forest tree occupying an extensive area of 1.2 million hectares in Chile (Veblen et al. 1996; Araya and Oyarzún 2000). It has a narrow distribution of 33,859 ha in Argentina, from 36° 49' S to 40° 11' S (Sabatier et al. 2011) along five lake basins (Lácar, Quillén, Ñorquinco, Moquehue and Lagunas de Epulauquen) and a river margin (Aluminé river; Gallo et al. 2000; Sabatier et al. 2011; Figure 1). The distribution pattern of these forests has been markedly modelled by glacial processes that affected northern Patagonia. Palynological and palaeo-climatic studies described a non-continuous ice cap at these latitudes (Rabassa and Clapperton 1990). Accordingly, many species could have survived in multiple glacial refugia (Markgraf et al. 1995; Veit and Garleff 1995) and gone on to recolonise the area in post-glacial times. Genetic studies in *N. obliqua* forests (Azpilicueta et al. 2009) and other South American temperate tree species (e.g. Marchelli et al. 1998; Premoli et al. 2000; Bekessy et al. 2002; Pastorino and Gallo 2002; Allnut et al. 2003; Azpilicueta et al. 2009; Marchelli et al. 2010) give support to this multiple refugia hypothesis.

Natural hybridisation is common among species of the *Nothofagus* genus. *N. obliqua* hybridises with *N. alpina*, especially in sympatric forests (Donoso et al. 1990; Gallo et al. 1997; Marchelli and Gallo 2000; Gallo 2004). In addition, *N. × leonii* Espinosa (Donoso and Landrum 1979; Grant and Clement 2004) is recognised as the hybrid between *N. obliqua* and *N. glauca*. Finally, hybridisation between *N. obliqua* and *N. macrocarpa* has been suggested (Vázquez and Rodríguez 1999).

Robles from Lagunas de Epulauquen

Forests in Lagunas de Epulauquen, growing at 36° 49' S, 71° 04' W and 1,500 masl, in an area of 1,501 ha (Sabatier et al. 2011), are considered the northernmost distribution of *N. obliqua* in Argentina. The nearest co-specific Argentinean forests are located at a distance of 220 km, at Moquehue lake (Sabatier et al. 2011), and are

separated by mountain chains, which contribute to the geographical isolation of Lagunas de Epulauquen.

Roble trees from Lagunas de Epulauquen differed from *N. obliqua* trees from other Argentinean populations in leaf size and margin, as well as in branching intensity (Figure 2). To an even greater extent, the bud shape (Figure 3A,B) and cotyledon size (Figure 4) of nursery-grown seedlings from Lagunas de Epulauquen were also different to those from other Argentinean populations of *N. obliqua*. These observations cast doubts on the accepted taxonomic status of the roble population of Lagunas de Epulauquen.

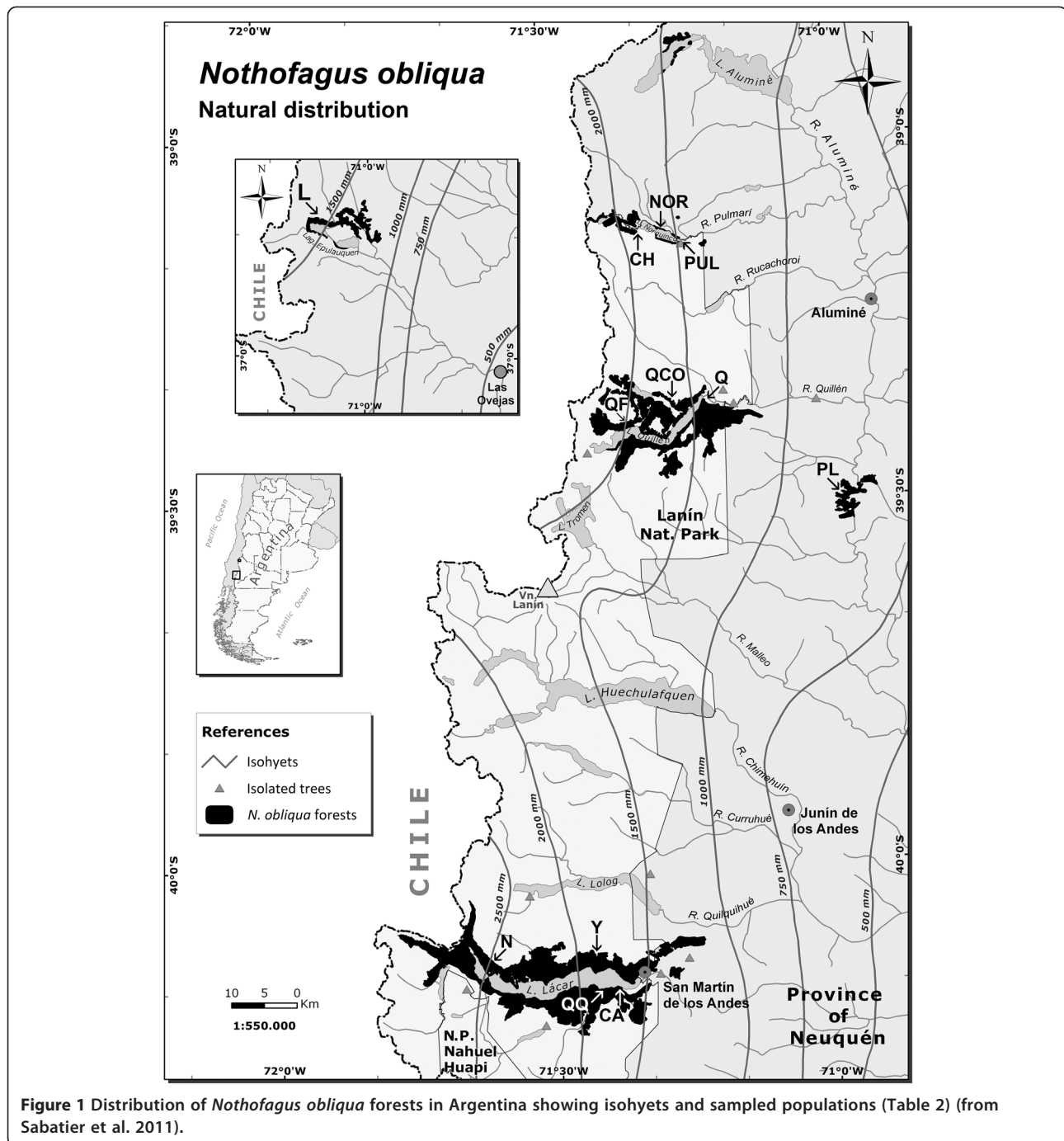
The main goal of this study is to review previous results and present new evidence - based on biochemical, molecular, morphological and architectural data - in order to postulate the hypothesis that the origin of the Lagunas de Epulauquen roble population is different to that of other Argentinean populations of *N. obliqua*.

Previous evidence supporting the distinctiveness of robles from Lagunas de Epulauquen

In Chile, *N. obliqua* forests grow approximately 50 km west of the Lagunas de Epulauquen population, with high mountains in between. Based on pollen fossil analysis, Markgraf (1987) postulated the occurrence of a post-glacial introgression within the Lagunas de Epulauquen region, coming from the west. Therefore, a historical connection between the two sides of the Andes Mountains at this latitude can be inferred.

Because of its maternal inheritance in the majority of angiosperm species (Harris and Ingram 1991), chloroplast DNA allows direct estimation of seed-mediated dispersal. Furthermore, the low mutation rate of the chloroplast (Wolfe et al. 1987), which determines its highly conservative nature, helps in the inference of glacial refugia and post-glacial migration routes of glacial-affected species. Azpilicueta et al. (2009) identified two different cpDNA haplotypes in the Lagunas de Epulauquen population. One of these haplotypes was found in two Chilean populations from similar latitudes (Altos de Vilches at 35° 34' S and Embalse Bulilleo at 36° 22' S), supporting the connection postulated by Markgraf (1987). The other cpDNA haplotype found at Lagunas de Epulauquen was fixed in all the Argentinean populations of *N. obliqua* growing north of Lanin volcano (39° 30' S) (Azpilicueta et al. 2009).

The occurrence of natural hybridisation between *N. obliqua* and other congener species (see *N. obliqua*, previous section) suggested a possible hybrid origin for Lagunas de Epulauquen individuals, and their identification as possible *N. × leonii* hybrids was even proposed (Gallo et al. 2000). The proximity of potentially hybridising *Nothofagus* forests in the west (Chile) reinforced this hypothesis. *N. obliqua*, together with *N. glauca*



(Donoso and Landrum 1979; San Martín and Donoso 1995; Le-Quesne and Sandoval 2001), *N. alpina* and *N. macrocarpa* (Vázquez and Rodríguez 1999), grow in Chile in Embalse Bulilleo, less than 60 km from Lagunas de Epulauquen. Accordingly, species-specific isozyme markers revealed a very high proportion of *N. alpina* alleles in the robles of Lagunas de Epulauquen. *Adh* locus (alcohol dehydrogenase, E.C.1.1.1.1) showed the highest

frequency for *Adh-2* allele - up to then considered as species-specific for *N. alpina* (Gallo et al. 1997; Marchelli and Gallo 2000) - suggesting the hybrid origin of trees in this population (Azpilicueta and Gallo 2009). This allele had a frequency of 25%, in contrast with frequencies of <14% in sympatric populations where hybrids are more likely to occur (Azpilicueta and Gallo 2009). This result was unexpected given the absence of *N. alpina*



Figure 2 Adult tree from the Lagunas de Epulauquen population.

within the area. In addition, Lagunas de Epulauquen showed the highest number of private microsatellite alleles of all Argentinean populations (five; Azpilicueta et al. 2013), two of which were shared with *N. alpina*. In conclusion, both isozyme and microsatellite markers supported the possible ancestral hybrid origin of Lagunas de Epulauquen individuals with *N. alpina*. The other *Nothofagus* species growing in the Lagunas de Epulauquen population - *Nothofagus antarctica* (G. Forst.) Oerst. and *Nothofagus pumilio* (Poepp. & Endl.) Krasser (Di Martino et al. 2005) - belong to the sub-genus *Nothofagus*, and no reports of hybridisation with *N. obliqua* exist.

New evidence supporting the distinctiveness of robles from Lagunas de Epulauquen

Biochemical markers: isozymes

As mentioned, *N. obliqua* also potentially hybridise with *N. macrocarpa* (Vázquez and Rodríguez 1999). In order to provide information about this possible origin for the robles from Lagunas de Epulauquen, four coastal and northern *N. obliqua* Chilean populations were analysed at *Adh* locus (for methodological details, see Azpilicueta and Gallo 2009). These populations overlap in distribution with *N. macrocarpa* (Ravenna 2002), whereas neither *N. alpina* nor *N. glauca* occur in the sampled area. While the Alto Colorado population - located at the southern limit of the *N. macrocarpa* distribution - exhibited only *Adh-1* fixed allele, the three northernmost populations (Til Til, Lampa and Alhué) showed the highest frequencies of the allele *Adh-2*, reaching up to >50% (Table 1). The particularly high frequency of the allele *Adh-2* may be indicative of *N. macrocarpa* gene introgression into the *N. obliqua* genome. Thus, this would also constitute evidence for a possible ancestral hybridisation origin of the robles from Lagunas de Epulauquen, this time with *N. macrocarpa*.

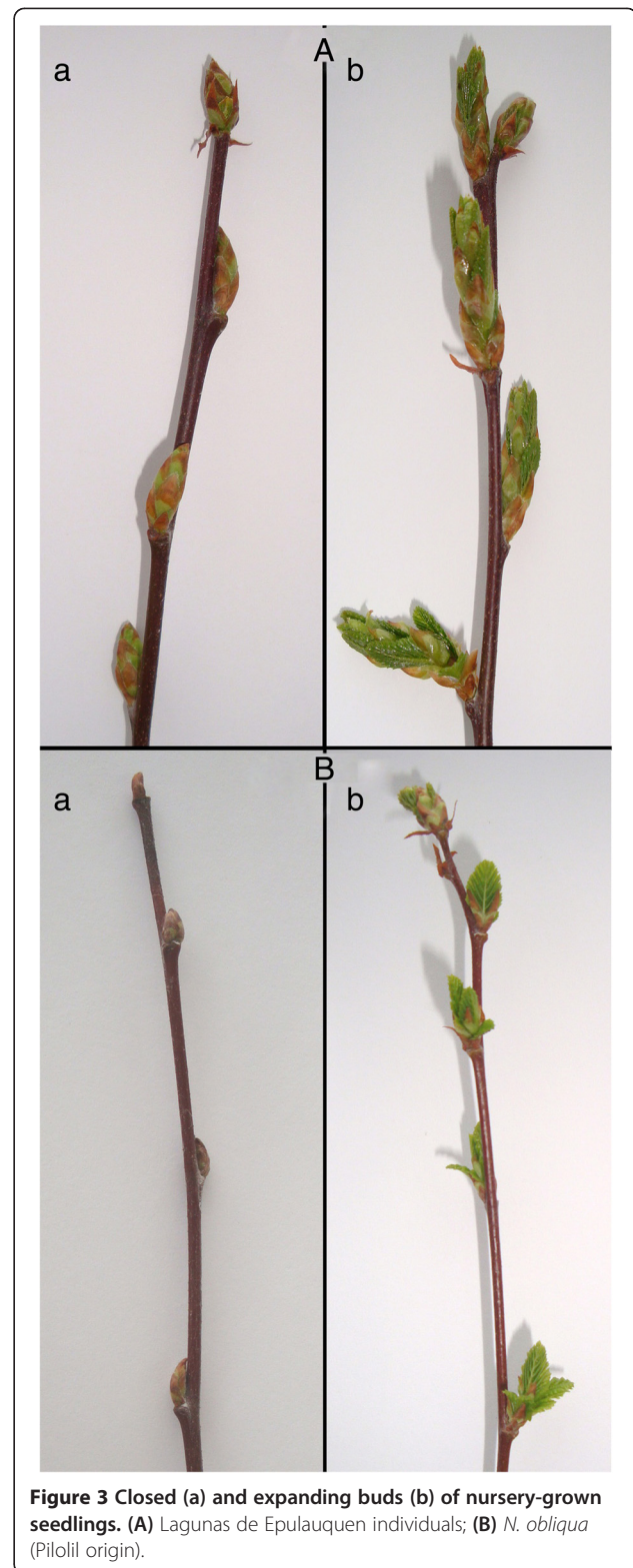


Figure 3 Closed (a) and expanding buds (b) of nursery-grown seedlings. (A) Lagunas de Epulauquen individuals; (B) *N. obliqua* (Pilolil origin).

Seed characters

Seed weight and percentage of filled seeds (all *Nothofagus* species typically have a great proportion of empty seeds



Figure 4 Seedlings from seeds sown in the greenhouse on the same date and under the same environmental conditions. Left: Lagunas de Epulauquen origin; right: Quila Quina origin (Lácar lake watershed).

due to their common parthenocarpic capacity; Poole 1950) were analysed in eight Argentinean populations of *N. obliqua* and germination capacity in five of these populations, including the Lagunas de Epulauquen population (F Barbero, PhD thesis in evaluation). We used between 13 and 15 open-pollination families *per* population (Table 2). Seed weight was measured in four replicates of 100 seeds per family, whereas percentage of filled seeds was determined based on 50 seeds per family in each population, by dissection and direct observation. Germination capacity was tested with four replicates of 100 seeds per family, under controlled temperature cycles of 25°C (for 16 h) and 15°C (for 8 h) over 40 days. Germination capacity was determined as the number of germinated seeds at the end of the experiment.

The Lagunas de Epulauquen population had a higher mean seed weight than the other provenances (2.24 g per 100 seeds, as compared to <1.00 g for the others; $P < 0.001$) (Figure 5A). The percentage of filled seeds was also significantly higher ($P < 0.05$) in Lagunas de Epulauquen (57%) than in the other populations (4% to 31%). Accordingly, a higher germination capacity was detected in Lagunas de Epulauquen (60%) in comparison with the other populations (13% to 21%; $P < 0.001$; Figure 5B).

Leaf characters

Four Argentinean *N. obliqua* natural populations - including Lagunas de Epulauquen (Table 2) - were essayed in a common garden experiment (INTA EEA Bariloche, 41° 7' 23" S, 71° 14' 58" W, 780 masl) (F Barbero, PhD thesis in evaluation). Ten families *per* population were analysed in a single-tree plot design with 24 replicates (960 individuals). At 2 years of age and before autumnal senescence, three leaves per sapling were collected from the distal one third of the crown, scanned with 300-dpi resolution, dried for 48 h at 72°C and finally weighed (precision = 0.0001 g). Leaf area (LA) was measured using the program ImageJ (URL: <http://rsbweb.nih.gov/ij/download.html>), and specific leaf area (SLA) was computed as follows:

$$SLA = \frac{LA \text{ (cm}^2\text{)}}{\text{Dry mass (g)}}$$

Lagunas de Epulauquen individuals exhibited the highest mean LA and lowest mean SLA (Figure 6A,B). Significant differences were found between the four populations analysed for LA ($P < 0.001$) and SLA ($P = 0.027$) after applying an analysis of variance using a likelihood

Table 1 Geographical location and allele frequency at *Adh* locus

Population	Country	Latitude S	Longitude W	N	<i>Adh</i> locus frequency	
					<i>Adh</i> -1 allele	<i>Adh</i> -2 allele
Lagunas de Epulauquen	Argentina	36° 49'	71° 04'	120	0.742	0.258
Til Til	Chile	33° 07'	70° 58'	42	0.476	0.524
Lampa	Chile	33° 16'	70° 55'	77	0.734	0.266
Alhué	Chile	33° 57'	71° 01'	65	0.508	0.492
Alto Colorado	Chile	34° 17'	71° 46'	73	1.000	0.000

Lagunas de Epulauquen is compared with four coastal populations of roble in northern Chile where *Nothofagus macrocarpa* is also found. N, sample size (number of individuals analysed).

Table 2 Geographical and altitudinal characteristics of the origins of roble populations evaluated

Population	ID	Latitude S	Longitude W	Altitude (masl)	Analyses
Lagunas de Epulauquen	L	36° 49'	71° 04'	1,500	slga
Ñorquinco Chumpiru	CH	39° 09'	71° 15'	1,200	g
Ñorquinco Seccional	NOR	39° 09'	71° 15'	1,071	sg
Ñorquinco Pulmarí	PUL	39° 09'	71° 12'	1,081	g
Quillén Fondo de Lago	QF	39° 26'	71° 25'	1,100	g
Quillén Corral de Bueyes	QCO	39° 22'	71° 17'	1,140	g
Quillén Casa Guardaparque	Q	39° 21'	71° 13'	1,100	sg
Pilolil	PL	39° 30'	70° 57'	836	slg
Yuco	Y	40° 09'	71° 30'	930	slg
Quila Quina	QQ	40° 10'	71° 26'	983	sga
Nonthué	N	40° 08'	71° 37'	680	s
Catritre	CA	40° 10'	71° 24'	650	sl

g, growth traits; a, architectural features; s, seed characters; l, leaf morphological characters.

ratio test (LRT). The Lagunas de Epulauquen population was different from all the other three populations for LA and from one of them for SLA.

Architectural features

Trunk growth and branching were compared for a total of 244 8-year-old roble individuals from Lagunas de Epulauquen and a southern Argentinean population (Quila Quina 40° 10' S, 71° 26' W), grown from germination in a common garden (Table 2). For Lagunas de Epulauquen, seven open-pollination families were differentiated (L1 to L7), consisting of 21 to 25 individuals per family. For Quila Quina, two families (QQ1 and QQ2) and two groups of families (QQ3 and QQ4)

were differentiated, which had 13 to 43 individuals per family or group of families. For each individual, the length (with a measuring tape), basal diameter (with digital callipers) and number of green leaves of the last but one annual shoot of the trunk (i.e. the stem portion extended in the year preceding the year of measuring) were registered. For each node of each annual shoot, the phyllotactic pattern and the presence/absence of axillary branch were recorded. In *Nothofagus* spp., phyllotaxis may adopt one of two conditions: alternate distichous (leaves arranged in two lines along the stem) or alternate tristichous (leaves arranged along three lines). Regarding branching, we recorded the following: presence/absence of an immediate branch (i.e. a branch

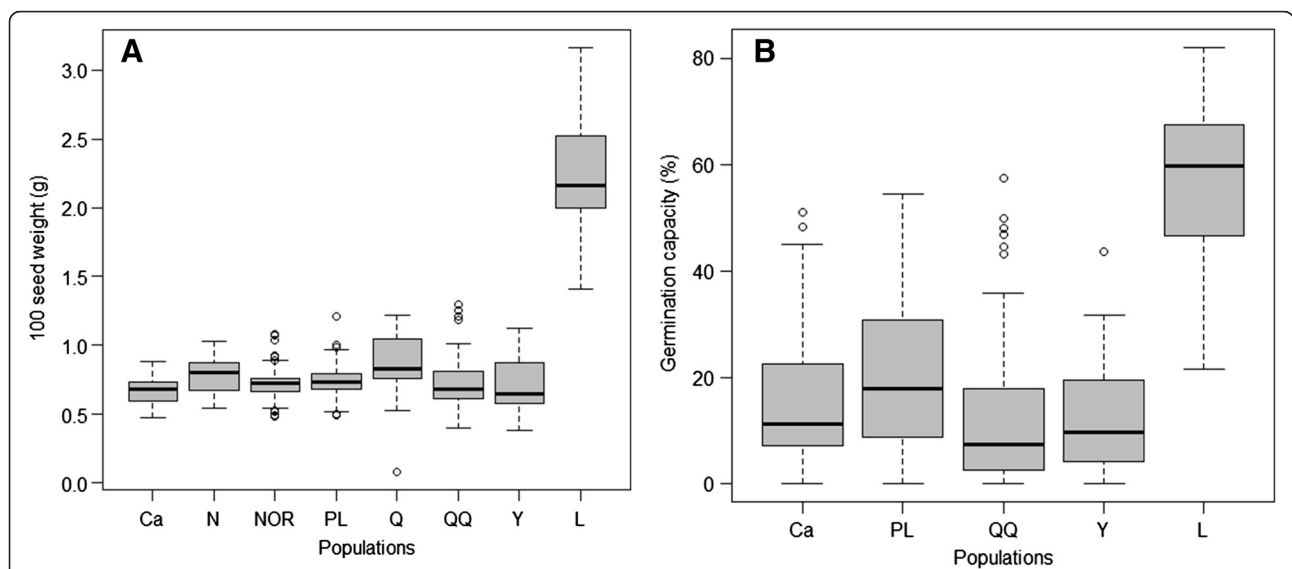
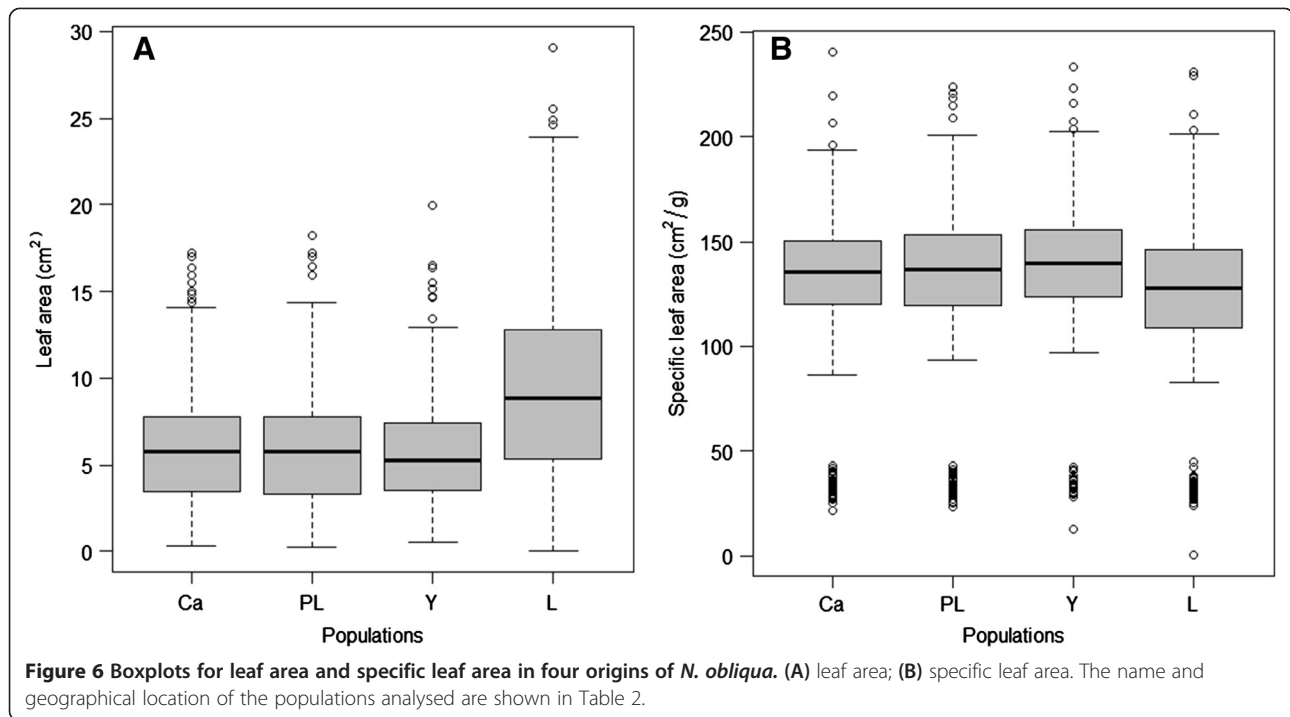


Figure 5 Boxplots of dispersion and symmetry for the characters. (A) Weight of 100 seeds from eight Argentinean *Nothofagus obliqua* origins, including Lagunas de Epulauquen; (B) germination capacity of five Argentinean origins of *N. obliqua*, including Lagunas de Epulauquen. The complete name and geographical location of the populations analysed are shown in Table 2.



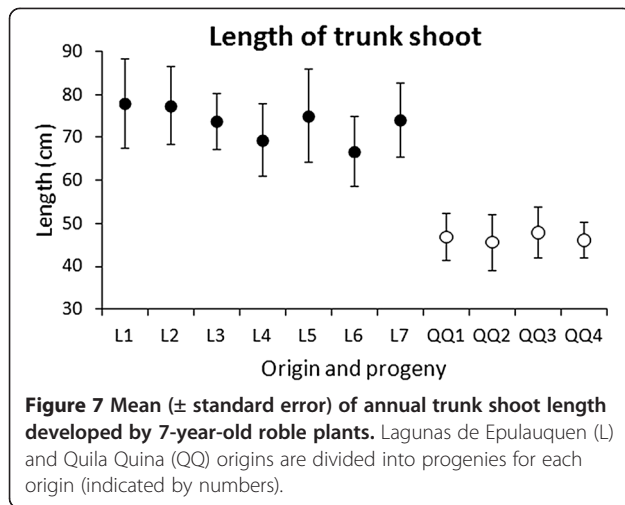
developed as the annual shoot extended) or a delayed branch (i.e. a branch developed 1 year after the extension of the bearing annual shoot). Analysis of variance for unbalanced designs was applied to all annual shoots so as to compare origins (Lagunas de Epulauquen and Quila Quina, fixed factor) and families (random factor, nested within each origin). Comparisons between origins regarding phyllotaxis and branching were performed graphically. For each stem node, numbered from the stem's proximal end, we computed the proportions of nodes with distichous and tristichous phyllotaxis and the proportions of nodes with immediate and delayed branches.

The mean basal diameter of the shoots did not differ between origins ($F = 1.1, P > 0.1$) (Table 3). Both the length (Figure 7) and the number of leaves of the annual shoots were higher for Lagunas de Epulauquen than for Quila Quina ($P < 0.001$ and $P < 0.001$, respectively; Table 3). Annual shoots had a higher length/diameter ratio (i.e. more slender shoots; $P < 0.001$) and length/number of leaves relationship (i.e. longer mean internode length; $P < 0.001$) for Lagunas de Epulauquen than for Quila Quina. Significant family effects on variations in diameter ($P < 0.01$) and in length/diameter relationship ($P < 0.05$) were detected. Phyllotaxis was predominantly

Table 3 Morphological attributes of annual shoots of 7-year-old roble plants

Shoot trait	Population	Mean	S.E.	Var	Min	Max
Basal diameter (mm)	Lagunas de Epulauquen	9.2	0.17	4.58	5.5	18.0
	Quila Quina	8.8	0.21	4.21	5.6	16.2
Total length (cm)	Lagunas de Epulauquen	73.4	1.72	450.43	22.0	128.0
	Quila Quina	46.6	1.32	160.11	23.0	73.5
Number of leaves	Lagunas de Epulauquen	25	0.5	38.7	10	42
	Quila Quina	22	0.4	15.5	14	35
Length/diameter (cm/mm)	Lagunas de Epulauquen	8.1	0.18	4.68	2.5	13.0
	Quila Quina	5.4	0.15	1.99	2.3	9.2
Mean internode length (cm)	Lagunas de Epulauquen	2.9	0.04	0.19	1.8	4.3
	Quila Quina	2.1	0.04	0.14	1.4	3.1

Mean, standard error (S.E), variance (Var), minimum value (Min) and maximum value (Max) from Lagunas de Epulauquen (152 trees) and Quila Quina (92 trees) are shown.



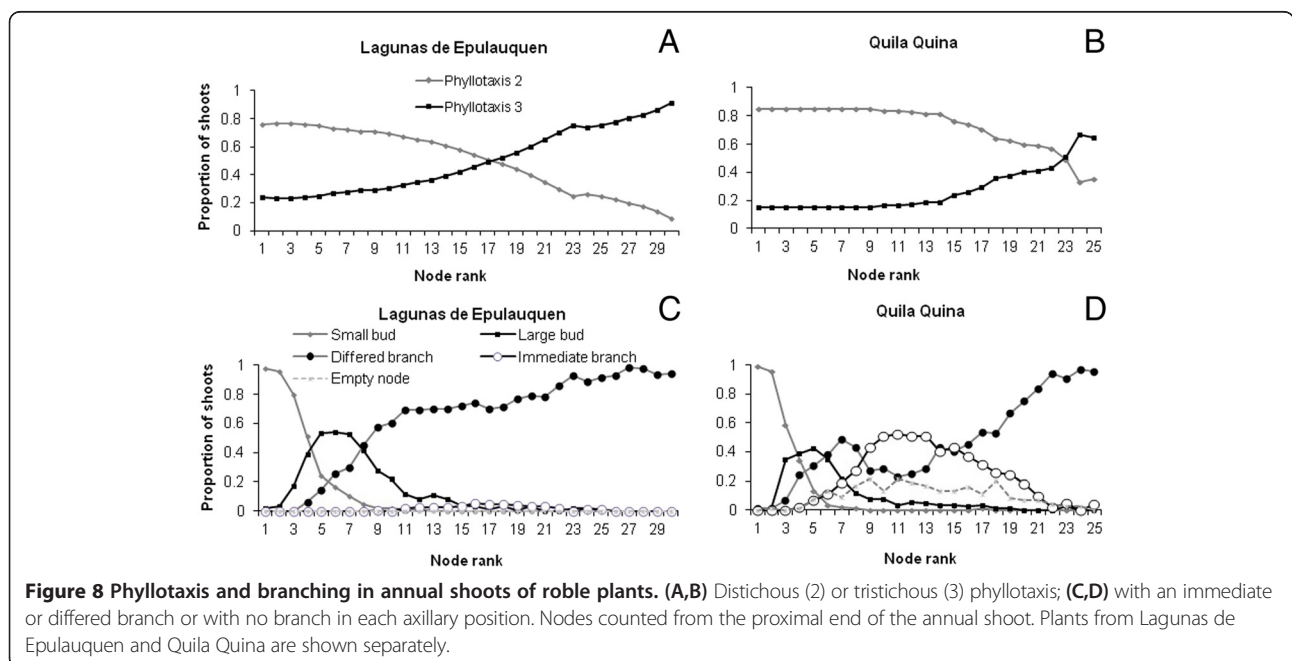
distichous for the proximal end of the annual shoots of both origins; a progressively higher proportion of annual shoots - especially those of Lagunas de Epulauquen - tended to have tristichous phyllotaxis toward the shoot's distal end (Figure 8A,B). For both origins, the proportion of branched nodes, in particular those with a delayed branch, increased from the fourth most proximal node to the annual shoot's distal end (Figure 8C,D). A high proportion of nodes with immediate branches and a corresponding decrease in the proportion of delayed branches were registered in intermediate nodes for annual shoots of Quila Quina (Figure 8C,D). For Lagunas de Epulauquen, in contrast, the proportion of annual shoots with immediate branches was very low.

Survival and height growth in field trials

During 2004, a provenance field trial - including the Lagunas de Epulauquen population - was installed at Lácar lake, Argentina (40° 07' S to 71° 28' W), beneath a mixed forest of *N. obliqua*, *N. alpina* and *Nothofagus dombeyi* (Mirb.) Oerst. The site is characterised by volcanic soils and a mean precipitation of 2,200 mm/year. The essayed populations and their geographical locations are shown in Table 2. We used a randomised complete block design (RCBD) with a plot size of nine individuals. Survival and height were measured at the first and fourth years from trial installation. Both height measures were used to calculate growth rate relative to the measurement at the first year and later transformed with the logarithm 10 for variance analysis; for multiple mean comparisons, we used a Tukey test.

Tree survival was high (94% and 91% at the first and fourth years after installation, respectively), which was as expected due to the optimal environmental conditions for *N. obliqua* in the trial site. The population effect gave rise to significant differences for the first year ($P < 0.001$) and for the fourth year ($P < 0.001$) regarding total height and log relative growth ($P < 0.001$; Figure 9A,B,C, respectively).

Lagunas de Epulauquen had the lowest total height (at both the first and fourth years) and presented significant differences to the other analysed populations, except for Pulmarí (PUL) and Quillén Fondo de Lago (QF), which correspond to the species' northern distribution areas. On the other hand, Lagunas de Epulauquen exhibited the highest relative growth rate, significantly different



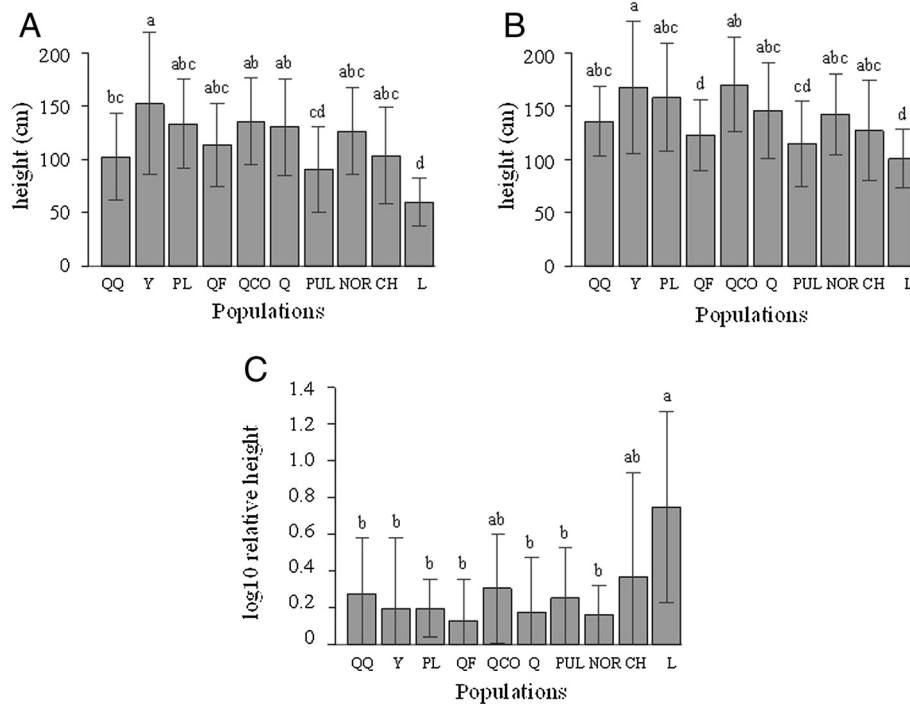


Figure 9 Mean height values (\pm standard error) by population. (A) at the first and (B) fourth years after plantation and (C) \log_{10} of the relative growth in height between the first and fourth years. Equal letters above the bar indicate statistically similar means ($\alpha = 0.05$). The name and geographical location of the populations analysed are shown in Table 2.

from all the other populations except Chumpiru (CH) and Quillén Corral de Bueyes (QCO).

Discussion

The evidence for the genetically distinctive character of robles in the Lagunas de Epulauquen population compared to other Argentinean *N. obliqua* forests is supported in the present revision by both previous and new information. Lagunas de Epulauquen individuals show differences at biochemical, molecular, morphological and architectural levels. Molecular (Azpilicueta et al. 2009) and palynological (Markgraf 1987) data suggested that the Lagunas de Epulauquen population could have originated as an introgression from the western side of the Andean Cordillera during post-glacial re-colonisation. The presence in Lagunas de Epulauquen of a chloroplast DNA haplotype private to western populations reinforces this hypothesis. In agreement with this, Markgraf et al. (2009) dated the forests of this region as 5,000 years BP, while an older origin was postulated for western refugia (Heusser 1983; Heusser et al. 1999; Villagrán 1991; Villagrán et al. 1995). The co-existence of *N. glauca*, *N. macrocarpa*, *N. alpina* and *N. obliqua* to the west suggests the possibility of a hybrid origin for robles from Lagunas de Epulauquen. Therefore, post-glacial history together with ancient hybridisation processes could stand

as the main reasons for the present distinctiveness of the roble population in Lagunas de Epulauquen.

The geographical isolation of the Lagunas de Epulauquen population predicts genetic drift and inbreeding processes. However, a high level of genetic variation was detected with all genetic markers (isozymes: Azpilicueta and Gallo 2009; chloroplast DNA: Azpilicueta et al. 2009; microsatellites: Azpilicueta et al. 2013). It is possible that past hybridisation increased genetic diversity by providing new genetic variants. Moreover, long-distance pollen gene flow can rapidly restore genetic diversity (Hampe et al. 2013). Although, at least for *N. alpina*, most pollen disperses at short distances, a high potential for long-distance dispersal was reported (Marchelli et al. 2012).

The high frequency of the allele *Adh-2* found in coastal and northern Chilean *N. obliqua* populations where *N. macrocarpa* grows casts doubt on the origin of this allele. Neither *N. alpina* nor *N. glauca* grow within this northern area; therefore, the *Adh-2* allelic variant could come from *N. macrocarpa*. The phylogenetic affinity between *N. macrocarpa* and *N. alpina* can explain the occurrence of common alleles in these two species. Vázquez and Rodríguez (1999) reported a closer morphological affinity between *N. macrocarpa* and *N. alpina* than between these two species and *N. obliqua*. Apparently, *N. macrocarpa* has been wrongly associated with *N. obliqua*, since both morphological and ecological (altitudinal niche)

data support a closer relationship between *N. macrocarpa* and *N. alpina* (Vázquez and Rodríguez 1999). However, an alternative interpretation must be considered. Among the drawbacks of isozymes is the inability to detect variants in the DNA sequence that produces isozymes with no changes in electromorph mobility. Thus, the *Adh-2* allelic variant observed in gels of *N. obliqua*, *N. macrocarpa* and *N. alpina*, although identical, could be the expression of different DNA sequences. The sequencing of the gene coding for the Adh enzyme should help in the precise identification of *Adh-2* allele and its species origin.

The large seed size observed in Lagunas de Epulauquen could be a consequence of genecological variation, as found for *N. obliqua* in Chile (Donoso 1979). The largest seed size of the northernmost *N. obliqua* populations was explained as an adaptation to xeric environments within a wide latitudinal species cline. However, since *N. macrocarpa* was only later recognised as a separate species, it is possible that the sample in Donoso's study consisted of seeds from the two species (or included hybrids with *N. macrocarpa*). The results of our study could then be explained by clinal variation together with ancient hybridisation with *N. macrocarpa*.

The differences regarding leaf characters (leaf area and specific leaf area) between robles from Lagunas de Epulauquen and *N. obliqua* plants from other Argentinean populations reinforced the distinctiveness of the former population. Previous non-systematic field and nursery observations have now been confirmed based on these results.

Clear differences in length, number of leaves, mean internode length and the branching pattern of annual shoots were found between robles from Lagunas de Epulauquen and those from Quila Quina. With regard to these traits, Quila Quina plants resembled those from other areas within the distribution of *N. obliqua* in Argentina (e.g. Puntieri et al. 2006). In annual shoots of *N. alpina*, as in those of roble plants from Lagunas de Epulauquen, the frequency of development of immediate branches is relatively low and internodes are long compared to those of annual shoots in similar architectural positions on *N. obliqua* plants (Barthélémy et al. 1999). Recent observations confirmed that the architectural traits that distinguished nursery-grown Lagunas de Epulauquen plants from Quila Quina ones also occur in field conditions. Architectural studies incorporating the closely related *Nothofagus* entities considered in the present study (*N. macrocarpa*, *N. glauca* and *N. × leonii*), little known in this regard, may provide a clearer picture of the evolution of morpho-architectural traits in plants.

Height growth analysis showed that Lagunas de Epulauquen individuals exhibited low stem height - at both the first and fourth years - but, notwithstanding, the highest height growth rate compared to *N. obliqua* plants from other origins. All results coming from quantitative trait

analyses may be associated with environmental conditions. In this regard, it must be highlighted that the roble population at Lagunas de Epulauquen is located at a relatively high altitudinal range compared to other roble populations from Argentina: from 1,500 to 1,700 masl, as opposed to the 600 to 1,200 masl range for other roble populations in Argentina. This environmental feature, together with the current genetic isolation of this population, leads us to put forward a hypothesis involving adaptation processes. New quantitative traits, especially phenological ones, should be considered in order to explore this alternative.

Conclusions

The glacial history of Patagonia - with valley-type glaciers at northern latitudes leaving ice-free areas and re-colonised southern regions - probably promoted the high genetically divergent evolution of *N. obliqua* forests as proposed by Donoso et al. (2004). The profusion of races, varieties, ecotypes and sub-species found nowadays in *N. obliqua* forests supports this idea. The results of the present study also provide evidence supporting the idea that the isolated roble population at Lagunas de Epulauquen shared (unlike other roble populations in Argentina) a common glacial history with *Nothofagus* populations from low-ice-impact areas in Chile, which could have resulted in a distinctive evolutionary process.

Future analyses of vegetative and reproductive characters in *N. alpina*, *N. glauca* and *N. macrocarpa* together with other *N. obliqua* populations could probably help in determining the taxonomic identity of the *Nothofagus* trees in Lagunas de Epulauquen. Architectural variation during early ontogenetic stages as well as phenological traits should be included in these studies. Sequencing of ITS nuclear ribosomal gene (internal transcript unit) and comparison with the results obtained by Manos (1997) and Acosta and Premoli (2010) should provide additional and valuable information for taxonomic identification through phylogenetic reconstruction.

The conservation value of Lagunas de Epulauquen

The Lagunas de Epulauquen area is of remarkable conservation value due to the diversity of its flora, which includes species from xeric environments (e.g. *Mulinum spinosum*), species typical of Valdivian forests (e.g. *Lomatia ferruginea*) and endemic species (e.g. *Puya alpestris*) (Alfonso and Prinna 2009; Di Martino et al. 2005). Most of the Lagunas de Epulauquen ecosystem - with a surface area of 7,450 ha - has been included within a provincial protected area called Reserva Lagunas de Epulauquen (Neuquén province), created in 1973. However, the limits of this reserve do not follow ecological criteria and, as a consequence, some *N. obliqua* forests lie outside the protected area, on private land. The limits of the protected

area should be re-considered and sustainable management policies should be applied so as to promote forest regeneration. Conservation of this population's genetic pool could be the key to securing its adaptative capacity and, consequently, its persistence, especially considering the ongoing global climate change.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MMA conceived and designed the study and prepared the first version of the manuscript, MMA and PM carried out the molecular genetic studies and their analysis, FB and MJP carried out the seed and leaf character analyses, JP carried out the architectural analysis, AMM and MMA carried out the fitness analysis and LAG coordinated the research group and helped in the design of the study; all authors helped in writing the manuscript. All authors read and approved the final manuscript.

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Author details

¹INTA (Instituto Nacional de Tecnología Agropecuaria), EEA Bariloche, Modesta Victoria 4450, 8400 Bariloche, Argentina. ²CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), 8400 Bariloche, Argentina. ³UNRN (Universidad Nacional de Río Negro), Av. San Martín 2650, 8430 El Bolsón, Argentina.

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