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RESEARCH ARTICLE

Genetic variability of *Aristotelia chilensis* ("maqui") based on AFLP and chloroplast microsatellite markers

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Abstract *Aristotelia chilensis* (Molina) Stuntz (Elaeocarpaceae) also known as "maqui" is a dioecious tree species native to Chile and neighbouring zones of Argentina. Its fruit is collected from the wild by locals for consumption, and recently, as a raw material for industrial processing because of its high antioxidant capacity. As a consequence of its increasing demand, sustainable production is required. To study intraspecific diversity patterns we therefore analysed 58 accessions, growing in the Experimental Station of Universidad de Talca and originating from eight wild populations, using AFLPs and chloroplast microsatellites. Only 5% of the variability could be attributed to the provenance, whereas 95% was found between individuals of the same population. A significant correlation between genetic differentiation and geographic distances was detected ($r = 0.51$). Bayesian analysis revealed four main genetic groups, which are not correlated to the provenances. Two chloroplast microsatellite primers revealed two haplotypes of which one was detected in individuals from all the populations, whereas the other was only present in the two northernmost populations. The genetic variability found for this species provides an excellent basis for further selection and breeding.

Keywords *Aristotelia chilensis* · Maqui · Wild populations · Haplotypes · Genetic differentiation · cpDNA · Genetic structure

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Introduction

Aristotelia chilensis (Molina) Stuntz, Elaeocarpaceae, is a wintergreen, dioecious shrub or tree native to Central and Southern Chile, including the western borders of Argentina (Rodríguez 2005) where it is commonly growing in sclerophyllous scrubland or deciduous forests between latitudes 30–46°S.

The dark purple fruits, commonly called "maqui", are mainly dispersed by birds (Hoffmann et al. 1992). For Mapuche and Huilliche indigenous populations of Chile, maqui is one of the sacred plants used in

religious ceremonies as well as being used for medicinal treatments (De Mösbach 1992; Mølgaard et al. 2011).

In recent years fruit of *A. chilensis* has caught wider attention for its strikingly high antioxidant capacity and potential health benefits *inter alia* reported by Araya et al. (2006), Céspedes et al. (2008), Schreckinger et al. (2010), Fuentealba et al. (2012), Jara et al. (2012), Rojo et al. (2012), and Hidalgo et al. (2014) which triggered a rising demand for this wild collected "superfruit" on the international market. To supply this increasing demand for maqui-berries as a raw material for food, cosmetic or pharmaceutical industry long-term sustainability of the production and the development of homogeneous fruit quality are urgently needed.

Up to now, no commercially cultivated fruit production of *A. chilensis* has been undertaken. Thus, the growing demand for maqui berries requires the domestication of this species to avoid genetic erosion and decimation of the wild populations caused by the increasing, non-sustainable wild collection. Such domestication requires basic information regarding the genetic structure of the natural populations, which can, amongst other things, guide the selection of accessions with different genetic background.

Initial domestication steps of this wild species started in 2007 and included screening of wild populations and morpho-phenological studies about fruit production of maqui (Vogel et al. 2014). As a basis for selection, cultivable *A. chilensis* germplasm was collected from wild populations over a range of more than 700 km, including locations of different altitudes and climatic zones. The accessions were planted in the Experimental Station of Universidad de Talca to study their performance and provide material for genetic studies of variability among accessions and provenances. In spite of the different predominating environmental conditions of the wild populations, no significant differences between provenances could be found for morphological characteristics or natural compounds, such as polyphenol or anthocyanin concentrations (Vogel et al. 2014; González et al. 2015). Instead, a very high phenotypic variation between accessions of any provenance could be observed.

Fredes et al. (2014) reported, for the same species, significant genetic diversity based on ISSR-PCR analysis and on anthocyanin content of four genetic groups. On the other hand, Bastías et al. (2016) recently found a panmictic genetic structure in wild populations of *A. chilensis* using SSR markers, suggesting non-significant groups between maqui samples. The present study explores the genetic variability of accessions from different wild populations (provenances) distributed from Central to Southern Chile using AFLP and chloroplast microsatellite markers to understand the genetic structure of wild populations over a wide distribution range of the species.

It could be supposed that with growing geographic distance between *A. chilensis* wild populations genetic distance would increase, and genotypes would adapt to different environmental conditions during evolution.

However, *A. chilensis* is a dioecious species and berries are eaten by birds suggesting the potential for wide distribution of the seeds and hence reducing inter-population or providence diversity. The present study aims to detect genetic structure of this wild species and thus help the optimization of a selection strategy. Besides, genetic fingerprints obtained by molecular markers would also allow identification of individuals of our maqui gene bank.

Materials and methods

Plant material

To implement an *Aristotelia chilensis* gene bank, fifty-eight accessions from eight wild populations (provenances), were collected in 2008 (Table 1). These accessions were propagated vegetatively by cuttings, and clones thus established in the Experimental Station of Universidad de Talca, Central Chile (122 m a.s.l.).

Nuclear and chloroplast DNA extraction

Young leaves from established plants in the Experimental Station were taken in summer 2011 and desiccated with silica gel until extraction of DNA in the laboratory of Forest Genetics and Forest Tree Breeding, Büsgen-Institute, Georg-August-University Göttingen. Nuclear and chloroplast DNA were extracted from dry leaves using the Qiagen DNA isolation Plant Mini Kit (Qiagen GmbH, Hilden, Germany).

Table 1 Geographical data of the provenances of the analyzed accessions

Provenance	Coordinates (S/W)	Altitude (m a.s.l.)	Number of sampled individuals included in the study
San Fernando	34°41′/70°50′	530	10
Romeral	34°57′/70°57′	495	9
San Clemente	35°34′/71°22′	275	4
Mulchén	37°40′/72°01′	329	2
Curacautín	38°21′/71°56′	607	7
Villarrica	39°16′/71°59′	190	7
Entrelagos	40°40′/72°33′	165	9
Puerto Montt	41°36′/72°37′	92	10

AFLP analysis

All accessions were genotyped using amplified fragment length polymorphisms (AFLP). The analysis was performed according to Vos et al. (1995) with slight modifications. Total genomic DNA was digested simultaneously with the restriction enzymes *Eco*RI and *Mse*I. Pre -amplification reactions were performed using the pre-selective primer pair *Eco*RI +A/*Mse*I +G under following cycle conditions: 2 min at 72 °C followed by 20 cycles of 10 s at 94 °C, 30 s at 56 °C, 2 min at 72 °C, and finally 30 min at 60 °C. Selective PCR reactions were carried out with the primer combinations *Mse*I + -GAA/*Eco*RI ACA and *Mse*I + GGT -/*Eco*RI + A-CA.The *Eco*RI primers were fluorescently labelled (6-FAM, HEX). A touchdown PCR was applied with following cycling conditions: 2 min at 94 °C, 10 cycles each consisting of 10 s at 94 °C, 30 s at 65 °C by subsequently reducing the annealing temperature about 1 °C in each cycle followed by 23 cycles with a stable annealing temperature of 56 °C, and a final extension step of 30 min at 60 °C. Electrophoresis of diluted amplification fragments was carried out on a capillary ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, CA) using an internal GS 500 Rox^{TM} size standard (Applied Biosystems). Absence (0) or presence (1) of bands from 70 to 450 bp were scored with the software packages Genescan 3.7 and Genotyper 3.7 (Applied Biosystems), and a binary matrix representing the AFLP profile of each sample was constructed.

Chloroplast SSR markers

Ten universal consensus chloroplast microsatellite primers, *ccmp1-ccmp10* (Weising and Gardner 1999), were first tested in one randomly chosen individual of each provenance to amplify corresponding cpDNA regions of *Aristotelia chilensis* samples. Then all 58 samples were tested with the two polymorphic primers *ccmp5* and *ccmp6*. The PCR profile consisted of an initial denaturation step at 95 °C for 15 min followed by 35 cycles at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min, ending with 20 min at 72 °C. The PCR amplification was performed in a final volume of 15 µL, containing 1 × PCR buffer (0.8 M Tris–HCl pH 9.0, 0.2 M $(NH_4)_2SO_4$, 0.2% w/v Tween-20), 2.5 mM $MgCl_2$, 1U Hot Start Taq polymerase (Hot Firepol, Solis BioDyne, Tartu, Estonia), 0.2 mM dNTP (Thermo Fisher Scientific, USA), 0.5 µM *ccmp* forward, 0.5 µM *ccmp* reverse, and 2 µL of DNA (ca. 10 ng). Forward primers are fluorescently labelled (6-FAM, HEX).

Diluted amplification products were electrophoretically separated on a capillary ABI 3100 Genetic Analyser (Applied Biosystems) with an internal GS 500 Rox^{TM} size standard (Applied Biosystems). The fragment sizes were scored with the software packages Genescan 3.7 and Genotyper 3.7 (Applied Biosystems).

Data analysis

For each provenance percentage of polymorphic loci (%P), allelic richness (Na), number of effective alleles (Ne) and Shannoń s information index (I) were estimated from AFLP profile using GenAlEx version 6.5 (Peakall and Smouse 2006, 2012). An analysis of molecular variance (AMOVA) for binary data allowed hierarchical division of genetic variation between and within provenances. Pairwise genetic differentiation between provenances (Φ_{PT} values),

Table 2 Genetic diversity indexes of *Aristotelia chilensis* provenances based on AFLP

Provenance	N	% P	Na ± SD	Ne ± SD	I ± SD
San Fernando	10	53.8	1.15 ± 0.06	1.27 ± 0.02	0.26 ± 0.02
Romeral	9	78.8	1.66 ± 0.04	1.37 ± 0.02	0.37 ± 0.01
San Clemente	4	42.1	0.99 ± 0.06	1.29 ± 0.02	0.25 ± 0.02
Mulchén	2	26.6	0.72 ± 0.05	1.27 ± 0.03	0.18 ± 0.02
Curacautín	7	72.2	1.54 ± 0.05	1.44 ± 0.02	0.39 ± 0.02
Villarrica	7	66.8	1.38 ± 0.06	1.39 ± 0.02	0.35 ± 0.02
Entrelagos	9	54.1	1.20 ± 0.06	1.30 ± 0.02	0.28 ± 0.02
Puerto Montt	10	61.0	1.25 ± 0.06	1.28 ± 0.02	0.28 ± 0.02
Mean	7.3	56.9	1.24 ± 0.02	1.33 ± 0.01	0.30 ± 0.01

n = number of studied individuals; % P = percentage of polymorphic loci; Na = allelic richness; Ne = number of effective alleles; I = Shannon's Information Index (Lewontin 1972); SD = Standard deviation

genetic differentiation (linearized Φ_{PT}) and log of geographic distance were generated from AMOVA. Log of geographic distance was correlated with genetic differentiation (linearized $\Phi_{PT)}$ in a Mantel Test (Mantel 1967). All calculations were performed by GenAlEx version 6.5 (Peakall and Smouse 2006, 2012) and carried out with 9999 permutations.

A phylogenetic tree was constructed using the neighbour joining method (Saitou and Nei 1987) implemented in Phylip (Phylogeny Inference Package) Version 3.695 (Felsenstein 2005) based on 1000 bootstraps. Genetic data, without defining a priori groups, were subjected to a Bayesian clustering analysis assuming admixture, using STRUCTURE version 2.3.2 software (Pritchard et al. 2000). The number of tested provenances (K) ranged from K = 1 to 10 with 5 runs per each K, a burn-in of 100,000 followed by 1,000,000 Monte Carlo Markov Chain iterations. The number of genetic clusters was determined based on the ΔK criterion of Evanno et al. (2005) using STRUCTURE Harvester (http://taylor0.biology.ucla.edu/structureHarvester/) (Earl and von Holdt 2012).

Chloroplast haplotype frequencies and genetic diversity within each provenance were estimated using the software HAPLOTYPE ANALYSIS version 1.05 (Eliades and Eliades 2009; Finkeldey and Murillo 1999).

Results

Both AFLP primer combinations showed polymorphism for 259 fragments among 58 accessions of *Aristotelia chilensis* coming from the eight different wild populations (provenances). Table 2 summarizes the genetic indices for the provenances, with the highest percentage of polymorphic loci, allelic richness and Shannon Index being displayed by Romeral and Curacautín, and lowest values by Mulchén and San Clemente, but it should be noted that they are only represented by two and four individuals, respectively. The correlation between geographic distance and genetic differentiation among provenances is significant ($r = 0.51$; $p \leq 0.01$) indicating isolation-by-distance (Fig. 1).

The AMOVA revealed 95% of the variance within provenances and 5% among them (Table 3). Similarly, the neighbour-joining tree shows a low degree of genetic relationship among individuals based on geographical provenance (Fig. 2).

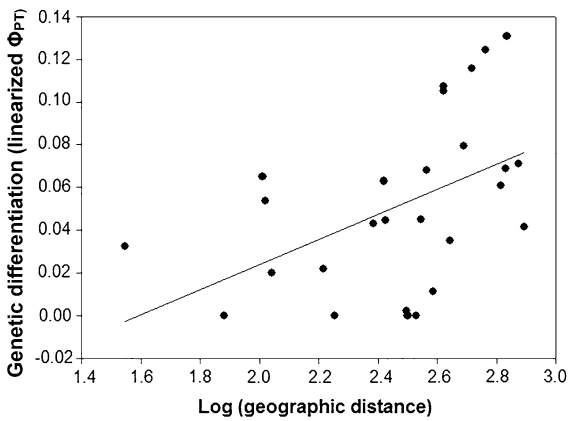

Fig. 1 Correlation between geographic distance and genetic differentiation according to Mantel (1967) with r = 0.51 and $p < 0.01$

Table 3 Analysis of molecular variance (AMOVA) within and between eight provenances of 58 *A. chilensis* accessions based on 259 AFLP markers

Source of variation	Degrees of freedom	Sum of squares	Mean square (MS)	Estimated variance	Percentage of variation (%)	Φ_{PT}
Between provenances	7	294.25	42.04	1.64	5	0.051*
Within provenances	50	1517.89	30.36	30.36	95	*
Total	57	1812.14	–	32.00	100	

* $p < 0.01$; Φ_{PT} value is based on standard permutation across the full data set

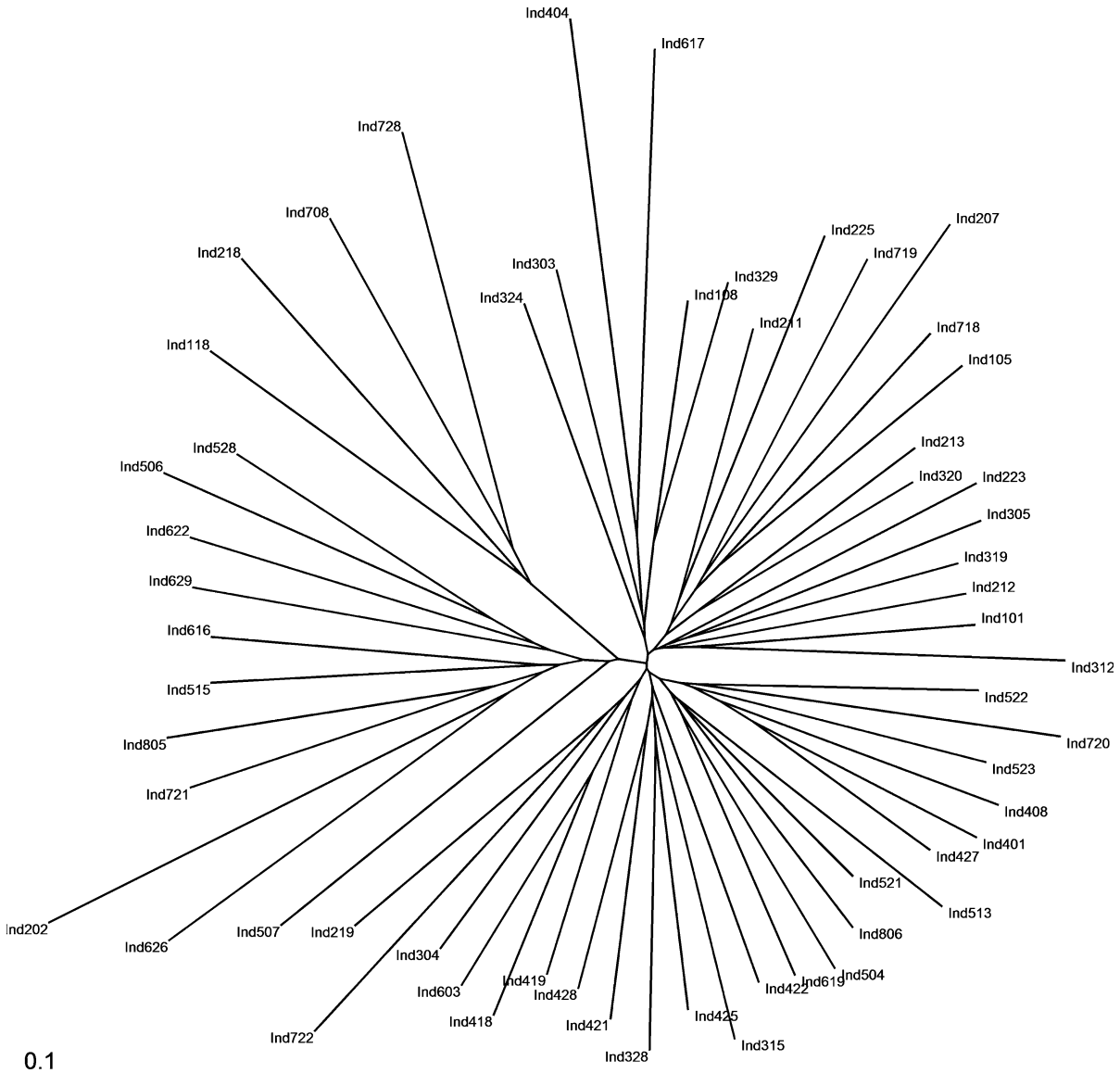

Fig. 2 Phylogenetic tree of the 58 accessions based on neighbour joining clustering method (Saitou and Nei 1987). Individuals from same provenances are indicated after "Ind" by numbers: *1* San Clemente, *2* Romeral, *3* San Fernando, *4* Puerto Montt, *5* Entrelagos, *6* Villarrica, *7* Curacautín, *8* Mulchén

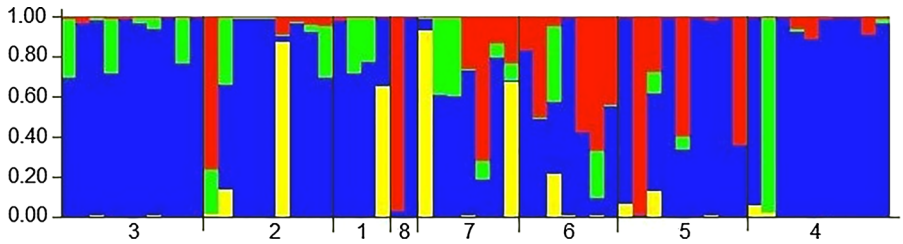

Fig. 3 Bayesian analysis cluster plot obtained for 58 *A. chilensis* accessions originating from eight wild populations. The most plausible grouping of all investigated genotypes ($k = 4$) is presented from left to right arranged from North to South, respectively. Each *vertical bar* refers to an individual representing assignment to the detected genetic clusters. Provenances are indicated by numbers: *1* San Clemente, *2* Romeral, *3* San Fernando, *4* Puerto Montt, *5* Entrelagos, *6* Villarrica, *7* Curacautín, *8* Mulchén

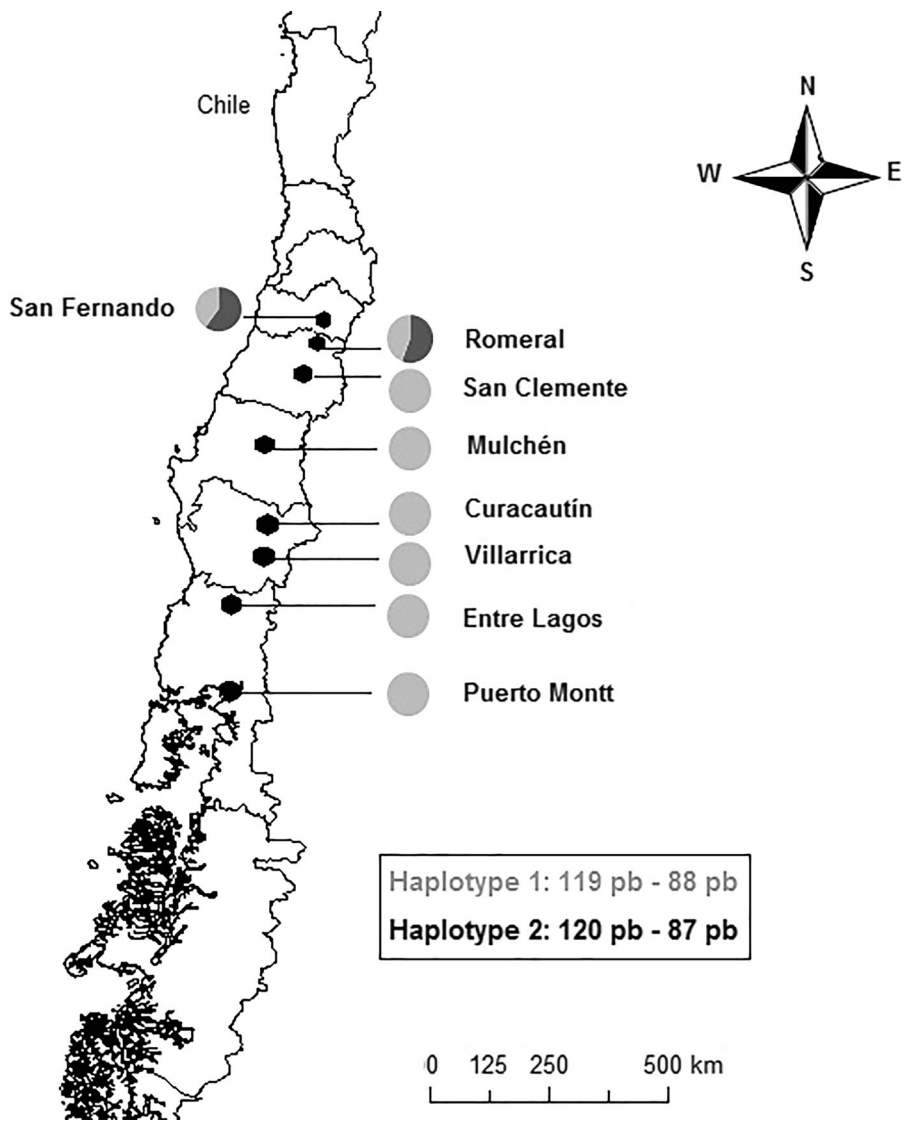

Fig. 4 Map of the 8 studied provenances of *Aristotelia chilensis* in Chile and their respective chloroplast haplotypes based on chloroplast microsatellites

The Bayesian analysis revealed four main genetic groups (Fig. 3) clustering accessions independent of their provenances.

Ccmp5 and *ccmp6* were the only two polymorphic primer pairs out of ten tested universal primers. Fragment lengths revealed for chloroplast loci were 119–120 pb for *ccmp5* and 87–88 pb for *ccmp6*. Two haplotypes were detected: haplotype 1 was found in 47 accessions and present in all provenances, whereas haplotype 2 was found in only 11 accessions which originated from the two northern most populations, San Fernando and Romeral (Fig. 4).

Discussion

AFLP analysis of 58 *A. chilensis* accessions showed a generally little genetic structure corresponding to the geographic location of their provenances, despite a Mantel test indicating a significant correlation between genetic differentiation and geographic distance. Most of the variation was found within the provenances, and a Bayesian analysis revealed the presence of four genetic groups that were not related to the geographic origin of accessions. Since the genetic variation detected in our investigation covered an area extending over about 750 km, it suggests a relative high gene flow between populations. These results are not surprising, as *Aristotelia chilensis* is known to be a pioneer species that colonizes burnt or cleared areas, and its seeds are commonly dispersed by birds (Hoffmann et al. 1992; Vogel et al. 2008), which would stimulate the genetic exchange between populations. Supporting this, Smith-Ramírez et al. (2013) reported seed dispersal by birds on Juan Fernandez Island where maqui is considered to be an invasive species.

Fredes et al. (2014) reported polymorphism of ISSR markers in four Chilean wild populations of *A. chilensis*, associated strongly with the sampled geographic areas, while the genetic groups based on SSR markers found by Bastías et al. (2016) were non-significant. In accordance to the latter authors, our results based on AFLP also exhibit a very slight effect of the sampled provenances on the total genetic variability and most variability between individuals within populations. Although the relation between genetic and geographic distance was significant, values scattered around the regression line.

Chloroplast microsatellite markers, also known as cpSSR, have proven to be a powerful tool for phylogeographic studies because they are uniparentally inherited without recombination and often linked. They are considered to be more effective indicators for population subdivision and differentiation than nuclear markers (Petit et al. 2005; Ebert and Peakall 2009). Interestingly, analysis of chloroplast microsatellite loci showed two distinct haplotypes that coexist only in the northernmost provenances (San Fernando and Romeral).

The set of high-resolution molecular markers now available, such as those used in the present study together with the independently developed and recently reported markers (Fredes et al. 2014; Bastías et al. 2016), compose suitable tools to provide basic information for further selection of accessions. The wide genetic variability between plants is an excellent base for selection of outstanding genotypes and development of varieties suitable for cultivation. Moreover, the polymorphic ALFP and microsatellite markers, either chloroplast or nuclear, would permit the identification of the studied individuals, among them recently selected mother plants to develop *A. chilensis* varieties for commercial cultivation (Vogel et al. 2016).

Conclusions

The present AFLP studies indicate a high genetic variability within the studied provenances of *Aristotelia chilensis*. Although the correlation between genetic differentiation and geographic distances was significant, only 5% of the total variation is due to the provenance. Four main genetic groups were detected representing all provenances. Two different haplotypes were found by using chloroplast microsatellite loci, with one ubiquitous haplotype in all provenances and the presence of the other in the two northernmost provenances. The large genetic variability provides an excellent basis for further selection and breeding of this species.

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Compliance with ethical standards

Conflict of interest None.

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