Research Article

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Anthocyanin profiling of wild maqui berries (*Aristotelia chilensis* [Mol.] Stuntz) from different geographical regions in Chile

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Abstract

BACKGROUND: Maqui (*Aristotelia chilensis*) is a Chilean species which produces small berries that are collected from the wild. Anthocyanins, because of their health benefits, are the major focus of interest in maqui fruit. For this study, we examined anthocyanin and phenolic content of maqui fruits from individuals that belonged to four geographical areas in Chile, and used DNA marker analysis to examine the genetic variability of maqui populations that had distinctly different fruit anthocyanin content.

RESULTS: Twelve primers generated a total of 145 polymorphic inter simple sequence repeat–polymerase chain reaction (ISSR-PCR) bands. ISSR-PCR showed different banding patterns for the individuals evaluated, confirming that maqui populations belonged to different genotypes. Maqui fruit from four different geographical regions during two consecutive growing seasons showed high total anthocyanin (6.6–15.0 g cy-3-glu kg^{-1} fresh weight (FW)) and phenolic (10.7–20.5 g GAE kg^{-1} FW) contents and different anthocyanin profiles.

CONCLUSION: Three maqui genotypes exhibited significantly higher anthocyanin content than the others, as measured by pH differential method and high-performance liquid chromatography–mass spectrometry. Significant genetic diversity was noted within each ecological population. ISSR-PCR analysis provided a fingerprinting approach applicable for differentiation of maqui genotypes.

Keywords: delphinidin-3,5-diglucoside; delphinidin-3-glucoside; genetic diversity; maqui genotypes; ISSR-PCR

INTRODUCTION

In recent years, international interest in the berries of South America has increased mainly because of their potential health benefits and consumer demand for novel exotic fruit.[1] Among these berries, maqui fruit (*Aristotelia chilensis* [Mol.] Stuntz, Elaeocarpaceae) from Chile has been singled out as an exceptionally rich source of anthocyanins and natural antioxidants.[2] These fruits were reported to have higher total polyphenolic content and antioxidant activity than other recognized, polyphenol-rich small fruit species such as blackberries (*Rubus* spp.), blueberries (*Vaccinium* spp.), raspberries (*Rubus idaeus* L.) and strawberries (*Fragaria* × *ananassa* L.).[3,4] Phenolic extracts of maqui fruits have also shown antioxidant activity *in vivo*[5] and furthermore a standardized anthocyanin-rich formulation from maqui has shown anti-diabetic activity *in vitro* and *in vivo*.[6] The main phenolic compounds in maqui fruits are anthocyanins, where eight types of anthocyanin in the form of delphinidin and cyanidin glycosides were reported.[6–9]

Maqui is a Chilean native dioecious species (female and male individuals) that grows naturally as an evergreen bush or tree from Limarí (30° S latitude, Coquimbo Region) to Aysén (45° S latitude, Aysén del General Carlos Ibáñez del Campo Region), Chile, from the coast to 2000 m above sea level.[10] It is a pioneer species that colonizes across varied environments and has great capacity for regeneration in pine forests of central and southern Chile,[11] forming wild populations called 'macales'. In native forests in Central Chile, maqui is a secondary species where populations of quillay (*Quillaja saponaria* Mol.), litre (*Lithrea caustica* [Mol.] Hook. et Arn.), and peumo (*Cryptocarya alba* [Mol.] Looser) are predominant.[12] Maqui fruits are collected from the wild from December to February,[13] because commercial plantations of maqui do not yet exist. Recently, the identification of elite genotypes with potentially enhanced health benefits has been prioritized as an important objective for anthocyanin-rich species such as strawberries,[14,15] blackberries[16] and cranberries

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(*Vaccinium macrocarpon* Ait.).[17] To facilitate this kind of research, the introduction of DNA-based markers has been essential, permitting the direct comparison of different genetic material independent of environmental influences.[18] Inter simple sequence repeat (ISSRs) markers[19] have been used successfully on a number of polyphenol-rich small fruit species such as wild blueberries (*V. angustifolium* Ait)[20] and strawberry cultivars.[21]

Significant advances in anthocyanin characterization and health benefits of maqui fruit have been achieved, but no previous studies have examined the influence of genotype and the environment on the phenolic content and antioxidant activity of maqui fruit. Owing to the wide distribution of this species in Chile, a diversity of genotypes adapted to different growing regimes, with different concentrations of polyphenols and different inherent levels of bioactive potential, could be expected. Because anthocyanins are the major focus of health-protective properties contained in maqui fruit, it is useful to identify the best maqui genotypes for anthocyanin profile and content.

The objective of this study was to evaluate the differences in individual and total anthocyanin content, total polyphenols, and the antioxidant activity of maqui fruits from four different geographical regions during two consecutive growing seasons in Chile. DNA marker analysis was applied to examine the genetic diversity of maqui populations in an attempt to explain the potential variability in fruit anthocyanin content.

EXPERIMENTAL

Plant material

Four wild populations from different geographical regions in Chile were selected for this study. During the flowering stage in 2010, both male and female individuals were differentiated according to flower morphology in each site of sampling. A total of 56 individuals were identified (six female and two male individuals for Puchuncavi, twelve female and eight male individuals for Paredones, eleven female and nine male individuals for Talca and five female and three male individuals for Pucon). The individuals remained labeled to perform sampling of leaves (genetic analysis) and fruits (chemical analysis).

Leaf sampling and DNA isolation for ISSR analysis

All sites of sampling were visited at least twice to collect leaves during sprouting. New leaves from the 56 individuals were kept separately in cryotubes and frozen in liquid nitrogen in the field and then stored at −80 °C before analysis. Genomic DNA was extracted from the fresh leaves using the DNeasy Plant Mini Kit (QIAGEN Inc., Valencia, CA, USA).

Fruit sampling

A total of 18 individuals (50% of the female individuals of each population) for each growing season (2010–2011 and 2011–2012) were chemically analyzed. For this, maqui deep purple-ripe fruit samples for each individual ($n = 3$) were taken randomly from the upper, middle and lower canopy, stored at 4 °C and processed within 24 h after collection. The fruit mean weight was estimated from 30 fresh fruit samples. Slurries were used to determine total soluble solid (SS) content by refractometry and to determine moisture content (%) by drying in an air-forced oven (WTE, Germany) at 45 °C for 24 h.

Geographical and climatic information

Growing conditions for each site of sampling are detailed in Table 1. Growing degree-days and cumulative rainfall were recorded from October to February (from full flowering stage to fruit harvest) for 2010–2011 and 2011–2012. Minimum and maximum air daily temperatures and temperature oscillation were recorded for 30 days before fruit harvest. This information was provided by the Chilean Meteorological Service from weather stations adjacent to the respective sites of sampling.

Table 1. Growing conditions in the four different sampling regions for maqui berry in Chile

	Puchuncavi		Paredones		Talca		Pucon	
Geographical area	2010–2011	2011–2012	2010–2011	2011–2012	2010–2011	2011–2012	2010–2011	2011–2012
Georeference	32°44′ S, 71° 24′ W		34° 36′ S, 76° 53′ W		35° 26′ S, 71° 11′ W		39° 21′ S, 71° 46′ W	
Altitude	109 m		137 m		817 m		398 m	
Annual mean temperature[a] (°C)	14.0b		13.6b		14.6b		11.2a	
Annual mean rainfall[a] (mm)	372.5a		700.0b		689.3b		1157.4c	
Soil type	Clay loam		Clay loam		Clay loam		Clay loam	
Soil pH	6.2		6.5		6.5		6.0	
Harvest time (mm-dd)	01–05	01–06	01–10	01–12	01–15	01–20	02–15	02–20
Growing degree days[b] (°C)	1141.4	1160.9	1105.4	1173.4	1142.6	1195.6	1132.0	1145.6
Cumulative rainfall[b] (mm)	17.0	5.2	43.5	7.5	78.0	6.5	359.4	66.8
Berry weight (100 berries, g)	10.8a	10.2ab	13.6c	12.6bc	12.8bc	12.4abc	11.2ab	11.4abc
Moisture content (%)	73.1a	70.5ab	73.3a	72.1ab	72.9a	70.7ab	70.4ab	69.9ab
Soluble solids (°Brix)	17.5ab	17.3b	19.2a	18.5b	19.5a	18.9b	17.2b	17.0bc
Minimum[c] (°C)	14.0c	14.0c	11.1ab	9.4a	11.7b	13.9c	9.4a	10.0ab
Maximum[c] (°C)	25.4b	25.1b	25.1b	21.0a	28.5c	29.9c	21.7a	23.0b
Temperature oscillation[d] (°C)	11.4a	11.2a	14.1abc	11.6a	16.9c	16.0c	12.3ab	13.1bc

[a] Mean values recorded from 2002 to 2013.
[b] Recorded from full flowering stage to fruit harvest.
[c] Mean minimum and maximum temperatures recorded during 30 days before fruit harvest.
[d] Mean temperature oscillation was calculated by the difference between maximum and minimum daily temperature. Values within the same row followed by different letters were significantly different at $P < 0.05$.

Table 2. Inter simple sequence repeat (ISSR) primers (numbers follow those in UBC set 9, no. 801–900) and their sequence, polymorphic bands (number and size range) and the corresponding resolving power generated in 56 maqui genotypes

Primer code	Sequence	Polymorphic bands		Resolving power
		No.	Size range (bp)	
Dinucleotide, 3′ mono-anchored				
810	$(GA)_8$ T	12	350–1200	17.6
812	$(GA)_8$A	15	300–2000	23.6
815	$(CT)_8$G	14	320–1800	19.9
825	$(AC)_8$ T	13	400–3000	19.0
Dinucleotide, 3′ di-anchored				
834	$(AG)_8$YT	11	250–2000	16.4
835	$(AG)_8$YC	11	280–2000	16.5
836	$(AG)_8$YA	11	200–1800	13.6
840	$(GA)_8$YT	11	300–3000	16.6
849	$(GT)_8$YA	11	350–3000	16.3
854	$(TC)_8$RG	7	400–2000	6.6
855	$(AC)_8$YT	6	450–2500	5.5
857	$(AC)_8$YG	9	400–3000	8.9

ISSR primers, polymerase chain reaction (PCR) amplification and electrophoresis

Twelve primers (Table 2) with clear amplification patterns after electrophoresis of PCR products on agarose gel were tested for a set of 56 individuals. Different template DNA concentrations and $MgCl_2$ were tested to optimize the PCR yield and clarity of amplification patterns. The optimized amplification reaction mixture (25 µL) contained 10 ng DNA template, PCR buffer (500 mmol L^{-1} KCl, 100 mmol L^{-1} Tris–HCl pH 8.8), 2.5 mmol L^{-1} $MgCl_2$, 24 pmol primer, 160 µmol L^{-1} of each dNTP, 1.25 U *Taq* DNA polymerase (Invitrogen, Sao Paulo, Brazil) and nuclease-free water (Ambion®). PCR were performed with a 96-well programmable thermal cycler (Veriti®, ABI Applied Biosystems) using an initial 'hot start' at 94 °C for 3 min, followed by 40 cycles of 30 s at 94 °C, 40 s at 50 °C and 90 s at 72 °C. The reaction was terminated with a final extension at 72 °C for 10 min. Amplified fragments, along with a 100 bp DNA ladder (Thermo Scientific, CA, USA) were separated by electrophoresis in 2.5% agarose gels prepared with 1× Tris–acetate–ethylenediaminetetraacetic acid (TAE) buffer. DNA samples were incubated with GelRed™ nucleic acid stain (5000×) (Biotium, Inc., Hayward, CA, USA) in a gel electrophoresis chamber for 60 min. All DNA fragments were visualized and photographed under UV light. Each sample was amplified with each PCR primer pair at least twice and congruence between replicates was verified.

Preparation of maqui extracts for chemical analysis

A ground sample (1.5 g) of whole fruits was mixed vigorously with 6 mL of acidified methanol (0.1% HCl) for 1 h and centrifuged for 15 min at 3000 rpm. Supernatant was collected in a 25 mL volumetric flask. The residue was extracted twice more with 6 mL acidified methanol. Supernatants were collected and standardized to a final volume of 25 mL.

Determination of total polyphenols (TP)

TP were determined by the Folin–Ciocalteu method[22] and gallic acid was used as the standard, according to a calibration curve. The results were expressed as grams of gallic acid equivalent per kilogram fresh weight (g GAE kg^{-1} FW).

Determination of total anthocyanins (TA)

TA were determined by pH differential method.[23] Results were expressed as grams of cyanidin-3-glucoside equivalent per kilogram fresh weight (g cy-3-glu kg^{-1} FW) using a molar extinction coefficient of 26 900 cm^{-1} mol^{-1} and a molecular weight of 449 g mol^{-1}.

Determination of anthocyanin profiles by liquid chromatography–mass spectrometry (LC-MS)

Maqui extracts from the growing season 2011–2012 were concentrated, freeze dried and shipped on dry ice to the Plants for Human Health Institute, North Carolina State University. Before high-performance liquid chromatography (HPLC) injection the freeze-dried samples were redissolved in 90% MeOH at a concentration of 5 mg mL^{-1} and were filtered through 0.2 µm PTFE membrane filters (VWR International, Atlanta, GA, USA). The samples for HPLC analyses were prepared in triplicate.

To verify anthocyanin compound identity, 2 µL filtered samples were analyzed by LC-MS with an ion trap time-of-flight mass spectrometry (IT-TOF) instrument (Shimadzu Scientific Inst., Columbia, MD, USA) equipped with a Prominence HPLC system (SIL-20A HT autosampler, LC-20 AD pump system, SDP-M20A photo diode array detector). LC separation was performed using a C_{18} reverse-phase column (Shim-pack XR-ODS column, 50 mm × 3.0 mm i.d. × 2.2 µm, Shimadzu, Scientific Instruments, Columbia, MD, USA) with a binary solvent system comprising 0.1% formic acid in H_2O (A) and 0.1% formic acid in methanol (B). A step gradient of 0%, 30%, 60%, 90%, 0% and 0% of solvent B at 0, 30, 45, 50, 51 and 60 min, respectively, at a constant flow rate of 0.35 mL min^{-1} was applied. The heat block and curved desolvation line (CDL) were maintained at 200 °C. Nitrogen gas was used as nebulizer and drying gas with the flow rate set at 1.5 mL min^{-1} and 10 mL min^{-1}, respectively. The electrospray ionization (ESI) source voltage was set at 4.5 kV and the detector was set at 1.5 V. The instrument was calibrated to <5 mg kg^{-1} error in mass accuracy with an external standard of sodium trifluoroacetate solution. Ionization was performed using a conventional ESI source in positive ionization mode. Data were acquired at 200–1500 *m/z* range. Shimadzu's LC-MS solution software (Shimadzu Scientific Instruments) was used for system control and data analysis. The identification of anthocyanins was based on comparison with published data,[7–9,24] mass spectral data and commercial standards of cyanidin-3-glucoside, delphinidin-3-sambubioside-5-glucoside, delphinidin-3-sambubioside and delphinidin-3-glucoside (Polyphenols, Norway).

Anthocyanin quantifications was conducted on a 1200 LC with photo diode array detector (DAD) (Agilent Technologies, Santa Clara, CA, USA) using a Supelcosil-LC 18 column (250 × 4.6 mm, 5 µm) (Supelco, Bellefonte, PA, USA) using 5% formic acid in H_2O (A) and 100% methanol (B) as mobile phases according to the method previously reported.[6,25] Quantification of anthocyanins was performed from the peak areas recorded at 520 nm with reference to the calibration curve obtained with cyanidin-3-*O*-glucoside.

Determination of antioxidant activity

2.2-Diphenyl-l-picrylhydrazyl (DPPH) radical-scavenging activity

The DPPH method was conducted according to Brand-Williams *et al.*[26] and the results were expressed as EC_{50} (milligrams of sample that bleached 50% of the DPPH methanolic solution) by the construction of a kinetic curve of each sample.

Ferric reducing ability of plasma (FRAP)

The FRAP method was conducted according to Benzie and Strain[27] and the results were expressed as millimoles of Fe^{2+} equivalent per kilogram fresh weight (mmol E Fe^{2+} kg^{-1} FW).

Data analysis

Climatic and phytochemical data were analyzed using one-way ANOVA test for means comparisons. The differences between the 18 maqui individuals ($n = 3$) and between the four geographical regions in two consecutive years for each parameter were calculated according to the Tukey HSD (honest significant differences) multiple-comparison test ($P \leq 0.05$). Correlations between phytochemical parameters were also calculated. Analyses were performed with SAS 9.2 for Microsoft Windows (2009; SAS Institute Inc., Cary, NC, USA).

The amplified DNA fragments (bands) were scored as present (1) or absent (0), and the data matrix of the ISSR banding patterns of the four populations was assembled for further analysis. For genetic analysis, resolving power (Rp)[28] was calculated following the formula $Rp = \sum Ib$, where $Ib = 1 - (2 \times [0.5 - p])$, where p is the proportion of the 56 genotypes containing the band. The Ib value was calculated for the 12 ISSR primers. The Shannon diversity index (I) was calculated in order to estimate genetic diversity using the software GenAlEx 6.5.[29] In order to examine whether the total anthocyanin variation of maqui populations was influenced by genetic diversity (I) a correlation between both parameters was calculated. An UPGMA (unweighted pair group method with arithmetic mean) tree was constructed based on the genetic distance[30] among the different populations to determine genetic relationships of the populations using DendroUPGMA (http://genomes.urv.cat/UPGMA/) applying Pearson's coefficient with default settings.[31]

RESULTS AND DISCUSSION

Environmental variation between maqui berry growing regions

In this study, four maqui wild populations from different geographical regions were chosen and evaluated for phytochemical content. Three of the regions (Puchuncavi, Paredones and Talca) are located in Central Chile (32–35° S latitude) and the fourth region (Pucon) was located in Southern Chile (39° S latitude) (Table 1). The four sampling sites have different altitudes, and climatic differences were inherent due to the maritime (Ocean Pacific) and altitude (Andean Mountain) influences on microclimates.

Many environmental factors may affect flavonoid biosynthesis in plants, including light, temperature, altitude, soil type, water, nutritional status, microbial interactions, pathogenesis, wounding, defoliation, plant growth regulators and various developmental processes.[32] Nevertheless, the existing literature suggests that (among these environmental variables) temperature has the greatest influence on anthocyanin biosynthesis in some species such as grapes.[33–35] According to adjacent weather station data, annual mean temperature was significantly lower in Pucon (11.2 °C) than the sampling sites (Puchuncavi, Paredones and Talca) located in central Chile (13.6–14.6 °C). Each of the four sampling sites featured similar soil texture (clay loam) and pH (6.0–6.5), and no external irrigation or fertilization was used in any of these wildcrafted sites. Although Pucon had the highest annual mean rainfall (1157.4 mm) of the four sampling sites, and also had the highest cumulative rainfall during fruit development and maturation (359.4–66.8 mm), the fruits from this region did not have significantly higher weight than fruits from the other regions.

Genetic variability in maqui populations

DNA marker analysis was used in this study to determine the genetic variability of wild maqui populations and to potentially help explain the variability in the accumulation of anthocyanins in maqui berry fruits. Table 2 represents the selected primers and the maximum number of reproducible polymorphic bands produced by each primer. The survey of the primers revealed the presence of polymorphisms in the amplified DNA fragments in a range 200–3000 bp. The amplification of ISSR markers was consistent between repeated PCR runs. Representative banding patterns obtained with primer 815 are shown in Fig. 1, suggesting the existence of genotypic differences among the 56 maqui individuals. The number of polymorphic bands produced ranged from a minimum of six for primer 855 to a maximum of 15 for primer 812. The 12 primers generated a total of 145 polymorphic ISSR-PCR bands. The Rp values varied between 5.5 for primer 855 and 23.6

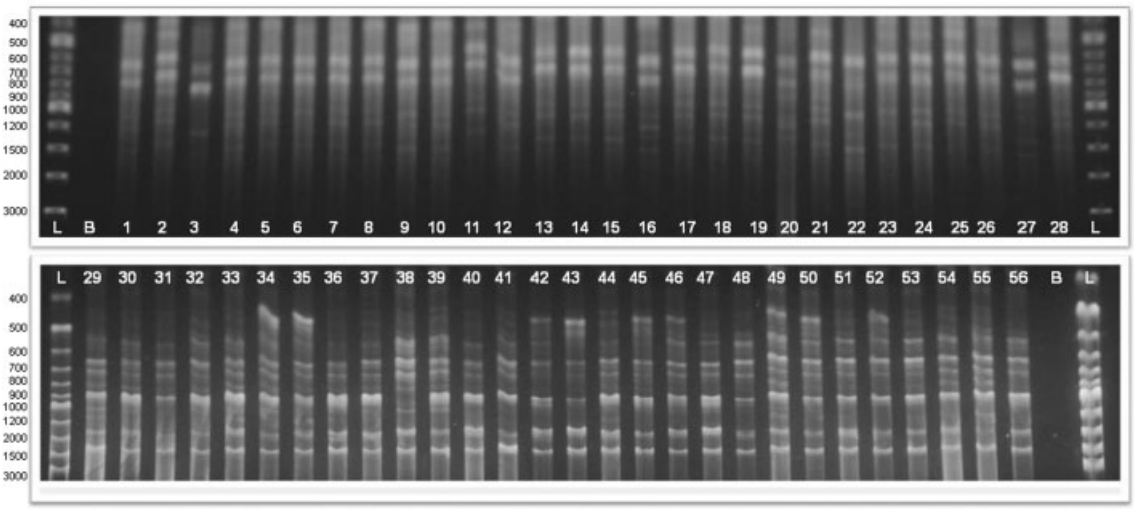

Figure 1. Inter simple sequence repeats (ISSR) banding pattern of 56 maqui genotypes generated by primer 815. L, standard molecular size (100 bp ladder); B, blank.

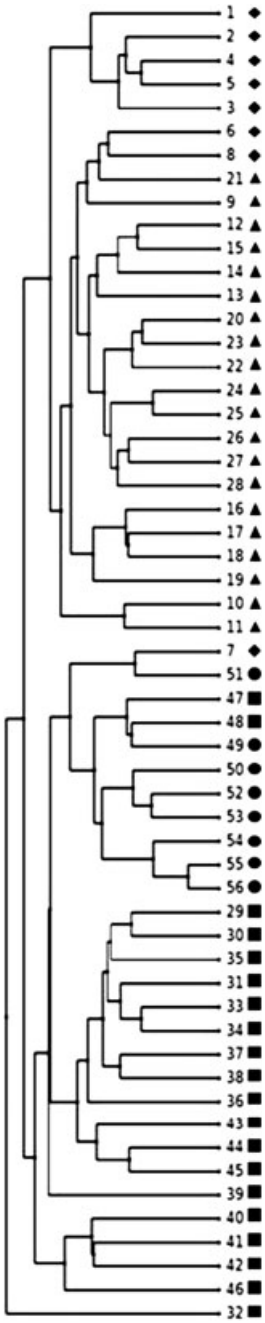

Figure 2. A UPGMA dendrogram illustrating genetic relationships of the 56 maqui genotypes from four different geographical areas based on the genetic distance coefficient. Numbers indicate the corresponding individuals. 1–8, Puchuncavi (◆); 9–28, Paredones (▲); 29–48, Talca (■); 49–56, Pucon (●).

for primer 812. Primers with higher Rp values were generally able to distinguish more genotypes and showed a higher number of polymorphic bands (Table 2).

The Shannon diversity index (I) showed significant genetic diversity among populations. The Talca population exhibited the highest level of genetic diversity ($I = 0.388 \pm 0.024$), whereas the Pucon population showed the lowest level of genetic diversity ($I = 0.226 \pm 0.024$). Figure 2 shows the UPGMA dendrogram, illustrating genetic relationships of the 56 maqui genotypes from the four different geographical areas based on the genetic distance coefficient among different populations. Although the UPGMA dendrogram differentiates the four sampling sites/geographical regions (Puchuncavi cluster: genotypes 1–5; Paredones cluster: genotypes 9–28; Talca cluster: genotypes 29–48; and Pucon cluster: genotypes 49–56), some maqui genotypes were included in clusters where they do not come from. This may be explained by potential gene flow along the wide distribution where maqui grows. Maqui seeds can be dispersed by birds (*Elaenia albiceps* D'Orbigny and Lafresnaye).[36] The wild maqui populations chosen for this study also corresponded to 'macales', or places were maqui individuals colonize after forest lodging; therefore some of the seeds were likely disseminated in the soil around the transplanted forest trees.

Total anthocyanins (TA) and total polyphenols (TP) in maqui genotypes

Significantly higher TA content measured among the 18 genotypes for phytochemical analyses were recorded for genotypes #10 (14.8 g cy-3-glu kg^{-1} FW), #29 (15.0 g cy-3-glu kg^{-1} FW) and #33 (14.8 g cy-3-glu kg^{-1} FW) and these TA contents were more than two times greater than those genotypes that fell at the low end of the scale (Table 3). According to our results, maqui fruit demonstrated higher TA content than fruits recognized for high TA content such as blueberry cultivars (0.2–4.8 g cy-3-glu kg^{-1} FW).[37]

The highest coefficient of variation for TA content was measured for Talca (18.1%) sampling site, suggesting that genotype had a significant influence on anthocyanin level. The Shannon diversity index had a high correlation with coefficient of variation for TA ($R = 0.75$, $P \leq 0.05$). This means that genetic diversity contributed to the variation of TA content, explaining the highest variation for TA content in Talca genotypes. Nevertheless, maqui fruit with the highest anthocyanin content (genotypes #29 and #33) do not correspond to genotypes that have very different genetic distance coefficients (data not shown) in comparison to individuals from Talca.

The TA content was significantly higher in Paredones and Talca than Puchuncavi and Pucon, showing the geographical area effect. Nevertheless, no significant differences between growing seasons 2010–2011 and 2011–2012 were found (Fig. 3). As described above, among the environmental factors, air temperature is the main factor influencing anthocyanin content in fruits,[32] and higher air temperatures were observed in Puchuncavi, Paredones and Talca than in Pucon during the last 30 days of fruit maturation (Table 1). Among sampling sites from central Chile, maqui fruit from Paredones and Talca showed the highest TA content in the range of 10.3–14.8 and 10.5–15.0 g cy-3-glu kg^{-1} FW, respectively, which may be attributed to the significant temperature oscillation during fruit maturation (Table 1). A similar trend was observed in grapes, where lower night temperature (15 °C) resulted in greater anthocyanin accumulation than for grapes grown at a constant temperature of 30 °C.[38]

As was observed with TA content, significant differences in TP content were found among the different genotypes and also between the different geographical regions (Table 3). The TP content for maqui fruits was significantly higher for Paredones (16.0 g GAE kg^{-1} FW) and Talca (16.2 g GAE kg^{-1} FW). The TP data did not appear to be a valid parameter in distinguishing the most anthocyanin-rich genotypes under conditions of this study, due to TP contributions from other phenolics in the crude extracts as measured by the Folin–Ciocalteu method. Although this method overestimates the phenol content due to the presence of L-ascorbic acid,[22] maqui would not present this interference

Table 3. Total anthocyanin (TA), total phenolic (TP) and antioxidant activity (FRAP and DPPH) of 18 maqui genotypes

Genotype	TA (g cy-3-glu kg^{-1} FW)	TP (g GAE kg^{-1} FW)	FRAP (mmol E Fe^{2+} kg^{-1} FW)	EC_{50} (mg FW)
Puchuncavi				
1	8.6 ± 0.4ab	14.7 ± 1.0abc	213.3 ± 23.2cdef	1.6 ± 0.2bcde
2	8.5 ± 0.2ab	13.3 ± 0.3abc	171.3 ± 20.6abcd	1.8 ± 0.1cde
3	8.7 ± 0.1ab	13.8 ± 0.8abc	161.5 ± 10.2abcd	1.6 ± 0.1abcd
4	7.3 ± 0.3a	16.1 ± 0.1cde	163.2 ± 44.0abcd	1.4 ± 0.1abc
Mean	8.3 ± 0.6A	14.5 ± 1.2A	177.3 ± 24.4B	1.6 ± 0.2AB
CV	7.7	8.4	13.7	10.4
Paredones				
9	11.0 ± 1.4b	15.3 ± 2.0bcd	203.5 ± 7.5bcdef	1.7 ± 0.2cde
10	14.8 ± 0.8c	20.5 ± 1.2e	227.6 ± 22.3def	1.2 ± 0.2a
11	10.3 ± 0.9b	14.7 ± 2.3abc	191.5 ± 46.2abcde	1.8 ± 0.2cde
12	10.6 ± 1.6b	14.3 ± 2.3abc	172.0 ± 50.8abcd	1.7 ± 0.1cde
13	10.8 ± 1.7b	14.9 ± 2.5abcd	193.7 ± 7.9abcdef	1.5 ± 0.3abcd
Mean	11.5 ± 1.9BC	16.0 ± 2.6B	197.7 ± 20.3B	1.5 ± 0.3A
CV	16.2	16.0	10.3	16.3
Talca				
29	15.0 ± 1.4c	19.7 ± 1.9e	262.5 ± 6.8f	1.2 ± 0.1ab
30	10.5 ± 1.0b	13.6 ± 1.2abc	158.3 ± 13.2abc	1.7 ± 0.3cde
31	10.8 ± 1.0b	14.1 ± 1.3abc	137.1 ± 12.0ab	1.7 ± 0.1cde
32	11.3 ± 1.0b	14.5 ± 1.2abc	204.2 ± 7.9bcdef	1.5 ± 0.2abcd
33	14.8 ± 1.2c	19.2 ± 1.6de	243.6 ± 4.0ef	1.2 ± 0.1a
Mean	12.5 ± 2.3C	16.2 ± 3.0B	201.1 ± 53.6B	1.4 ± 0.3A
CV	18.1	18.5	26.7	17.6
Pucon				
49	6.6 ± 0.3a	10.7 ± 0.8a	156.1 ± 8.7abc	2.0 ± 0.1e
50	7.1 ± 0.4a	10.8 ± 0.8a	147.2 ± 0.8abc	2.0 ± 0.1e
51	8.5 ± 0.1ab	12.0 ± 1.2abc	160.6 ± 3.5abcd	1.9 ± 0.1de
52	7.2 ± 0.5a	11.1 ± 0.1ab	131.0 ± 2.7a	1.8 ± 0.1cde
Mean	7.3 ± 0.8AB	11.1 ± 0.6A	148.7 ± 13.0A	1.9 ± 0.1B
CV	11.1	5.3	8.8	5.4

Mean values ($n = 6$) and standard deviation in the same column that are followed by different lower-case letters indicate significant differences between genotypes, and different upper-case letters indicate significant differences between sampling sites ($P \leq 0.05$). Analyses were performed in two consecutive growing seasons (2010–2011 and 2011–2012). cy-3-glu, cyanidin-3-glucoside; GAE, gallic acid equivalent; FW, fresh weight; EC_{50}, milligrams of sample that bleached 50% of DPPH methanolic solution; CV, coefficient of variation within each sampling site.

because L-ascorbic acid has not been detected in fruit according to Miranda-Rottmann *et al.*[3]

Anthocyanin profiles in maqui fruit genotypes

The anthocyanin glycosides and total anthocyanin content measured in maqui genotypes by HPLC were calculated as cyanidin-3-glucoside equivalents at 520 nm (Table 4). A representative chromatogram obtained by HPLC-DAD at 520 nm of maqui fruit and the corresponding anthocyanin profile is shown in Fig. 4. Maqui genotypes investigated in this study accumulated eight major anthocyanins, including: del-3-sa-5-glu; del-3,5-diglu; cy-3-sa-5-glu + cy-3,5-diglu; del-3-sa; del-3-glu; cy-3-sa; and cy-3-glu. These findings agree with previous reports on maqui berry fruits.[6–9] According to LC-MS analysis, maqui fruit from Puchuncavi (genotypes 1–4) had cy-3-sa and del-3-sa in very low concentrations that were under our limit of detection (LOD) of 5 mg kg^{-1} on LC-MS, and limit of quantitation (LOQ) of 0.05 µg by HPLC. Three anthocyanin peaks (Fig. 4; peaks 1, 10, 11) with absorption of 520 nm were present in some of the genotypes in quantifiable amounts.

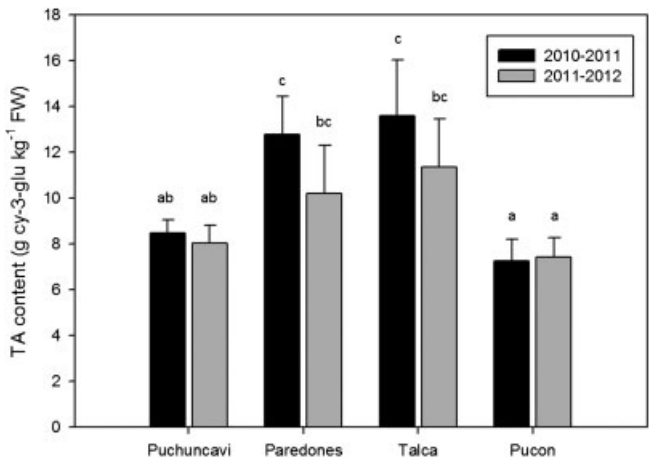

Figure 3. Total anthocyanin (TA) content of 18 maqui genotypes from four geographic regions in two growing seasons. Columns represent mean ± standard deviation. Columns with different letters were significantly different at $P < 0.05$.

Table 4. Relative anthocyanin glycoside content in 18 maqui fruit genotypes (expressed as g cy-3-glu kg^{-1} FW)

Genotype	ni	del-3-sa-5-glu	del-3,5-diglu	cy-3-sa-5-glu + cy-3,5-diglu	del-3-sa	del-3-glu	cy-3-sa	cy-3-glu	ni	ni	Total (TA)
Puchuncavi											
1	0.3 ± 0.03a	0.6 ± 0.05cde	2.6 ± 0.15abc	1.2 ± 0.12efg	0.1 ± 0.06ab	3.6 ± 0.17bcd	nd	1.6 ± 0.14f	nd	nd	9.9 ± 0.27bcde
2	0.3 ± 0.06a	1.1 ± 0.06f	3.0 ± 0.25abcd	1.5 ± 0.06 fg	nd	2.8 ± 0.15abc	nd	1.2 ± 0.08ef	nd	nd	10.0 ± 0.10bcde
3	0.4 ± 0.10a	1.5 ± 0.10g	4.7 ± 0.20fgh	1.5 ± 0.35g	nd	1.9 ± 0.15a	nd	0.5 ± 0.15ab	nd	nd	10.5 ± 0.65def
4	0.4 ± 0.10a	0.6 ± 0.06cde	3.1 ± 0.05abcd	0.7 ± 0.00abcd	nd	2.3 ± 0.15ab	nd	0.5 ± 0.20ab	nd	nd	7.6 ± 0.36a
Mean	0.3 ± 0.1A	0.9 ± 0.5B	3.4 ± 0.9B	1.2 ± 0.4B	nd	2.7 ± 0.7A	nd	0.9 ± 0.5C	nd	nd	9.5 ± 1.3A
CV	20.2	49.4	27.6	31.6	nd	27.9	nd	54.7	nd	nd	13.6
Paredones											
9	0.5 ± 0.00ab	1.8 ± 0.15h	3.8 ± 0.65defg	1.0 ± 0.00bcdef	0.5 ± 0.20cd	3.9 ± 0.06d	nd	1.0 ± 0.15cde	nd	nd	12.6 ± 1.20g
10	0.7 ± 0.10abcd	2.1 ± 0.06h	5.3 ± 0.40h	1.2 ± 0.06efg	0.6 ± 0.15d	5.4 ± 0.45e	0.1 ± 0.00a	1.1 ± 0.10de	nd	0.2 ± 0.00bc	16.6 ± 1.01h
11	0.7 ± 0.00abcd	0.4 ± 0.20bcd	3.4 ± 0.40bcde	0.6 ± 0.15ab	0.2 ± 0.06ab	4.2 ± 0.20d	0.1 ± 0.00a	0.8 ± 0.20bcde	nd	0.1 ± 0.06ab	10.5 ± 0.45cdef
12	0.7 ± 0.06abc	0.6 ± 0.06cde	3.2 ± 0.20abcd	0.8 ± 0.20bcde	0.1 ± 0.00ab	4.1 ± 0.20d	nd	1.1 ± 0.15de	nd	0.1 ± 0.00ab	10.6 ± 0.36def
13	0.8 ± 0.20abcd	0.6 ± 0.06cde	3.6 ± 0.55cdef	1.0 ± 0.15bcdef	0.1 ± 0.06ab	3.6 ± 0.65cd	nd	1.0 ± 0.06de	nd	nd	10.8 ± 0.90defg
Mean	0.7 ± 0.1B	1.1 ± 0.8C	3.9 ± 0.8C	0.9 ± 0.2A	0.3 ± 0.2B	4.2 ± 0.7B	nd	1.0 ± 0.1C	nd	nd	12.2 ± 2.6C
CV	16.2	73.2	21.7	25.6	75.5	15.6	nd	12.0	nd	nd	21.2
Talca											
29	1.8 ± 0.60e	0.7 ± 0.06de	4.8 ± 0.75gh	1.1 ± 0.15cdefg	0.2 ± 0.06ab	5.3 ± 0.70e	0.1 ± 0.06a	0.7 ± 0.10bcd	0.1 ± 0.06bc	0.3 ± 0.06c	15.2 ± 1.48 h
30	1.1 ± 0.10bcd	0.7 ± 0.06e	4.4 ± 0.40efgh	0.8 ± 0.06bcde	0.1 ± 0.06ab	3.7 ± 0.06cd	nd	0.5 ± 0.06ab	0.1 ± 0.06ab	0.1 ± 0.10ab	11.5 ± 0.30efg
31	1.2 ± 0.06cde	0.3 ± 0.06abc	3.6 ± 0.20cdef	1.1 ± 0.06cdefg	0.1 ± 0.06ab	3.7 ± 0.30cd	nd	0.6 ± 0.15abc	0.1 ± 0.00bc	1.1 ± 0.00d	11.7 ± 0.45efg
32	1.3 ± 0.30de	0.4 ± 0.06bc	3.8 ± 0.20defg	1.1 ± 0.06cdefg	0.1 ± 0.10ab	3.9 ± 0.06d	nd	0.6 ± 0.15abc	0.1 ± 0.06bc	1.1 ± 0.10d	12.4 ± 0.45fg
33	1.8 ± 0.35e	0.8 ± 0.15e	8.2 ± 0.60i	0.7 ± 0.15abc	0.2 ± 0.06ab	5.9 ± 0.25e	0.1 ± 0.06a	0.4 ± 0.06ab	0.2 ± 0.06c	1.5 ± 0.15e	19.7 ± 0.85i
Mean	1.5 ± 0.3C	0.6 ± 0.2A	5.0 ± 1.9D	0.9 ± 0.2A	0.1 ± 0.1A	4.5 ± 1.0B	nd	0.6 ± 0.1A	0.1 ± 0.0	0.8 ± 0.6	14.2 ± 3.5D
CV	23.4	36.2	37.8	19.5	57.1	22.8	nd	19.1	31.7	72.1	24.8
Pucon											
49	0.3 ± 0.06a	1.5 ± 0.15g	2.2 ± 0.15a	1.1 ± 0.06cdefg	0.3 ± 0.06bc	2.3 ± 0.15ab	0.1 ± 0.06a	0.8 ± 0.06bcd	nd	nd	8.4 ± 0.12abc
50	1.3 ± 0.06cde	0.1 ± 0.06a	3.7 ± 0.15defg	0.3 ± 0.06a	nd	3.7 ± 0.25cd	0.1 ± 0.06a	0.3 ± 0.06a	nd	0.1 ± 0.00ab	9.4 ± 0.21abcd
51	0.7 ± 0.15abc	0.2 ± 0.06ab	2.3 ± 0.30ab	1.1 ± 0.10cdefg	nd	2.8 ± 0.40abc	nd	1.2 ± 0.20ef	nd	0.1 ± 0.10ab	8.4 ± 1.00ab
52	1.0 ± 0.06bcd	0.6 ± 0.06cde	3.1 ± 0.06abcd	0.9 ± 0.00bcdef	nd	2.5 ± 0.06ab	0.1 ± 0.06a	0.8 ± 0.10bcde	nd	nd	8.9 ± 0.26abcd
Mean	0.8 ± 0.4B	0.7 ± 0.06A	2.8 ± 0.7A	0.8 ± 0.4A	nd	2.8 ± 0.6A	nd	0.8 ± 0.4B	nd	nd	8.8 ± 0.5B
CV	49.7	85.7	25.9	46.5	nd	21.3	nd	50.4	nd	nd	5.2

Mean values ($n = 3$) and standard deviation that are followed by different lower-case letters in the same column indicate significant differences between genotypes, and different upper-case letters indicate significant differences between sampling sites ($P \leq 0.05$). Analyses were performed in one growing season (2011–2012). Columns refer to the chromatogram shown in Fig. 4: del-3-sa-5-glu = delphinidin-3-sambudioside-5-glucoside; del-3,5-diglu = delphinidin-3,5-diglucoside; cy-3-sa-5-glu + cy-3,5-diglu = cyanidin-3-sambudioside-5-glucoside + cyanidin-3,5-diglucoside; del-3-sa = delphinidin-3-sambudioside; del-3-glu = delphinidin-3-glucoside; cy-3-sa = cyanidin-3-sambudioside; cy-3-glu = cyanidin-3-glucoside. Total (TA), total anthocyanin content by HPLC; ni, not identified; nd, not detected under the conditions of this method; CV, coefficient of variation within each sampling site.

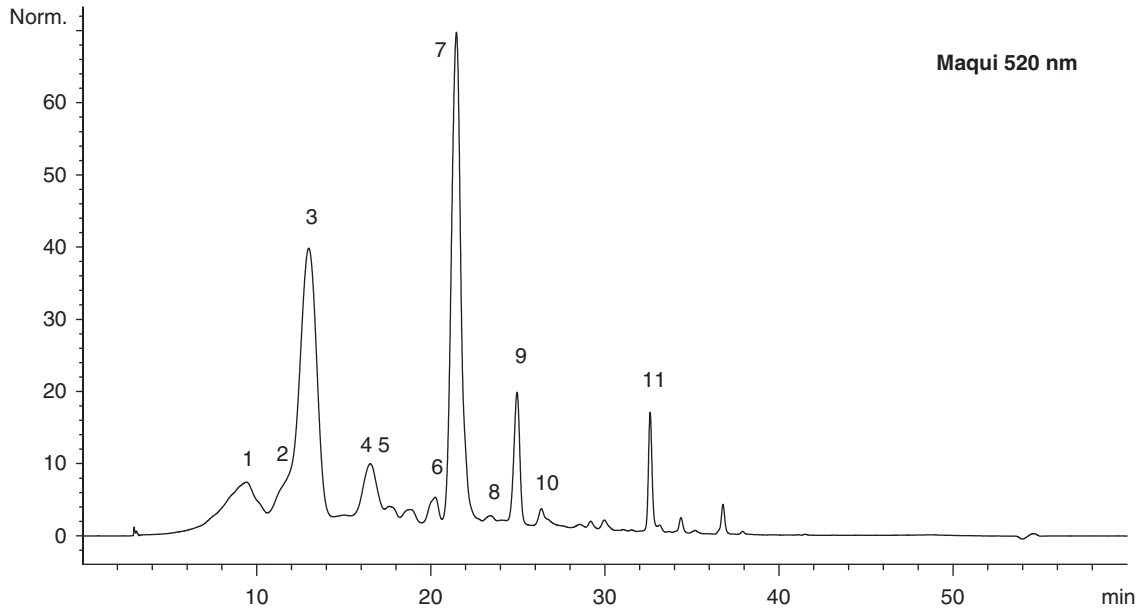

Figure 4. Representative HPLC-DAD chromatogram of anthocyanin compounds obtained at 520 nm. Peaks: 1 = not identified; 2 = delphinidin-3-sambudioside-5-glucoside (del-3-sa-5-glu); 3 = delphinidin-3,5-diglucoside (del-3,5-diglu); 4–5 = cyanidin-3-sambudioside-5-glucoside + cyanidin-3,5-diglucoside (cy-3-sa-5-glu + cy-3,5-diglu); 6 = delphinidin-3-sambudioside (del-3-sa); 7 = delphinidin-3-glucoside (del-3-glu); 8 = cyanidin-3-sambudioside (cy-3-sa); 9 = cyanidin-3-glucoside (cy-3-glu); 10–11 = not identified.

Both del-3-sa-5-glu and del-3-glu have been reported as the main anthocyanins in maqui berry.[6–9] In our results del-3-sa-5-glu did not appear as the main anthocyanin in any of the genotypes, whereas both del-3,5-diglu and del-3-glu were the main anthocyanins in 50% of the genotypes. The variations and ranges recorded in these results were expected given the different genotypes and environments used in this study.

Total anthocyanins estimated by HPLC quantification, as cyanidin-3-glucoside equivalents, were higher than those obtained by the pH differential method by an average of 1.2-fold (Tables 3 and 4). There was a high correlation ($R = 0.83$, $P \leq 0.05$) between the two methods for the determination of anthocyanin content. Previous reports have indicated that higher estimates are obtained using the HPLC method as compared to the pH differential method for cranberry[25] and elderberry.[39]

As was observed with pH differential method, the total anthocyanin levels estimated by HPLC were very different between the 18 maqui genotypes (ranging from 7.6 to 19.7 g-cy-3-glu kg^{-1} FW), where genotypes #10 (16.6 g cy-3-glu kg^{-1} FW), #29 (15.2 g cy-3-glu kg^{-1} FW) and #33 (19.7 g cy-3-glu kg^{-1} FW) recorded the highest anthocyanin content. The coefficients of variation within each sampling site were also very high, especially in Paredones (21.2%) and Talca (24.8%). The observed variability in each region may be a consequence of the genetic variability between the samples, each obtained from different genotypes from wild populations. Maqui is a dioecious plant[13] with cross-pollination where a high level of genetic variation should be expected. Maqui fruit has a diameter of 0.5 mm with three or four seeds.[10] Therefore the genetic variation can result in differential phytochemical accumulation among genotypes, including anthocyanin accumulation.

Several authors[15,37,40,41] have indicated that genotype variety is the major factor that affects the anthocyanin content in berry fruits; therefore it is possible to distinguish better genotypes within a specific fruit species that can be selected for future selection and breeding programs. Our data indicated that maqui genotypes could be distinguished based on their inherently high anthocyanin content and the different anthocyanin profile. The identification of different anthocyanin profiles in maqui fruit genotypes is important in finding specific types of anthocyanins. For example, del-3-sa-5-glu has been reported in few species;[7] therefore selection for genotypes with superior content of this anthocyanin type could be an important factor because of its superior anti-diabetic effect *in vivo*.[6]

Antioxidant activities in maqui berry genotypes

Significant differences in antioxidant activity by FRAP and DPPH method were found among the different genotypes and also between the different geographical areas, as shown in Table 3. For the DPPH method, results were calculated as EC_{50}; therefore the lowest EC_{50} values indicate the highest antioxidant activity. The genotypes which had the highest antioxidant activity by the FRAP method were #29 (262.5 mmol E Fe^{2+} kg^{-1} FW), #33 (243.6 mmol E Fe^{2+} kg^{-1} FW) and #10 (227.6 mmol E Fe^{2+} kg^{-1} FW). These results were in accord with the DPPH method, where genotypes #29 (1.2 mg), #10 (1.2 mg) and #33 (1.2 mg) were distinguishable by their high antioxidant activity. A high correlation ($n = 108$) between TP-EC_{50} ($R = 0.76$, $P \leq 0.05$), TP-FRAP ($R = 0.59$, $P \leq 0.05$), TA-EC_{50} ($R = 0.60$, $P \leq 0.05$) and TA-FRAP ($R = 0.53$, $P \leq 0.05$) was found. Similar results, where TP demonstrated a stronger correlation with antioxidant activity in red fruits, were obtained by Lohachoompol *et al.*[41] and Caliskan *et al.*[42]

It is important to consider both the bioactivity and bioavailability of health-relevant anthocyanins from different maqui genotypes in order to make the best recommendations to growers. Previous research has indicated that certain types of anthocyanins have potent antioxidant activity, in particular those with multiple OH groups, where activity increased with increasing number of hydroxyl substituents on the B ring.[43] However, other results showed that different structural features of anthocyanins had greater absorption efficiency in *in vitro* and *in vivo* models, conferring the importance of specific anthocyanins in some fruits

types.[44,45] Yi *et al.*,[45] using Caco-2 human intestinal cell monolayers, showed that cyanidin 3-glucoside and peonidin 3-glucoside had higher transport efficiencies than cyanidin 3-galactoside and peonidin 3-galactoside, respectively, indicating the higher bioavailability of glucose-based anthocyanins. On the other hand, Charron *et al.*[46,47] showed that non-acylated anthocyanins from juiced carrots, whole carrots and red cabbage are more bioavailable than acylated anthocyanins. Although *in vivo* bioavailability research for maqui anthocyanins is not available yet, these non-acylated, non-conjugated anthocyanin forms may prove to be more bioavailable. Nevertheless, further studies in this area are warranted to address the effects of anthocyanin localization in the plant matrix and the possibility to isolate this effect from the effects of anthocyanin structure.[46,47]

CONCLUSION

ISSR-PCR provides different banding patterns (fingerprints) for the 56 maqui individuals evaluated, confirming that the 18 maqui individuals studied for phytochemical analyses belonged to different genotypes. This study demonstrated that the 18 tested maqui genotypes all exhibited high anthocyanin content and different anthocyanin profiles, and three genotypes were distinguished by particularly high anthocyanin content. The antioxidant activity (FRAP and DPPH methods) correlated better with total polyphenols than total anthocyanins. Nevertheless, the genotypes with the highest anthocyanin content had the highest antioxidant activity. The superior genotypes can be used as resources for maqui selection and in breeding programs aimed at developing new maqui varieties with elevated anthocyanin content.

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