

Genetic diversity and population structure of Chilean blueberry *Gaultheria pumila* (L.f.) D.J. Middleton (Ericaceae)

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ABSTRACT

Gaultheria pumila (L.f.) D.J. Middleton is a native shrub of Chile that produces edible berry fruits. This species is related to the cultivated *Vaccinium* species; for this reason it is currently called Chilean blueberry locally. Although *G. pumila* has important attributes, it has been largely ignored, and remains an unexplored genetic resource. This study investigates the genetic diversity to support the efforts to domesticate the species. Sampling was carried out in 11 sites collected from four Regions of Chile. In total, 160 individuals were collected and analyzed using a set of 10 simple sequence repeats (SSRs) markers. The average observed heterozygosity was $H_o = 0.50$, while the expected heterozygosity was $H_e = 0.46$. The fixation index (F_{IS}) showed an average of -0.07 , and the proportion of differentiation among populations (F_{ST}) was 0.11 . The average level of polymorphic loci in all populations (PPL) was 96.97% . AMOVA showed that the genetic diversity among populations was very low ($\Phi_{PT} = 6\%$). Significant correlations were found between genetic and geographic distance. Multivariate and Bayesian analyses identified two genetic groups. These results will be very useful to support the efforts to domesticate and increase the value of this species.

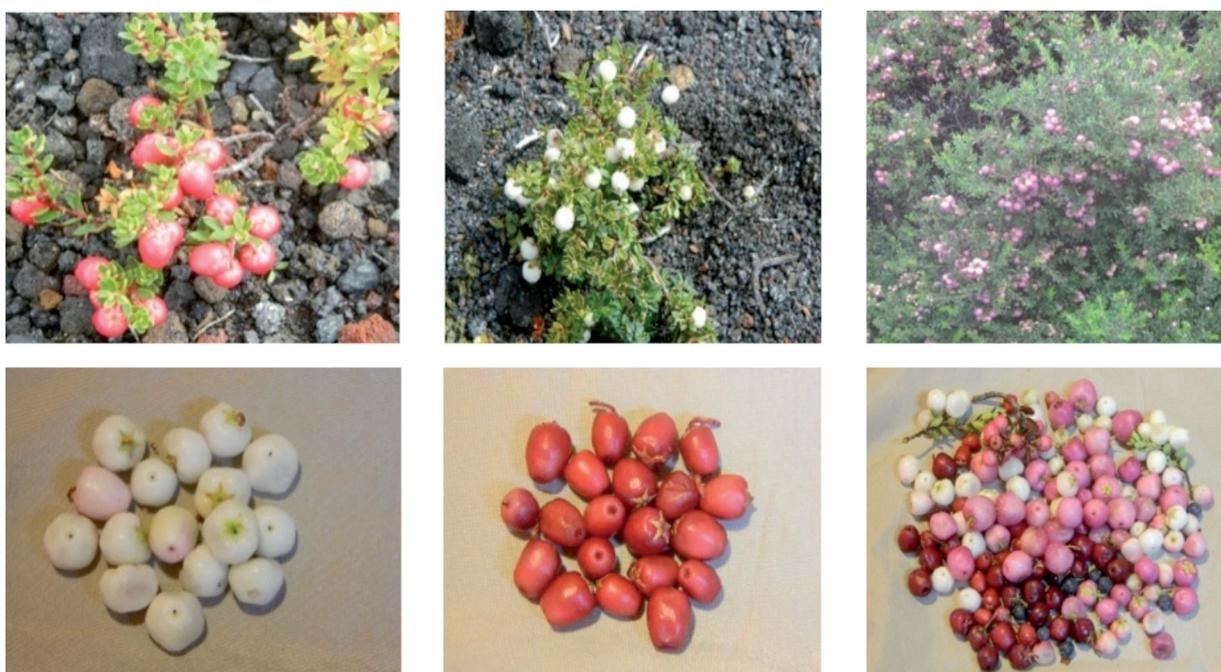
Key words: Allele frequency, AMOVA, *Gaultheria pumila*, genetic diversity, heterozygosity.

INTRODUCTION

The genus *Gaultheria* belongs to the family Ericaceae; it is adapted to tropical and temperate climates. It is important in terms of functional human nutrition, as many of its species contain high levels of flavonoids and simple phenols (Villagra et al., 2014) and also have different compounds with anti-rheumatic effects (Liu et al., 2013). *Gaultheria pumila* is diploid, but other species of *Gaultheria* are tetraploid or hexaploid (Mukhopadhyay et al., 2016). The chromosome numbers in some species of the tribe *Gaultherieae* are $2n = 22, 24, 26, 36, 44, 48, 66, 88, \text{ and } 96$, indicating that polyploid species have appeared frequently during its evolutionary history (Middleton and Wilcock, 1990; Lu et al., 2019). The distribution of this genus in the American continent ranges from Mexico to Argentina (Luteyn, 2002). In Chile, 12 species of this genus have been reported, including *G. pumila* (Teillier and Escobar, 2013), which are distributed from the Metropolitan Region (33° S lat) to the Magallanes y la Antártida Chilena Region (53° S lat). However, other authors express that *G. pumila*

is distributed from Valparaíso Region (Rodríguez et al., 2018). The species *G. pumila* is a dioecious evergreen shrub, commonly known locally as “chaura” and “mutilla del zorro”. Currently, chaura is considered as the Chilean blueberry due to its characteristics; its properties are similar to those of *Vaccinium* spp. It has been observed that *G. pumila* has a high degree of adaptability to extreme conditions and can even be found under snow in the winter or exposed to high temperatures and UV radiation during the summer. This species has a symbiotic mycorrhizal association (Medina et al., 2009); it is a colonizing plant, being one of the first to appear in areas affected by disasters (Luteyn, 2002). *Gaultheria pumila* is exploited by the native people for food and medicines (Dominguez, 2010). Fruits have different forms (globose or ovoid) with sizes between 6 and 12 mm in diameter and in white, pink and red colors (Figure 1). The fruits are very aromatic and tasty; they are flattened at the apex, fleshy, and have an intense flavor and different phytochemical content (Villagra et al., 2014). The phytochemical content of *G. pumila* depends on the genotype; red fruit have an average value of monomeric anthocyanin of 5.942 ± 0.422 g 100 g⁻¹, while pink and white fruits gave lower values of 3.854 ± 0.192 g and $0.626.2 \pm 0.41$ g (Villagra et al., 2014). Morphological differences are easily detected between individuals within the natural populations of *G. pumila*, so it becomes essential to study and identify phenotypes in germplasm collections for conservation, selection, and breeding (Ahmad et al., 2010). All these attributes of *G. pumila* make this species an interesting target for domestication, due to its potential use as a functional food. Unfortunately, little information is available about the propagation of this species out of its natural habitat, culture techniques, nutritional requirements, and even less about its genetic composition, thus this species remains an unexploited genetic resource. To take advantage of this resource species, it is necessary to develop basic studies on its genetic composition and population structure (Gepts, 2004). Molecular markers such as simple sequence repeats (SSRs) or microsatellites are a robust tool for population genetic studies in *G. pumila*. Today SSRs are available for several plant species to assess genetic diversity (Hoshino et al., 2012). In the family Ericaceae, SSRs have been used to perform molecular studies in *Rhododendron delavayi* (Wang et al., 2010), *R. simsii* (Wang et al., 2016) and *R. ferrugineum* (Delmas et al., 2011). In the genus *Vaccinium*, SSRs have allowed the evaluation of more than 1700 accessions from the National Clonal Germplasm Repository, Corvallis, Oregon, of the Agricultural Research Service of the United States Department of Agriculture (Bassil, 2012). This study aimed to determine the genetic diversity of *G. pumila* through its natural distribution. The purpose of this study was to support domestication programs and select potential genotypes for breeding programs.

Figure 1. Variability in morphometric traits in fruits of *Gaultheria pumila* collected in different sites in the volcano Villarrica, La Araucanía Region.



MATERIALS AND METHODS

Plant material and DNA extraction, PCR amplification and microsatellite analysis

Leaves of 160 shrubs of *Gaultheria pumila* were collected from 11 sites distributed in four of Chile's administrative Regions: Maule, La Araucanía, Los Lagos, and Magallanes y la Antártida Chilena (Table 1). Samples were collected from March to August 2013, from bushes separated by at least 10 m. Every site was georeferenced using the Global Positioning System (GPS). Leaf samples were immediately conserved in ice and transported to the laboratory, where they were stored at -80 °C until DNA extraction.

A sample of 200 mg of young leaves of *G. pumila* was placed in 2.0 mL tubes and frozen in liquid nitrogen. Leaf samples were macerated with the Precellis 24 and Cryolys systems (Bertin Technologies, Montigny-le-Bretonneux, France) and centrifuged at 6000 rpm in three cycles of 30 s. Total DNA extraction of all samples was performed using the cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle, 1990). Ten simple sequence repeat (SSR) loci were used according to Garcia-Gonzales et al. (2018); their main characteristics are shown in Table 2. Polymerase chain reaction (PCR) was carried out using the following steps: denaturation at 94 °C for 5 min; annealing for each primer pair (Table 2) for 45 s, extension at 72 °C for 45 s. This cycle was repeated 30 times, then a final extension at 72 °C

Table 1. Location of the sites where *Gaultheria pumila* was sampled within its range in Chile.

Regions	Nr samples	Sites	Geographical coordinates
Maule (Reserva Nacional Radal Siete Tazas)	10	Parque Inglés (PI)	35°29'420" S; 70°56'667" W
La Araucanía (Villarrica)	15	Parque de las Araucarias (PT)	39°21'570" S; 71°57'865" W
	7	El Playón (PZ)	39°34'463" S; 71°28'509" W
	22	El Mirador (EM)	39°23'209" S; 71°58'006" W
	15	Centro de sky (CK)	39°22'915" S; 71°55'565" W
	15	Parque Nacional Conguillío (CN)	39°34'400" S; 71°46'973" W
Los Lagos	9	Parque Nacional Puyehue (PN)	40°40'538" S; 72°07'103" W
	19	Camino Puyehue (CH)	40°40'599" S; 72°34'501" W
Magallanes y la Antártida Chilena	20	Punta Arenas (PA)	53°9'413" S; 71°1'437" W
	11	Reserva Nacional Magallanes (RM)	53°10'490" S; 71°10'120" W
	17	Reserva Nacional Laguna Parrillar (PL)	53°24'140" S; 71°15'099" W

Table 2. Characteristics of 10 microsatellite markers and specific primer pairs developed for *Gaultheria pumila*.

Locus	Primer sequences (5'-3')	Repeat motif	Allele size	Annealing temperature	GenBank accession
GP.7	F: CGCATTCACTCACCTCTCA R: TGGTTGGTGAAGGCTTTGGA	(ACTC)4	360	52.1	KX719822
GP.9	F: ACCCGCTCTAGATCCTCTCT R: AGGGGGAGTAATCAAGCCTC	(T)10	378-380	60.5	KX719823
GP.10	F: GGGTACGGCGTAGTGGTAAT R: TCGTACAAAACCGCCCTCAA	(AT)8	323-327	60.5	KX719824
GP.12	F: AGGATTATAGAGGCCAGGTGGA R: CAGAAGACGAAATCGAAGCCG	(CTT)4	198-201	52.1	KX719825
GP.13	F: AGAGTAAGAGCTCTCTCCGA R: GCAGACTCGAATCGGCAGTA	(ATT)4	161-278	52.1	KX719826
GP.14	F: GCATAGCCCGTGTGTCAAAC R: ACCGAAAGATCCGACCATCG	(ACGC)4	196-228	52.1	KX719827
GP.15	F: GGGCTGCTGCTCAATCAATT R: ACCCGCTTCAAGTCATGATGA	(T)10	347-351	52.1	KX719828
GP.16	F: GCTATTCTAGGGCCGGACC R: GCACAATACATAGATTCTGGATCGA	(AT)6	281-283	52.1	KX719829
GP.17	F: GAGAGAAATCCACCAGGGCA R: CAAGCGGACGACGTATACGA	(T)10	202-205	60.5	KX719830
GP.18	F: ACCGAAAGATCCGACCATCG R: GCATAGCCCGTGTGTCAAAC	(GCGT)4	192-228	52.1	KX719831

GenBank is a comprehensive public database of nucleotide sequences and supporting bibliographical and biological annotation; built and distributed by the National Center for Biotechnology Information (NCBI), a division of the National Library of Medicine (NLM), Bethesda, Maryland, USA (Benson et al., 2009).

for 30 min. PCR were carried out in a final volume of 20 μ L reaction mixture containing 10 ng DNA template, 0.15 mM each dNTP, 1 X Taq polymerase reaction buffer, 1.5 mM MgCl₂, 0.025 μ M forward primers, 0.1 μ M reverse primer, 0.1 μ M M13 primer and 0.35 U Taq DNA polymerase. PCR products were separated by capillary electrophoresis in an ABI 3130xl Prism Genetic Analyzer with POP-7 polymer (Life Technologies, Foster City, California, USA). Allele sizes were calculated with GeneMapper software v 4.0 (Applied Biosystems, Foster City, California, USA).

Statistical analysis

The genetic diversity index was estimated for each microsatellite locus and each sample. The POPGENE v. 1.31 software (Yeh et al., 1999) was used to calculate the genetic parameters (percentage of polymorphic loci, number of alleles, heterozygosity). The inbreeding coefficients for each locus (F_{IT} , F_{ST} , F_{IS}) were estimated by resampling using the jackknife method with FSTAT v. 2.9.3 (Goudet, 1994). Null allele (F Null) estimation was calculated using CERVUS (Kalinowski et al., 2007). Deviation from the Hardy-Weinberg equilibrium (EHW) and linkage disequilibrium (LD) were determined with GenePop v. 4.2 with the sequential Bonferroni correction carried out for all SSR loci. Analysis of molecular of variance (AMOVA) was applied to estimate the variance components of individuals among and within the sample sites using GenAlex software (Peakall and Smouse, 2006). The Mantel test was also carried out to determine the correlation between genetic and geographic distance. Analysis of population genetic structure was carried out through the Bayesian clustering method with the STRUCTURE 2.3.3 software (Pritchard et al., 2000) Genetic clusters ($K = 1-11$) were evaluated with ten runs per K value. The initial burn-in period was set to 100 000 with 1 000 000 MCMC iterations under the admixture model. The best K value was estimated by STRUCTURE HARVESTER software (Earl and vonHoldt, 2012).

RESULTS AND DISCUSSION

Genetic diversity analysis

Ten polymorphic SSRs were applied to evaluate 160 samples of *G. pumila* from the different natural populations (Table 2). The average of null allele frequency was very low for all loci (mean 0.10). However, SSR loci GP12 and GP16 showed significant heterozygosity; they also had the highest values of null alleles. The neutral test for all loci showed no evidence of positive selection. In the analysis of all possible binary combinations, none showed significant linkage disequilibrium (LD) after applying the Bonferroni correction ($p < 0.00003046$). The average number of alleles (N_a) was 4.20; the highest value was observed in locus GP18 (10) and the lowest value in locus GP7 (1.0). The mean of observed heterozygosity (H_o) and expected heterozygosity (H_e) were 0.50 and 0.46, respectively. H_o ranged from 0.0 (GP7) to 1.0 (GP17); H_e ranged from 0.0 (GP7) to 0.74 (GP13). The inbreeding coefficient (F_{IS}) showed negative values for the loci GP7, GP10, GP13, GP14, GP18, and five positive values for GP9, GP12, GP15, GP16, and GP17. The average F_{IS} value was -0.07 and was significantly different from zero ($P = 0.0007$). Values of proportion of differentiation among populations (F_{ST}) ranged from 0.00 (GP7) to 0.39 (GP16) (Table 3).

Table 3. Estimates of genetic diversity (per locus) in *Gaultheria pumila* based on 10 SSR loci. Observed number of alleles (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e), inbreeding coefficient (F_{IS}), proportion of differentiation among populations (F_{ST}), Hardy-Weinberg equilibrium (HW), frequency of null alleles F (Null).

Locus	N_a	H_o	H_e	F_{IS}	F_{ST}	HW	F (Null)
GP7	1	0.00	0.00	-0.20	0.00	ns	0.00
GP9	2	0.38	0.31	0.27	0.04	*	0.40
GP10	4	0.98	0.57	-0.77	0.04	*	-0.25
GP12	3	0.03	0.51	0.93	0.11	*	0.88
GP13	6	0.94	0.74	-0.44	0.13	*	-0.13
GP14	8	0.39	0.34	-0.29	0.11	ns	-0.10
GP15	3	0.44	0.57	0.36	0.10	*	0.30
GP16	3	0.25	0.54	0.24	0.39	*	0.34
GP17	2	1.00	0.53	-0.80	0.12	ns	0.30
GP18	10	0.61	0.53	-0.21	0.04	ns	-0.09
Mean	4.2	0.50	0.46	-0.07	0.11		0.11

*Significant at $p = 0.01$; ns: nonsignificant.

The lowest percentage of polymorphic loci (83%) was found in the populations of Parque Inglés (PI) in the Maule Region and Parque de Araucarias (PT) in La Araucanía Region (Table 4). The mean value of observed heterozygosity ($H_o = 0.54$) was greater than the expected heterozygosity ($H_e = 0.47$). The H_o ranged from 0.46 in PT, La Araucanía Region, to 0.66 in the Reserva de Magallanes (RM), Magallanes y la Antártida Chilena Regions. The inbreeding coefficient (F_{IS}) was positive only for two populations, Parque Nacional Puyehue (PN; 0.03) and Camino Puyehue (CH; 0.16). Other populations showed negative F_{IS} values. The average F_{IS} value for *Gaultheria* was significantly negative (-0.16; $p = 0.01$), indicating excess heterozygosity.

SSR markers have been widely used to detect genetic diversity in the different blueberries of the family Ericaceae, especially in *Vaccinium*. However, this is the first time that this kind of study, using SSR markers, has been carried out in any wild species of the genus *Gaultheria*. Genetic diversity per locus in Chilean blueberry showed that H_o ranged from 0.0 to 1.0, and H_e ranged from 0.0 to 0.74. These results are similar to those reported in other Ericaceae, such as *Rhododendron simsii* (Wang et al., 2016) and *R. ferrugineum* (Delmas et al., 2011), where H_e and H_o showed a broad range of variation. An excess of heterozygosity was observed, which can be explained by its high levels of gene flow ($N_m = 2.06$) due to its sexual reproduction by outcrossing. In contrast, only four loci (GP9, GP12, GP15, and GP16) showed a significant heterozygote deficit; a high frequency of null alleles could explain these results in part. According to Oddou-Muratorio et al. (2009), heterozygous deficiency can be affected by null alleles, which are a common cause of apparent deviations from Hardy-Weinberg equilibrium at SSR loci and can overestimate the inbreeding coefficient.

The descriptive statistics for diversity genetic over all loci were moderate ($N_a = 4.20$, $H_o = 0.50$, $H_e = 0.46$). These results could be explained by the number of individuals sampled, low polymorphism of the SSRs, isolation by distance, or the influence of factors from forest fragmentation (Kramer et al., 2008). These variability levels are similar to those reported in species with high economic and ornamental values, such as cherry *Prunus pseudocerasus* Lindl. (Chen et al., 2015) and *Prunus persica* (Pérez et al., 2020). Bantawa et al. (2011) found a high level of polymorphism (68.52%) in *Gaultheria fragrantissima* using RAPD. This level of polymorphism in this related species was later confirmed using ISSR markers (82.82%) (Apte et al., 2006).

In *G. shallon* (Wilkin et al., 2005), the percentage of polymorphic loci detected with amplified fragment length polymorphism (AFLP) reached 89%. These similarities with *G. pumila* could be explained by the fact that they are all open-pollinated species, typically inhabiting extreme environmental conditions. These authors considered that this level of genetic diversity could be an excellent basis to begin domestication based on the selection of wild genotypes. Some wild species have been domesticated, such as *Vaccinium macrocarpon* (Fajardo et al., 2013), *V. sieboldii*, *V. ciliatum*, *V. oldhamii*, *V. bracteatum* (Hirai et al., 2010), and other species such as grape (Emanuelli et al., 2013) as well as Chilean native species such as *Fragaria chiloensis* (Carrasco et al., 2013), and *Vasconcellea* sp. (Carrasco et al., 2009).

Table 4. Descriptive statistics for all loci for each population of *Gaultheria pumila*. Sites where samples were taken, number of alleles (N_a), observed heterozygotes (H_o), expected heterozygotes (H_e), inbreeding coefficient (F_{IS}), percentage of polymorphic loci (PPL), number of effective alleles (N_e), Shannon index (I).

Regions	Sites	N_a	H_o	H_e	F_{IS}	PPL	N_e	I
Maule	PI	3.16	0.62	0.45	-0.39	83%	2.07	0.77
La Araucanía	PT	2.50	0.46	0.38	-0.21	83%	1.75	0.60
	PZ	2.33	0.61	0.45	-0.39	100%	1.80	0.65
	EM	3.66	0.50	0.47	-0.06	100%	2.02	0.80
	CK	3.50	0.53	0.53	-0.008	100%	2.36	0.89
	CN	2.33	0.48	0.37	-0.32	100%	1.69	0.57
Los Lagos	PN	2.16	0.54	0.52	0.03	100%	1.90	0.68
	CH	3.00	0.47	0.56	0.16	100%	2.49	0.91
Magallanes y la Antártida Chilena	PA	2.83	0.49	0.45	-0.10	100%	1.89	0.72
	RM	2.83	0.66	0.50	-0.33	100%	2.14	0.79
	PL	3.16	0.58	0.51	-0.12	100%	2.08	0.84
	Mean	2.86	0.54	0.47	-0.16	96.97%	2.02	0.75

Maule Region: Parque Inglés (PI); La Araucanía Region: Parque de las Araucarias (PT), El Playón (PZ), El Mirador (EM), Centro de sky (CK), Parque Nacional Conguillío (CN); Los Lagos Region: Parque Nacional Puyehue (PN), Camino Puyehue (CH); Magallanes y la Antártida Chilena Region: Punta Arenas (PA), Reserva Nacional Magallanes (RM), Reserva Nacional Laguna Parrillar (PL).

The inbreeding coefficient (F_{IS}) had an average negative value and showed an excess of heterozygosity that could be due to the immigration process or absence of selfing (Balloux, 2004). These negative values suppose that the reproductive biology of the species probably causes the excess of heterozygosity since it is open pollinating. Gene flow is also due to the mobility of wild animals that eat fruit and disperse their seeds, causing a continuous mixture in the different demes. In the 4 years of this study we observed pollinator insects, especially *Bombus* spp., as well as wild animals, such as rabbits, birds, lizards and foxes feeding on the fruits. This species is also a food source for foxes in the wild; *G. pumila* is commonly named mutilla del zorro.

Population structure and genetic differentiation

The analysis of molecular variance (AMOVA) showed that a high proportion of the variation was observed within populations (94%) with the remaining variance (6%) being attributed to the genetic differences between populations (Table 5).

The Mantel test showed a significant positive correlation between genetic distance and geographic distance (correlation coefficient $R_{xy} = 0.718$, $p = 0.010$). These results would suggest that there is isolation by distance. The organization between populations, based on Nei's genetic distance, found the maximum value (0.55) between the populations of Parque Nacional Conguillío (CN) and RM, and the minimum genetic distance (0.01) between the populations of CN and Playón (PZ).

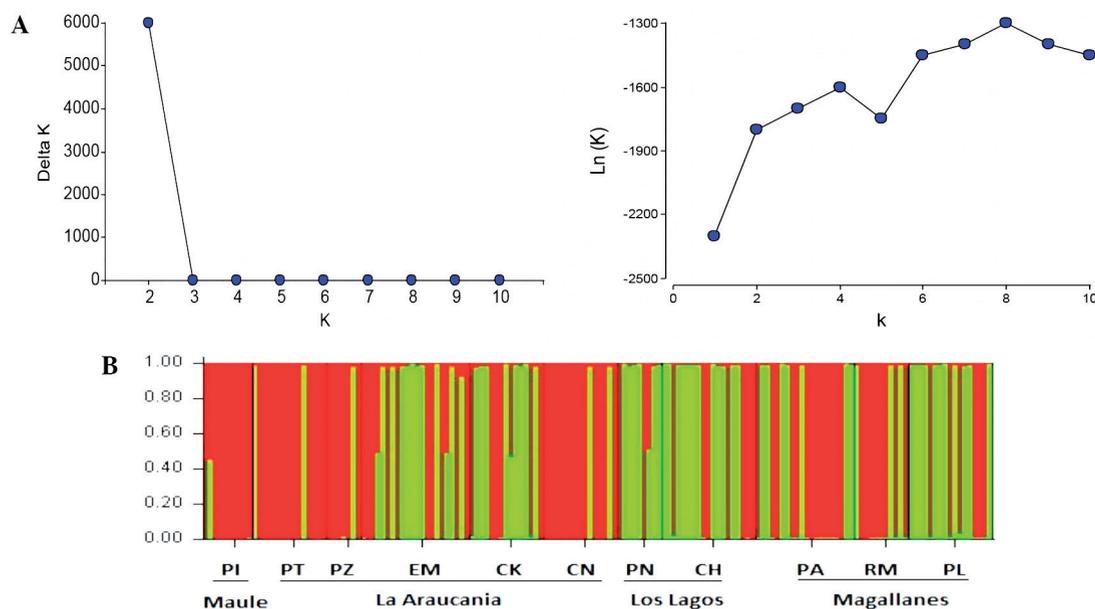
The Bayesian clustering indicated that the maximum ΔK was obtained at $K = 2$ (Figure 2). The K values between 3 and 11 showed complex admixture that did not fit the criteria to be considered as genetic groups. The two genetic groups identified clustered individuals from all population collection points except in PI in Maule Region. The populations from La Araucanía to Magallanes Regions showed a high degree of admixture (Q values < 0.8), based on results obtained from Bayesian model-based structure analysis of 160 samples of *G. pumila*. The sites evaluated were sampled from the Maule to Magallanes y la Antártida Chilena Regions of Chile, covering part of its distribution. The northern population was collected from the Maule Region, and the Southern population was collected from the Magallanes Region, the limits of the species distribution. The high level of variation within populations indicating that most of the genetic variation is explained by the genetic exchange among individuals. There was no evidence of genetic differentiation due to geographically isolated groups.

These results coincide with previously reported observations that found a high level of phenotypic variation in natural populations of this species (Villagra et al., 2014). This pattern of distribution of the genetic variability is very similar to that detected for "maqui" (*Aristotelia chilensis* [Molina] Stuntz) (Salgado et al., 2017), a wild berry native to Chile which is currently subject to an intense domestication process. Indeed, it was found in this species that 95% of the genetic variability was within populations and 5% between populations, remarkably similar to *G. pumila* in Chile. Bayesian-based analysis of genetic grouping detected only two groups in *G. pumila*. The first and second group contains individuals from the populations belonging to Maule, La Araucanía, Los Lagos, and Magallanes Regions, which suggests a consistent mixture of alleles, showing a constant dynamism that does not allow differentiation. However, positive correlation between distance and genetic distance was detected by the Mantel test, suggesting lower genetic connectivity between the populations belonging to extreme Regions. Consequently, the isolation of these populations can affect the pollen dispersal and therefore the gene flow.

Table 5. AMOVA in different natural populations of *Gaultheria pumila*, Chile.

Sources of variation	df	Sum of squares	Est. variance	Percentage variation	Fixation index	P value
Among populations	10	18.836	0.062	6%		
Within populations	149	148.562	0.997	94%	0.058	0.001
Total	159	167.398	1.059			

Figure 2. Genetic groups estimated by Bayesian analysis. A) ΔK estimates of the posterior probability distribution of the data for a given K. $\ln(K)$ obtained is a structure analysis and an *ad hoc* quantity distribution. B) Estimated population structure and genetic clusters of the 11 sites of *Gaultheria pumila* and 160 individuals with K = 2.



Maule Region: Parque Inglés (PI); La Araucanía Region: Parque de las Araucarias (PT), El Playón (PZ), El Mirador (EM), Centro de sky (CK), Parque Nacional Conguillío (CN); Los Lagos Region: Parque Nacional Puyehue (PN), Camino Puyehue (CH); Magallanes y la Antártida Chilena Region: Punta Arenas (PA), Reserva Nacional Magallanes (RM), Reserva Nacional Laguna Parrillar (PL).

CONCLUSIONS

The species shows a moderate level of genetic diversity and a very low level of genetic differentiation, indicating a high degree of genetic connectivity and gene exchange throughout its distribution. These results can be used to continue the domestication programs that have begun for the species, giving support to the selection of individuals from two genetic groups identified in this study.

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