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Genetic and morphological analysis of *Berberis microphylla* G. Forst. accessions in southern Tierra del Fuego

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Abstract

Calafate (*Berberis microphylla* G. Forst.) is a spontaneous shrub grown in the Patagonian region, from which berries are traditionally harvested for different purposes. This study aims to investigate on the spatial genetic structure of calafate populations grown in southern Tierra del Fuego and their morphological and genetic diversity. A first step of the research focused on 23 putative populations screened by a landscape genetic approach based on 82 geo-referenced and Random Amplified Polymorphic DNA marker characterized plants. The second phase regarded the analysis of the morphological characteristics of fruits, shoots and leaves observed on a subset of 39 plants. Taking into account multiple similarity between pairs and sub-sets of accessions, the observed differences have been associated to geographical and environmental conditions. The results allowed to postulate the existence of homogeneous populations within the studied plant sets and to formulate hypothesis on the evolution of *B. microphylla* in that area. No association between genetic and morphologic distances of the accessions has been observed.

Keywords: Underutilized species, calafate, genetic resources

Introduction

The *Berberis* L. genus has a large distribution from Neuquén to Tierra del Fuego (Job 1942; Orsi 1984), being well represented in Patagonia by 16 species of native shrubs (Orsi 1984; Bottini et al. 1993). However, according to a later classification of the genus (Landrum 1999), the number of species is lower than previous studies cited by Orsi (1984). In fact, Landrum (the species *B. buxifolia*, *B. microphylla* and *B. heterophylla* under *B. microphylla* G. Forst., postulating that the differences among them may fluctuate as to retain its range of species. However, although this is the last classification, it contradicts morphological, biochemical and molecular studies of the *Berberis* genus made by Bottini et al. (1999, 2000, 2007). This situation is confirming the complexity of the classification of the genus, as suggested by a study based on the *matK* and *rbcL* DNA barcoding loci applied on Indian *Berberis* species (Roy et al. 2010).

Berberis microphylla G. Forst. (calafate) often grows in differentiated environments in Tierra del Fuego such as coastal scrubs, *Nothofagus* forest margins and clearings, moister areas in grass steppes, and along streams and rivers (Moore 1983). It is an

evergreen, spiny and erect medium size shrub, with a reproductive pattern based on both seedling recruitment (Arena & Martínez Pastur 1994) and clonal development by rhizomes (Arena & Martínez Pastur 1995; Arena et al. 1998). This species belong to the so-called group of minor or underutilized fruit tree species that are relevant for diversification of agro food production. It is classified as a non-timber forest product (Tacón Clavaín 2004), particularly interesting since its black-blue fruits can be consumed fresh and processed in marmalades and jams (Orsi 1984), in non-alcoholic beverages, and in ice creams. It is an important source of alkaloids, i.e. berberines, and phenolic compounds such as anthocyanins with medicinal and tinctorial functions (Pomilio 1973; Shaffer 1985; Fajardo Morales et al. 1986; Fajardo Morales 1987; Pozniakovskii et al. 2003; Arena et al. 2012; Ruiz et al. 2013, 2014; Ramirez et al. 2015; Reyes-Farias et al. 2015). A recent research indicated that *B. microphylla* is appreciated by local rural populations also as fuelwood (Cardoso et al. 2015). At present, commercial barberry orchards are being planned due to its economical potential related to flavour, taste and nutraceutical properties of the fruits.

Some aspects of the phenological phases, fruit composition, postharvest and production, and the annual cycle together with the morphological variation were already studied in natural populations of this species (Arena et al. 2003, 2011; Arena & Curvetto 2008; Arena, Giordani, et al. 2013; Arena, Zuleta, et al. 2013; Arena & Radice 2014; Rodoni et al. 2014).

Random Amplified Polymorphic DNA (RAPD) markers, even if recognized as limited in terms of repeatability, constitute, nevertheless, a reliable and rapid cheap methodology to study the diversity especially in non-previously genetically investigated populations of perennial plant species (Costa et al. 2015; Jena et al. 2016; Verma et al. 2016). Furthermore, this technique requires small amounts of DNA, a relevant aspect when working on wild plants growing in remote areas and under harsh climatic conditions. Landscape genetics is a relatively new approach which aims to study the interaction between landscape aspects and evolutionary processes (Manel et al. 2003); Geneland software (Guillot, Mortier, et al. 2005) has been recently used in various studies on forest genetics (Paffetti et al. 2012; Vernesi et al. 2012; Piotti et al. 2013). It allows to divide a sample into a certain number of groups in such a way that each group is genetically homogeneous taking into consideration its spatial distribution.

The objective of this research is to analyse putative natural populations of *Berberis microphylla* G. Forst. of Tierra del Fuego island taking into account geographical and environmental growing conditions, morphological traits and genetic profiles in order to elucidate population structure also in relation to the propagation strategy of this species.

Materials and methods

Plant sampling and spatial distribution

A total of 82 adult shrubs were sampled and georeferenced from several different southern areas of the Argentinian part of Tierra del Fuego island, representing 23 putative populations; the number of plants for each population ranged from 1 (single sparse trees) to 7 (Müller 2011). Among the 82 specimens, a subset of 39 plants representing 12 of the 23 putative populations was randomly defined in order to perform a morphological description (Table I and Figure 1). The distance between pairs of specimens within each putative population were calculated with ArcGIS software (ESRI, CITA) after having loaded the coordinate data of each specimen.

DNA genotyping and genetic analysis

Leaf samples were collected from the 82 selected plants during spring time and used to extract the total

genomic DNA. Total DNA was isolated from freeze-dry samples (50–200 mg as starting material) using the DN easy plant kit (QIAGEN, Germany) and following the manufacturer's specifications to perform molecular analyses by RAPD methods. The main components of lysis buffer were β -mercaptoethanol and polyvinylpyrrolidone, which are very effective for trapping polyphenols and other secondary metabolites resulting from DNA extraction. The amplification patterns were analysed with a scanner densitometer (model GDS2000; Ultra-Violet Products, Ltd., Cambridge, UK) (Paffetti et al. 1996). Three 10-mer random primers (1253, 1247 and RF2), with a G + C content ranging from 60 to 80% were selected from an initial set of 20 primers on the basis of their reproducibility and stability; similarly 94 markers, ranging from 100 to 750 bp, were selected taking into account their stability and reproducibility among the 150 markers generated by the three used random primers. Such procedure has been adopted taking into account previous results reported for other woody species with high contents of polyphenols and secondary metabolites (Paffetti et al. 1999). RAPD analyses were performed following the PCR conditions reported by Badenes et al. (2004); each DNA sample was amplified three times in order to assess the stability of the profile. A 6 μ l aliquot of each reaction mixture was analysed by electrophoresis on an agarose (2%, wt/vol) gel. The amplification patterns were analysed with a scanner densitometer (UVP scanner, Photo-Capt, Vilbert, Lourmat, France). The amplification products were transformed in a vector of presence and absence of RAPD markers (1 indicated that a band was present on a gel, and 0 indicated that a band was not present). Pair-wise comparisons of genotypes, based on both unique and shared polymorphic products, were used to generate similarity Jaccard's coefficients (Jaccard 1908). These were used to construct a dendrogram adopting the unweighted pair-group method with arithmetical averages (UPGMA) employing PAST software (Hammer et al. 2001) and based on 9999 permutations.

General estimates of genetic diversity, marker frequencies and *F*-statistic from RAPD data were calculated using the option for dominant markers of SPAGeDi 1.3a (Hardy 2003). Statistical significance was determined by Jackknifed estimators (Sokal & Rohlf 1995) after 20,000 permutations. The RAPD data were also analysed using Geneland software. We inferred population structure using a Bayesian Monte Carlo Markov Chains method implemented in the Geneland package vers 3.0 (Guillot et al. 2009) under the R Language and Environment for Statistical Computing software as described by Guillot, Estoup, et al. (2005), Guillot, Mortier, et al. (2005) and Guillot et al. (2008). Ten independent Monte Carlo Markov Chains runs were performed by Geneland

Table I. Putative populations of *Berberis microphylla* G. Forst. analysed in Tierra del Fuego island: number of specimens, location and altitude.

Population code and name	Number of plants ^a	Codes of analysed plants ^b	GPS (barycentre) S	GPS (barycentre) W	Altitude (m asl)
P1 – Campo PR CADIC	4 (3)	S107 , S108, S110 , S111	54 49 43 0	68 19 01 6	33
P2 – CADIC Casa Solar	3 (2)	S112, S113 , S114	54 49 31 5	68 19 01 6	31
P3 – Monte Olivia	5 (0)	S101, S102, S103, S104, S105	54 45 40 5	68 11 41 8	123
P4 – Playa Larga Alto	1 (1)	S97	54 48 46 5	68 12 25 5	40
P5 – Playa Larga Intermedia	2 (0)	S26, S27	54 4837 6	68 12 52 2	23
P6 – Playa Larga Baja	4 (2)	S30, S93, S94 , S95	54 48 39 4	68 12 42 2	13
P7 – Playa larga pre albardón	3 (0)	S22, S23, S24	54 48 34 5	68 12 55 2	19
P8 – Estancia Tunel	1 (0)	S55	54 49 19 4	68 10 55 5	40
P9 – Camino Parque-Club Hípico	6 (4)	S45, S46, S47 , S48 , S49 , S50	54 50 00 7	68 22 56 3	87
P10 – Acceso Rugby Club	3 (0)	S41, S42, S44	54 49 54 7	68 21 35 4	60
P11 – Subida pista sky – C.A.	5 (0)	S14, S15, S17, S18, S19	54 48 57 7	68 20 30 8	85
P12 – Camino Hotel Yámanas	2 (0)	S52, S54	54 50 21 4	68 21 08 4	19
P13 – Camino Hotel Los Cauquenes	3 (1)	S32, S34, S35	54 50 36 0	68 22 08 1	30
P14 – Frente Ray	1 (0)	S36	54 50 31 7	68 21 51 8	48
P15 – Bajada del Martial	3 (0)	S11, S12, S13	54 48 47 4	68 20 28 2	120
P16 – Margen lago Fagnano	5 (5)	S61 , S62 , S63 , S64 , S65	54 36 32 7	67 22 27 4	54
P17 – El quemado	3 (0)	S88, S89, S90	54 36 44 6	67 35 20 5	79
P18 – Ruta 3 pasando Escondido	5 (5)	S83 , S84 , S85 , S86 , S87	54 36 00 4	67 38 09 8	46
P19 – Ruta F o H	5 (1)	S66, S67, S68, S70, S71	54 21 28 1	67 26 22 5	89
P20 – Corazón de la Isla	7 (7)	S72 , S73 , S74 , S75 , S76 , S77 , S78	54 28 02 8	67 33 51 6	120
P21 – Vega antes de la reseva	3 (0)	S8, S9, S10	54 27 29 6	67 27 01 4	142
P22 – Puerta de la Reserva	4 (4)	S2 , S4 , S5 , S6	54 27 50 5	67 30 28 4	124
P23 – Vega de la reserva	4 (4)	S20 , S60 , S79 , S80	54 27 38 1	67 32 03 1	115

^aNumber of plants analysed by molecular markers; in brackets the number of plants characterized also by phenotypic traits.

^bIn bold the codes of plants characterized by phenotypic traits.

with the following settings: 1,000,000 iterations with 100 thinning intervals and a burn-in period of 250,000, using the correlated allele frequencies model. The maximum number of populations was set to 20. A map of posterior probabilities (membership) was obtained by Post Process Chain and Post Tesselation functions into Geneland by tesselling the landscape at a resolution of 1 m.

Morphological characterization

From a subset of 39 shrubs, data regarding plant phenotype were directly observed and/or measured as follows (Gambineri 2012):

Spines clusters (Spin): number of tips per cluster, perimeter (mm) and projection of area (m²) of each spine taken from 12 clusters per plant (three from apical, median and basal parts, respectively).

Leaves: major axis length (MaAL) and minor axis length (MiAL) (mm), perimeter (Perim) (mm), projection of area (Area) (m²), elongation (Elon) (ratio of the length of the major axis to the length of the minor axis), roundness (Roun) [$(4\pi \times \text{area}) / \text{perimeter}^2$] and compactness (Comp) (sqrt of $(4 \times \text{area} / \pi) / \text{major axis length}$), R (ColR), G (ColG) and B (ColB) colour coordinates (calculated by an electronic colorimeter (RGB-PCE Instruments, Lucca, Italy), on 20–40 leaves per plant from apical, median and basal parts of shoots. UTHSCSA ImageTool software (San Antonio, TX, USA) was applied on pictures taken on leaves and spines to measure biometric parameters on leaves and spines.

Fruits: fresh weight (FWFr) (g), dry weight (DWFr) (g) and percentage of dry weight on fresh weight (D-FFr) from 25 fruits per plant. The fruits were dried in an oven at 50°C for 7–10 days until constant weight was reached.

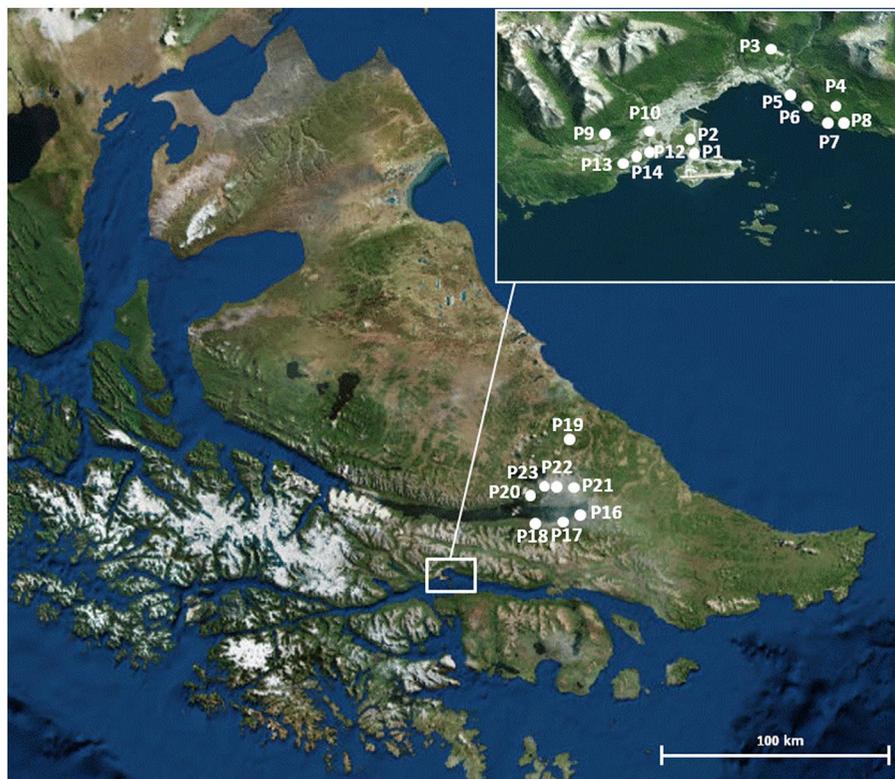


Figure 1. Geographic location of the studied populations of *Berberis microphylla* G. Forst. along Tierra del Fuego island.

Minimum, maximum, average, standard deviation and coefficient of variability (%) values were calculated for the whole set of plants and for each putative population in order to analyse the diversity of the subset of 39 specimens. Average values were standardized and used for the Principal Component Analysis and the Cluster Analysis (SPSS 20 and XLStat software's), two statistical multivariate approaches suitable for analysing relationships between individuals or groups of specimens within a population.

Environmental factors

The plants were selected to include diversified stands (e.g. lake and marine coast, hill, farmland and urban areas), comprising isolated, single-species patches and isolated, mixed species patches. Four parameters related to environmental conditions (namely elevation, light, wind and land slope) have been associated to each plant of the 23 putative populations. Elevation of stands was measured in m above sea level with a Garmin 12 100014600 2.6-Inch Portable and specimens were attributed to two classes of altitude (below and over 80 m). Light intensity was estimated as percentage of light received by each plant of a putative population by taking as reference the historical average photon flux of the period December–January ($2197.1 \pm 498.20 \mu\text{mol m}^2 \text{s}^{-1}$) (Martínez Pastur et al. 2007) and classifying the results into one

of the three groups (Low < 50%; Medium 50–85%; High > 85%). Wind intensity on plants was subjectively estimated taking into account their exposition to the predominant winds of the area; in relation to its mean value, each population was attributed to one of the following classes: Low (when plants were protected from the predominant winds by high, long and wide physical objects such as rocky wall formations, high tree colonies; Medium (when specimens were protected by medium size natural objects); High (when plants were totally unprotected by natural objects (e.g. flat and coastal areas; plants in open stands and absence of vegetal and/or rocky barriers). Land slope was measured and expressed in degrees for each plant stand; each population was ascribed to one of the following classes of slope declivity (Low < 15°; Medium 15–30° – High > 30°) in relation to its average.

Results

Plant sampling and spatial distribution

The results of plant sampling and spatial attributes of the stands of each putative population are shown in Tables I and II. The geographic distance matrix (data not shown) indicates that sampled plants were distant from few metres (≈ 3.5 m) up to ≈ 83 km; while distances between putative population ranged from 81 m to 82 km. Taking into account the whole

Table II. Spatial dispersal and environmental characteristics of the stands of the 23 putative populations of *Berberis microphylla* G. Forst. analysed in Tierra del Fuego island.

Population code and name	Number of pairs of specimens	Average distance among pairs of specimens (m)	CV%	Minimum distance among pairs of specimens (m)	Maximum distance among pairs of specimens (m)	Light intensity	Wind intensity	Slope declivity
P1 – Campo PR CADIC	6	29	54	8	51	High	High	Low
P2 – CADIC Casa Solar	3	21	31	15	27	High	High	Low
P3 – Monte Olivia	10	432	77	9	746	Medium	Medium	Low
P4 – Playa Larga Alto	1	25	–	–	–	High	High	High
P5 – Playa Larga Inter-media	1	23	–	–	–	High	Medium	Medium
P6 – Playa Larga Baja	6	37	81	5	66	Medium	Medium	Low
P7 – Playa Larga pre albardón	3	58	52	28	88	High	Low	Low
P8 – Estancia Tunel	0	–	–	–	–	Low	Low	High
P9 – Camino Parque-Club Hípico	15	49	47	4	100	High	High	Low
P10 – Acceso Rugby Club	3	16	44	8	22	Low	Low	Low
P11 – Subida pista sky – C.A.	10	123	71	12	219	Medium	Medium	Medium
P12 – Camino Hotel Yámanas	2	19	–	–	–	Medium	Medium	Low
P13 – Camino Hotel Los Cauquenes	3	37	19	30	45	Medium	Medium	Low
P14 – Frente Ray	0	–	–	–	–	High	High	Low
P15 – Bajada del Martial	3	21	62	5	28	High	High	Low
P16 – Margen lago Fagnano	10	109	57	22	211	High	Medium	Low
P17 – El quemado	3	94	78	9	141	High	High	Low
P18 – Ruta 3 pasando Escondido	10	25	52	8	52	High	High	Low
P19 – Ruta F o H	10	99	59	12	206	High	High	Low
P20 – Corazón de la Isla	21	46	63	5	98	High	Medium	Low
P21 – Vega antes de la Reseva	3	53	53	22	78	High	High	Low
P22 – Puerta de la Reserva	6	32	53	10	56	High	Medium	Low
P23 – Vega de la Reserva	6	60	77	6	90	Low	Medium	Low

set of analysed plants, the average of the distances between pairs of specimens within each putative population ranged from 16 m (P10) to 432 m (P3) with an average of 67 ± 89 m (coefficient of variation 133%). Most (33%) of putative populations showed an average distance between pairs of specimens of 25 m, while 29 and 24% of populations had average distances in the ranges 25–50 and 50–100 m,

respectively. Only 14% of cases of plant pairs were observed for distances longer than 100 m.

DNA genotyping and genetic analysis

The dendrogram obtained from the similarity matrix adopting Jaccard's coefficient and the UPGMA clustering method (Figure 2) shows a complex



Figure 2. Results of Geneland analysis of the population of *Berberis microphylla* G. Forst. along Tierra del Fuego island showing the spatial organization into five clusters (named C1, C2, C3, C4, and C5): (a) spatial organization of the population in proximity to Ushuaia city; (b) spatial organization of the population in sites around Escondido Lake.

pattern with low bootstrap values; nevertheless, the clustering has a good fit (cophenetic correlation equal to 0.815) with the distance matrix. Taking into account the dendrogram and the similarity matrix among genotypes (data not reported) in relation to the putative sampled populations indicated in Table I, on the 17 original populations with at least three plants, 12 of them show no matching clustering. On the other hand, populations P1, P5, P6, P16 and P17 have at least one pair of plants grouped in the same cluster, showing very low genetic distances among them. For instance, P17 is totally contained by one cluster, with three plants resulting identical (Jaccard's similarity index equal to 1); P16, holding five plants, resulted very well represented in the dendrogram, showing a

complex pattern with two identical plants (S61 and S64, and the remainder S62, S63 and S65 being more distant).

In order to perform the landscape genetic analysis, the plants sampled from 23 putative populations were considered as forming a single large population and then the significant possibility of a subdivision in genetically homogeneous groups was estimated by Geneland software. The population of calafate in Tierra del Fuego appears divided into five clusters lacking any geographical structure (Figure 3). The Cluster 5 shows a value of genetic diversity (D_S) among plants equal to 0, and a very low value of genetic divergence with the Cluster 1 ($F_{ST} = 0.064$) (Table III).

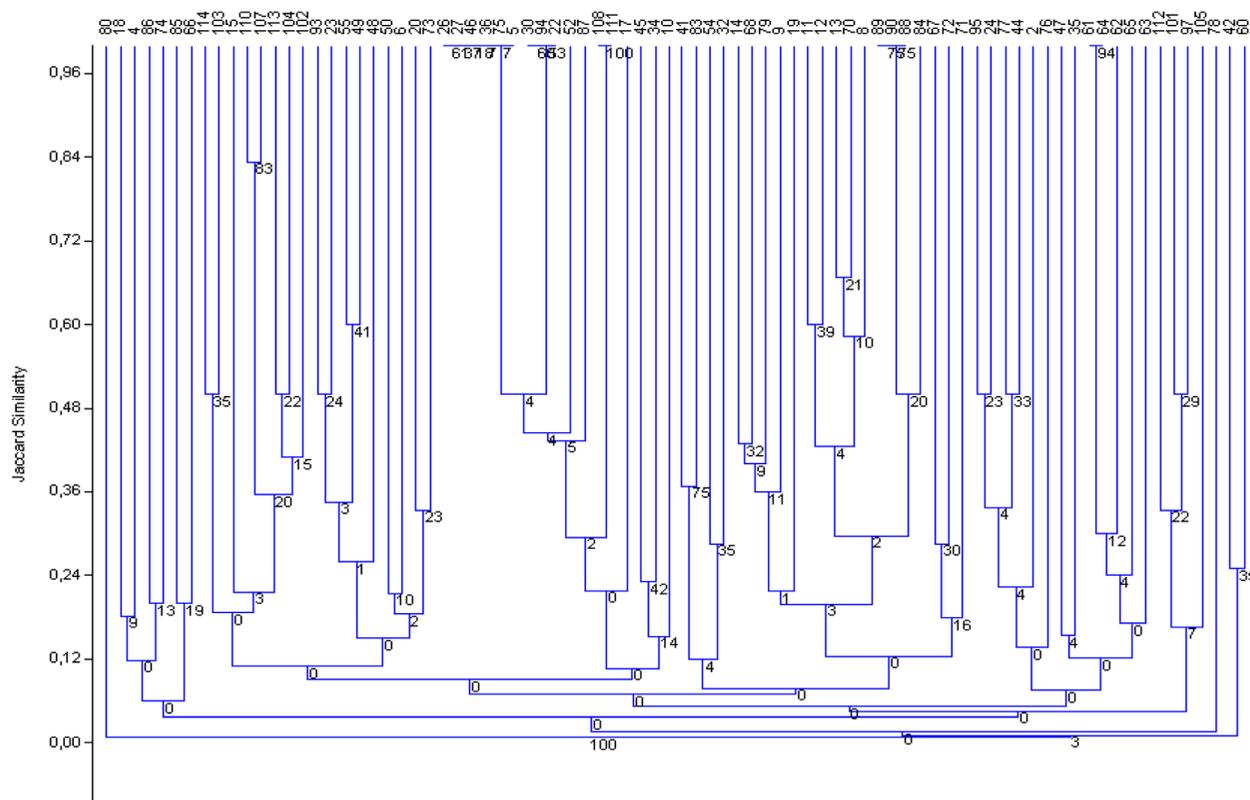


Figure 3. Dendrogram (UPGMA method) obtained from the RAPD data by Jaccard's coefficient (Jaccard similarity) of the 82 *Berberis microphylla* G. Forst. specimens individuated in Tierra del Fuego.

Note: Cophenetic correlation 0.815.

Table III. D_s (gene diversity corrected for sample size; Nei 1972) of five clusters and matrix of pairwise F_{ST} among clusters in the population of *Berberis microphylla* G. Forst. analysed in Tierra del Fuego (above diagonal).

Cluster	C1	C2	C3	C4	C5
	$D_s = 0.139$	$D_s = 0.098$	$D_s = 0.100$	$D_s = 0.052$	$D_s = 0.000$
C1	–	0.138	0.025	0.067	0.064
C2		–	0.189	0.351	0.453
C3			–	0.076	0.102
C4				–	0.337
C5					–

Morphological characterization

The morphological diversity from leaves, spines and shoots of the subset of 39 plants is summarized in Table IV. Some populations showed higher values of the coefficient of variability than those observed for the set of all the specimens. For instance, P1 showed the highest value for leaf area (CV 52.73%), P16 for leaf perimeter, P22 for roundness and compactness (Table IV), while P9 showed values of variation for all the traits lower than those found for the other populations. P19 and P22 plants showed the greatest number of spines per shoot, which resulted statistically different from the other populations. The average of leaf area ranged from 0.30 to 0.55 cm², respectively, for P1 and P19. Significant differences between the populations were observed also for leaf shape. Major axis length was greater on leaves of P18 and P22 plants while P22 plants showed

higher minor axis length of leaves than the values of populations P16, P19, P20 and P23. Fruit dry and fresh weight ranged from 0.05 and 0.17 g to 4.9 and 15.0 g, with average values of 1.76 ± 0.9 and 7.7 ± 3.7 g, respectively. The DW/FW ratio of fruits varied from 17.6 to 33.6% (mean value 23.7 ± 4.6).

The application of Principal Component Analysis on the whole standardized morphological data-set obtained from shoots, leaves and fruits showed that the first, second and third components expressed the 29.5, 19.6 and 16.3% of variability, respectively, for a total cumulative variability of 65.4%, indicating a medium level of correlation between the morphological variables. No significant values of correlation were observed among the variables of different organs, while most of dimensional traits (area, perimeter, axis length), shape indexes (roundness and compactness) and colour (R, G,

Table IV. Descriptive statistics of leaf parameters of *Berberis microphylla* G. Forst. related to the total amount of studied specimens (TS) and the eight presumed populations (P) with more than four plants.

Parameter	Statistics	TS	P1	P9	P16	P18	P19	P20	P22	P23
<i>n</i>		1953	187	225	241	201	46	372	179	218
Area (cm ²)	Minimum	0.04	0.04	0.08	0.04	0.10	0.15	0.07	0.12	0.05
	Maximum	1.48	1.48	1.13	1.06	0.84	0.42	0.97	1.01	0.78
	Average	0.47	0.55 ^a	0.51 ^a	0.40 ^b	0.43 ^b	0.30 ^c	0.42 ^b	0.45 ^{ab}	0.39 ^{bc}
	Standard deviation	0.23	0.29	0.19	0.20	0.15	0.06	0.17	0.2	0.13
	CV%	49.00	52.73	38.27	50.57	34.90	21.15	40.76	43	33.53
Perimeter (cm)	Minimum	1.16	1.62	1.16	1.18	1.66	1.61	1.21	1.45	1.46
	Maximum	14.06	12.22	12.80	11.16	10.50	7.10	13.81	12.91	9.78
	Average	4.02	3.99 ^{ab}	4.03 ^{ab}	3.83 ^{ab}	4.27 ^a	3.33 ^b	3.97 ^{ab}	4.22 ^a	3.70 ^{ab}
	Standard deviation	2.07	1.62	2.01	2.11	2.11	1.56	2.20	2.71	1.84
	CV%	51.34	40.46	49.77	55.07	49.43	46.72	55.50	64	49.67
Major axis length (cm)	Minimum	0.34	0.42	0.46	0.34	0.63	0.66	0.49	0.60	0.52
	Maximum	4.22	3.15	3.51	3.30	3.15	2.17	3.83	3.86	2.83
	Average	1.41 ^{ab}	1.40 ^{ab}	1.38 ^{ab}	1.35 ^{ab}	1.51 ^a	1.15 ^b	1.42 ^{ab}	1.44 ^a	1.30 ^{ab}
	Standard deviation	0.53	0.42	0.46	0.55	0.56	0.33	0.55	0.69	0.44
	CV%	37.44	30.01	33.36	40.79	37.00	28.79	39.21	48	33.39
Minor axis length (cm)	Minimum	0.21	0.22	0.22	0.21	0.21	0.32	0.21	0.25	0.26
	Maximum	2.82	2.41	2.32	2.49	2.40	1.52	2.82	2.47	1.93
	Average	0.73	0.74 ^{ab}	0.77 ^{ab}	0.68 ^b	0.70 ^{ab}	0.63 ^b	0.70 ^b	0.83 ^a	0.68 ^b
	Standard deviation	0.42	0.32	0.37	0.43	0.44	0.30	0.45	0.56	0.39
	CV%	57.62	42.91	48.57	64.04	62.46	48.75	64.74	67	58.19
Elongation	Minimum	0.13	0.18	0.14	0.13	0.10	0.41	0.13	0.29	0.18
	Maximum	9.93	0.95	0.89	0.93	0.96	0.82	0.93	0.95	0.94
	Average	1.24	0.52 ^{ab}	0.53 ^{ab}	0.49 ^{bc}	0.46 ^{bc}	0.53 ^{ab}	0.48 ^c	0.54 ^a	0.50 ^{bc}
	Standard deviation	1.04	0.16	0.14	0.15	0.16	0.10	0.14	0.12	0.13
	CV%	29.26	31.29	26.22	31.28	34.13	19.67	30.26	23	26.50
Roundness	Minimum	0.05	0.07	0.06	0.06	0.06	0.08	0.05	0.06	0.06
	Maximum	0.89	0.87	0.88	0.96	0.89	0.76	0.87	0.88	0.83
	Average	0.49	0.50 ^{ab}	0.54 ^{ab}	0.46 ^{ab}	0.42 ^c	0.50 ^{abc}	0.48 ^b	0.55 ^a	0.48 ^{ab}
	Standard deviation	0.24	0.22	0.25	0.24	0.21	0.25	0.22	0.29	0.22
	CV%	48.16	45.02	45.93	50.63	50.49	50.27	46.97	53	44.75
Compactness	Minimum	0.25	0.29	0.29	0.27	0.26	0.32	0.25	0.26	0.29
	Maximum	0.90	0.83	0.88	0.82	0.90	0.72	0.87	0.83	0.77
	Average	0.57	0.59 ^{ab}	0.61 ^a	0.56 ^b	0.53 ^b	0.57 ^{ab}	0.55 ^b	0.58 ^{ab}	0.56 ^b
	Standard deviation	0.14	0.12	0.15	0.14	0.14	0.13	0.14	0.16	0.12
	CV%	24.55	21.02	24.15	25.52	27.36	22.93	24.82	29	21.38
Spines	Minimum	1	1.00	1.00	1.00	1.00	3.00	1.00	3.00	1.00
	Maximum	3	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
	Average	2.82	2.88 ^b	2.77 ^b	2.68 ^b	2.73 ^b	3.00 ^a	2.79 ^b	3.00 ^{ab}	2.94 ^a
	Standard deviation	0.53	0.41	0.56	0.73	0.61	0.00	0.58	0.00	0.32
	CV%	19.00	14.00	20.00	27.00	22.00	0.00	21.00	0.00	11.00

Notes: *n* = number of leaves. Different letters for the average of each parameter indicate a statistical difference for $p \leq 0.05$.

B) of leaves resulted positively associated ($r > 7$) within each category (data not shown). A graphical relationship among original morphologic variables is shown in Figure 4, together with the dispersion of the 39 studied *B. microphylla* G. Forst. specimens plotted against the first two principal components.

The average Euclidean distance between the 39 analysed shrubs based on morphological traits resulted equal to 4.99, with the lowest value (1.16) observed for the couple S60 and S76 belonging to P23 and P20, respectively, and the highest (10.31) in the pair P22-S6 and P6-S94. The morphological dissimilarity dendrogram (Figure 5) showed three main aggregations. The widest cluster (I) held 19 specimens, and in particular all those belonging to populations P2 (S113 and S114), P18 (S83, S84, S85, S86, S87), P23 (S20, S60, S79 and S80). More in detail, all the specimens of P18 resulted agglomerated in a sub-cluster, while the specimens of P2 and P23 were dispersed in various sub-sets. Cluster II was not associated to specific populations

and cluster III held P2 and a relatively high number of specimens belonging to P9, P19 and P22.

Environmental factors

Seventeen of the 23 studied stands were located above 80 m a.s.l.; 15 of them were exposed to strong light intensity (85–100% of total photon flux), while 10 were highly exposed to predominant winds and 19 were placed on sites with low declivity (<15%).

Elevation

Taking into account the two classes of altitude of plant growth sites (below and above 80 m a.s.l), leaf elongation resulted significantly affected ($p < 0.05$; ANOVA), with average values of 0.51 ± 0.05 and 0.57 ± 0.12 for high and low sites, respectively. Among all the remainder morphological characteristics, altitude exerted a statistically significant effect ($p < 0.01$) only on the percentage of dry weight on

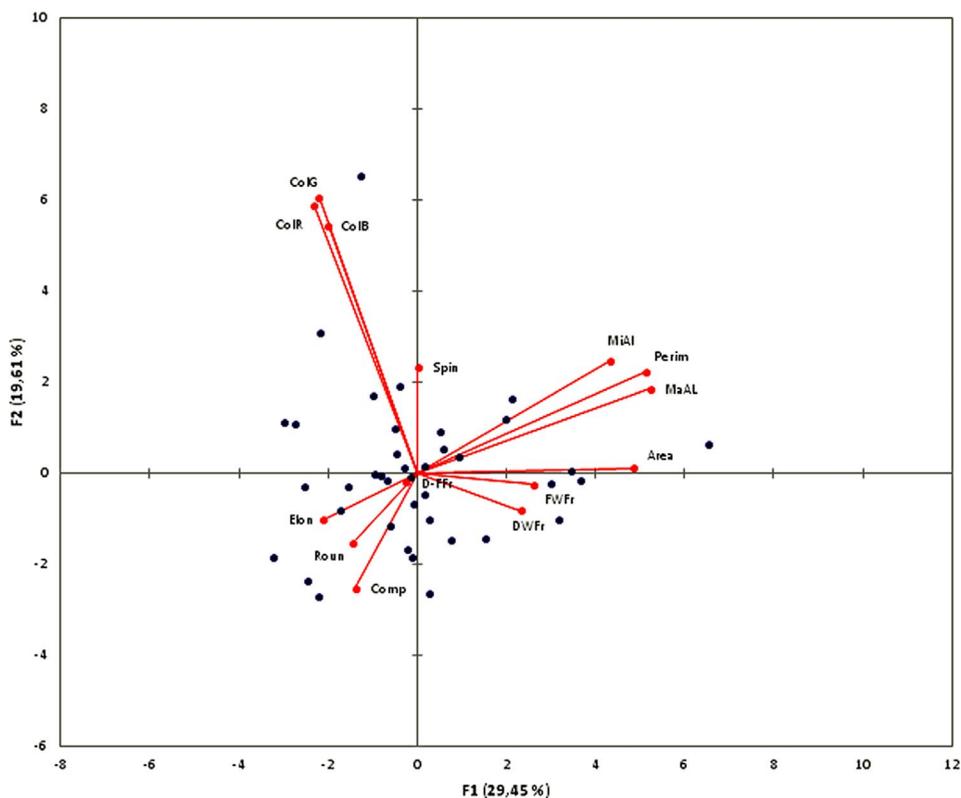


Figure 4. Morphological variables and *Berberis microphylla* G. Forst. specimens plotted on the two first Principal Components representing 49.6% of variability.

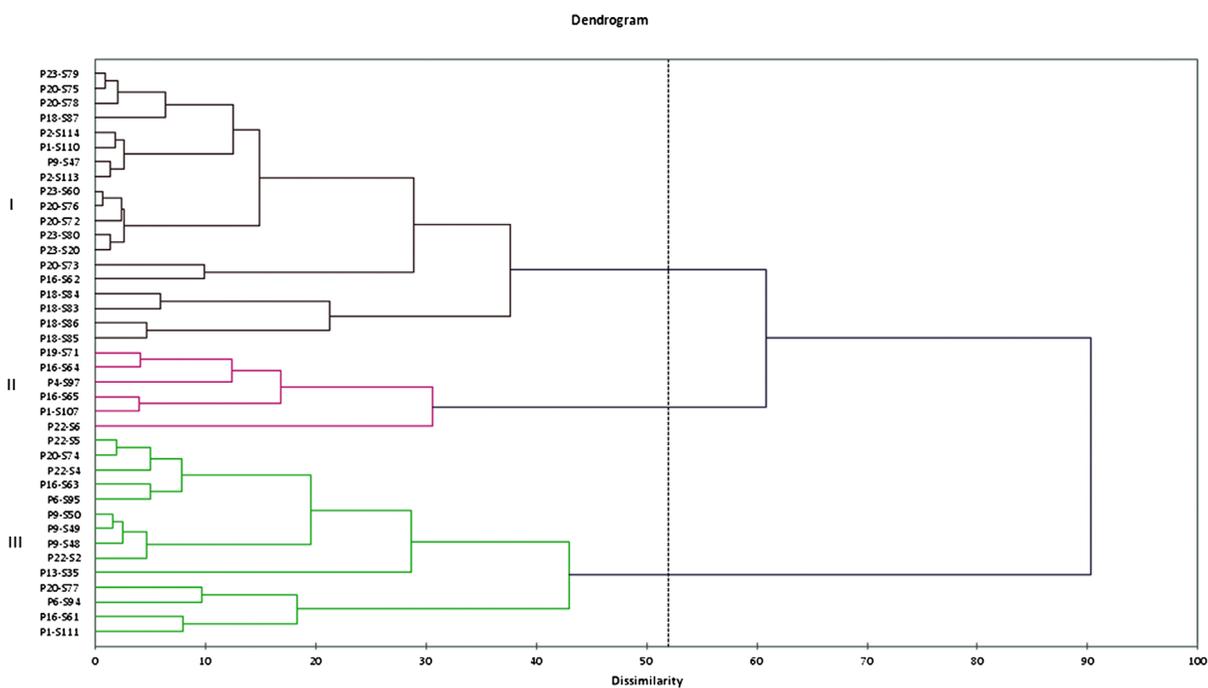


Figure 5. Dendrogram (Ward's agglomeration method) obtained from the Euclidean distance matrix calculated on morphological traits of shoots, leaves and fruits for 39 specimens of *Berberis microphylla* G. Forst. individuated in Tierra del Fuego. Notes: P = population; S = specimen.

fresh weight (D-FFr) (21.7 ± 4.17 and $25.9 \pm 4.12\%$ for high and low locations, respectively).

Wind

This factor influenced very significantly ($p < 0.01$) leaf compactness and the percentage of dry weight on fresh weight of fruits. The highest mean values for both parameters were observed on populations located in very windy areas (0.59 ± 0.02 and 28.1 ± 3.1 , against 0.56 ± 0.03 and 22.1 ± 4.0 , respectively).

Light intensity

Light intensity exerted a significant effect ($p < 0.05$; ANOVA) on leaf roundness and compactness, being 0.52 ± 0.04 and 0.48 ± 0.03 the average values for high and low sunlight intensity for the first parameter, while those for the second one resulted 0.59 ± 0.02 and 0.56 ± 0.02 .

Slope

Shrubs growing on flat areas were characterized by leaves with higher average values of perimeter (4.02 ± 0.63 mm), minimum axis length (0.73 ± 0.12 mm) against those located on slopes (3.28 ± 0.63 mm of perimeter and 0.47 ± 0.48 mm of minimum axis length) ($p < 0.05$; ANOVA); conversely, leaf elongation average values resulted higher (0.75 ± 0.08) in plants sited in inclined areas ($p < 0.01$; ANOVA).

Discussion

Genetic characterization related with geographic distance and site characterization

The topography of the obtained dendrogram shows the almost total absence of structuration in evident and significant clusters, with low bootstrapping values (Farias & Jaksic 2006), indicating a high level of genetic diversity among the studied samples. The observed low genetic distances among plants belonging to populations P1, P5, P6, P16 and P17 could be explained in part by the geographic distance among plants of the same population. For example, the geographic distance among plants 90 and 88 of P17 is close to 10 m, while the geographic distance among pair 108 – 111 of population P1 is about 40 m, and it resulted near to 140 m among plants 61 and 64 of P16. These results could suggest that even if the geographic distance among plants is not so small, the specimens of the mentioned populations could originate from vegetative propagation, one of the two colonization systems of this species together with sexual

reproduction. Nevertheless, the predominance of one of the two strategies of colonization may depend on different biotic and abiotic factors, as reported for *Berberis nervosa*, a species growing in similar climatic conditions in north America (Huffman & Tappeiner 1997). Conversely, the low genetic distances among plants 26 (P5), 27 (P5), 46 (P9), 36 (P14), 75 (P20) and 5 (P22) could not be explained by the geographic distance since the specimens and populations are quite faraway (e.g. P5 and P9 are 11 km apart).

Taking into account the results of Geneland analysis, no geographical structure was evidenced, nevertheless cluster C4 (including P1, P2, P3, P4, P5, P6 and P7) is solely located in the area of Ushuaia (Figure 2). Among the putative populations, P17 which is composed by only three plants, all with the same genotype, was represented by Cluster 5 ($D_s = 0.000$). In this case, clonal propagation strategy could be hypothesized, average plant distance is about 35 m and fire could have had a relevant influence on P17. In fact, P17 is located in an area called 'El quemado' = 'The burnt place', and fire may have emphasized asexual reproduction of this set of plants and hinder the development of seedlings. To our best knowledge, there is no information about the effect of fire events on *Berberis* seed germination. At this regard, Cavallero and Raffaele (2010) observed that fire affected the reproductive phenology of *B. buxifolia* shrubs which flowered and fruited only two years after fire had occurred, in contrast with other small trees that did not show any evidence of sexual reproduction in the burned area, implicitly demonstrating the vegetative origin of flowering shoots. The positive stimulation of fire on woody plant vegetative propagation has been hypothesized also in other environmental conditions and for different species (Hoffmann 1999).

The low F_{ST} value observed between C5 and C1 can be associated to a high gene flow between these two clusters. P16 (Cluster 2) is a putative population genetically isolated, with the highest levels of genetic divergence than all subsets (Table III). In fact, the botanical composition of the location is completely different from the other sites of plant sampling, with environmental characteristics allowing the growth of *Ribes* spp., with the maximum P content in the soils compared with the other populations and with high pH values. Interestingly, this cluster has the highest level of divergence with the Cluster 5 ($F_{ST} = 0.453$) despite its geographical proximity (Figure 3).

Morphological characterization related with geographic distance and site characterization

As previously mentioned, only 39 shrubs were morphologically characterized from the original set of

82 plants. The low genetic distance among plants 75 (P20) and 5 (P22) were not correlated with the morphological characterization and plants 75 and 5 resulted located in different clusters (namely I and III, respectively). Similarly, the low genetic and geographical distances among plants 61 and 64 of P16 were not correlated with the morphological distances.

All the plants of the populations P2, P18 and P23 were grouped in the same cluster I, while the two plants of the P6 were matched to the cluster III. The fact that all the plants of the mentioned populations were grouped in the same morphological cluster could indicate that the low geographic distance and the similar site characteristics could influence the plant morphology. However, plants of populations P1, P9, P16 and P20, which have a high geographical distance among them, are clustered in the same group, as occurs in the three groups. The marked morphological differences among genotypes of the same population could be explained in this case by the predominance of allogamy (Suárez 2015) and seedling recruitment on clonal expansion by rhizomes as the reproduction system of this species.

Environmental factors are external forces either living (biotic) or non-living (abiotic as climatic, physiographic and edaphic factors) that affect the life of the organisms. Environmental factors do not act individually, but many factors interact to influence the existence and success of an organism, known as interaction of environmental factors. The intensity, importance and time scale of factors, however, vary with organisms and ecosystem types (Agrawal 2007). Numerous studies show a matching of leaf physiology and morphology to environment. In addition, principles of water use efficiency, heat and gas exchange result in an optimal leaf form for a given environment (Bonan 2002). Leaf area of P1 was the broadest among all the studied populations; the number of spines per shoot resulted highest in P19 and P23 shrubs, while leaf area, perimeter and major axis length were minima in such populations. These results could be explained due to the different climatic conditions among the studied populations. Plants growing in P19 and P23 are exposed particularly to strong winds and consequently to lower temperatures, and, according to Bonan (2002), leafy plants growing in arid environment of deserts or cold arctic and alpine environments have small leaves. In part, this could be related to the influence of leaf dimension on leaf boundary layer resistance and the efficiency with which heat and moisture are transported away from a leaf. For a given wind speed, boundary layer resistance decreases with smaller leaf size or deeper lobes, decreasing the surface area of a leaf relative to its perimeter length, with a greater heat and moisture transfer. Small leaves provide

a thin boundary layer and result in efficient heat transfer (Bonan 2002), while large leaves have a thick boundary layer and inefficient heat transfer. These results could indicate the fact that the size and shape of leaves is an example of at least a compromise between leaf energy exchange and leaf temperature, and photosynthesis.

It is known that some abiotic and biotic factors change with the altitude, being the temperature and associated variables the most changing ones (Korner 2007; Massaccesi et al. 2008; Normand et al. 2009; Moretto & Martínez Pastur 2014), although the warmth index, Bailey's index, photosynthetically active radiation, coldness index, ultraviolet B and the annual precipitation dominantly affects the characteristic parameters of leaves and fruit (Luo et al. 2014). This phenomenon could explain the leaf elongation and dry fruit weight/fresh fruit weight variations found when comparing these traits in plants growing below and above 80 m asl as reported for *Malus* (Luo et al. 2014). In effect leaf elongation and dry fruit weight/fresh fruit weight ratio were 1.1 and 1.2 higher, respectively, in plants growing below 80 m asl compared with those growing at higher altitude. These results can be associated to the fact that most of the studied populations growing at low altitude are located in the southern areas of Tierra del Fuego where the climate is less rigorous than in the northern part.

Wind is an important environmental factor as it governs transpirational water loss from vegetation, dispersal and dissemination of seeds and pollination in plants. Wind velocity varies at different geographical situation and along vegetation types (Agrawal 2007), and it could explain that leaves of plants exposed to high wind velocity have a leaf compactness 1.1 higher than leaves of plants growing in site less exposed to winds. On the other hand, fruits of plants exposed to high wind velocity have a dry weight/fresh weight ratio of 1.3 higher than fruits of plants growing in sites less exposed to winds, probably due to a higher evapotranspiration of the plants growing in high wind sites.

Light environment constitutes a key factor in the performance of photosynthetic organisms, inducing dramatic changes in plant phenotypes. At the leaf-level, phenotypic acclimation to light conditions involves the adjustment of both morpho-functional (e.g. leaf size, leaf angle) and physiological (e.g. photosynthetic rates, pigment pools) traits. Despite the differences in their functioning, both types of traits are expressed in a coordinated fashion, and allow the expression of phenotypes suitable to particular environmental conditions (García-Verdugo et al. 2010). Leaf size and shape (via effects on boundary layers) also influence rates of photosynthesis and water loss (Read & Stokes 2006). The diversity of

shape suggests that there is not a unique ecological strategy that is dependent exclusively on leaf shape. Even within a single genus, leaf shape variation can be great and adaptations to optimize light interception are previously reported in angiosperms (Nicotra et al. 2011). In effect leaf roundness and compactness were 1.1 higher in plants growing with high light compared with those growing at lower light environments, which could be in accordance with the observed in *Quercus acutissima* where the elongation index decreased when the light intensity increased (Xu et al. 2009).

As mentioned above, environmental factors do not act individually, but many factors interact to influence the existence and success of an organism, known as interaction of environmental factors. Heat, moisture, air movement and light vary from hill to mountain slopes, valleys, and surface of the ground and beneath vegetation, thus creating a range of climate. Steepness of the slope is also important because of high speed air movement and poor soil drainage (Agrawal 2007), explaining the higher leaf perimeter in low slope sites (1.2 higher) compared with high slope sites.

A very low correlation ($r = 0.007$; $p > 0.05$; Mantel test) between the genetic and morphologic distance matrixes among the 39 specimens has been observed in our study. Weak relationships between distances estimated from molecular and morphological data have been observed in various studies adopting different sampling strategies and methods of analysis applied on different plant species grown under diverse selection pressure and environmental conditions (Fanizza et al. 1999; Elias et al. 2001; Hamza et al. 2011; Alves et al. 2013). Such results may be explained by a possible different effect of natural and human selective actions on molecular and morphological profile of plant specimens; furthermore, morphological traits are heavily affected by the environment, whereas molecular markers are not subject to such variation (Collard et al. 2005).

Conclusions

This research was developed using plant samples from putative populations growing in very different environmental conditions, and the comparison of phenotypic characteristics showed a wide diversity between and within the putative populations. These morphological differences observed on the tested plants were not associated to their genetic distances. No geographic structure was observed on the population, nevertheless some subpopulations obtained from Geneland analysis resulted absolutely uniform, indicating a prevalence of asexual propagation (i.e. C5 – P17), while C1 (which included seven putative

populations) showed a high genetic diversity. The genetic divergence was the lowest between subpopulations C1 and C3, notwithstanding the very different environmental conditions of their locations. On the contrary, P16 and P17, included in individual clusters, showed the greatest genetic divergence. However, a significant correlation among some morphological characters with environmental factors (wind, elevation, light and slope) could indicate that *B. microphylla* plants could exhibit a remarkable phenotypic plasticity. The achieved results represent a suitable tool for the selection of genotypes for the conservation of genetic resources and for breeding of calafate.

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