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BASES PARA LA DOMESTICACIÓN DE GAULTHERIA PUMILA LF, ERICACEAE. ESTUDIO DE DIVERSIDAD GENÉTICA Y PROTOCOLOS DE MULTIPLICACIÓN

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BASES PARA LA DOMESTICACIÓN DE GAULTHERIA PUMILA LF, ERICACEAE. ESTUDIO DE DIVERSIDAD GENÉTICA Y PROTOCOLOS DE MULTIPLICACIÓN

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A mi familia
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Introducción General

El género Gaultheria pertenece a la familia Ericaceae y su distribución en el continente americano esta reportada desde México hasta Argentina (Luteyn 2002, Hermann and Cambi 2006). En Chile se reportan doce especies dentro de este género, entre ellas está Gaultheria pumila (Teillier and Escobar 2013).

Gaultheria pumila (L) Middleton, sub familia Vaccinioideae, del orden Ericales, tribu Gaultherieae, es conocida comúnmente como "chaura o cola de zorro" y se distribuye desde la Región Metropolitana hasta la Región de Magallanes, encontrándola desde los bordes costeros hasta las faldas de la cordillera de los Andes.

G. pumila es un arbusto pequeño, dioico, de hojas alternas, coriáceas, de flores blancas, de polinización abierta al igual que otras especies de la familia Ericaceae (Cane et al. 1985, Luteyn 2002). La especie presenta características deseables, como el color, el sabor, el aroma de sus frutos y su alto contenido de antocianinas y pectinas, y un grado importante de adaptabilidad a diferentes condiciones ambientales.

Los frutos presentan diversos colores (blanco, rosado y rojo), formas (globosas u ovoides), y diámetros (6 a 12 mm). Estos son achatos en el ápice, carnosos, aromáticos, de sabor intenso y poseen alto contenido de flavonoides (Middleton 1992, Villagra et al. 2014). Mayormente el contenido de antocianinas está en los frutos de color rojo, reportando un promedio de 5.942 mg, seguido por los frutos de color rosa, con un promedio de 3.854 mg, y los frutos blancos tienen el menor contenido de antocianina con un promedio de 626.2 mg antocianinas monoméricas por 100 g de muestra (Villagra et al. 2014). Estos compuestos polifenólicos aportan con grandes beneficios para la salud humana (Ruiz et al. 2010, Schreckinger et al. 2010, Nile and Park 2013, Ramirez et al. 2015).
Los frutos son una fuente de alimento y medicina para los pueblos originarios Aónikenk, Sel’nam, Kawésqar, Yagan y Haush, en la Patagonia Austral de Chile (Domínguez Díaz 2010). Poseen alrededor de 50 semillas por frutos, sin embargo, se pudo determinar que tiene bajo potencial germinativo, lo que hace necesario recurrir a técnicas eficientes de multiplicación in vitro.

Este arbusto, cuya expresión fenotípica varía entre individuos dentro de la misma población, crece en suelos volcánicos, arenosos con bajos niveles de materia orgánica, por lo que muestra poca exigencia en calidad de suelos. La rusticidad que presenta permite que sea considerada como una especie colonizadora, al ser una de las primeras en aparecer en áreas afectadas por desastres naturales (Luteyn 2002).

Posee un amplio rango de adaptación, adecuándose a diferentes condiciones gracias a la plasticidad ecológica que presenta. Se han encontrado plantas que permanecen bajo la nieve en época invernal y en verano expuesta a altas temperaturas y de radiación solar, condición que para otras especies resulta ser una limitante (Larcher et al. 2010). Se le atribuye también un efecto de simbiosis micorrícica (Medina et al. 2008), con alto valor ecológico en los procesos de restauración de ecosistemas, ya que forma nuevos nichos ecológicos junto a especies como el colihue (Chusquea culeou) o lenga (Nothofagus pumilio).

Estos atributos hacen de esta especie un recurso fitogenético de alto valor, para su uso y aprovechamiento en diferentes escenarios productivos (agrícola-farmacéutico), como ha ocurrido en otras especies del genero Gaultheria (Apte et al. 2006, Liu et al. 2013, Michel et al. 2016). Sin embargo, y a pesar de su alto potencial, esta especie ha sido poco estudiada, permaneciendo aún como un recurso genético sin explorar, dejando de lado sus atributos y reduciendo la posibilidad de convertirla en una opción de consumo.
En este sentido, la identificación y selección de genotipos silvestres de chaura juega un rol importante en las pretensiones de domesticarla y adaptarla (Gepts and Papa 2002, Gepts 2004) y a su vez, pueden constituir la base científica y tecnológica para implementar programas de selección y mejora de la especie, siguiendo un modelo de desarrollo similar al aplicado para otras Ericaceas (Finn et al. 2003).

Los esfuerzos por descubrir y desarrollar nuevas especies son constantes, ya que de toda la agrobiodiversidad que alberga la tierra, solo un pequeño porcentaje es aprovechado ya sea mediante su cultivo o mediante explotación en su medio natural. Dentro de la familia Ericaceae, 4050 especies existentes, solo 28 son aprovechadas en forma de cultivo (Khoshbakht and Hammer 2008).


Estas iniciativas para aprovechar los beneficios de las especies nativas, se deben a la necesidad que existe de potenciar especies que puedan ser una opción para la producción agrícola, la agregación de valor y el desarrollo de nuevos suplementos alimenticios y medicamentos. En este contexto el presente trabajo se centra en determinar la diversidad y composición genética de las poblaciones naturales de G.
*pumila*, así como el desarrollo de tecnologías de propagación *in vitro*, con el objetivo de crear las bases científicas y metodológicas para desarrollar un programa de domesticación y aprovechamiento de la especie.
Referencias


Domínguez Díaz, E. 2010. Flora de interés etnobotánico usada por los pueblos


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Objetivos

Objetivo General

Determinar la diversidad y estructura genética de *G. pumila* mediante marcadores moleculares SSRs y establecer protocolos de multiplicación *in vitro*

Objetivos Específicos

a) Desarrollar marcadores moleculares SSRs específicos de *G. pumila* utilizando tecnología de última generación para el estudio de diversidad genética

b) Determinar la estructura y variabilidad genética de *G. pumila* a través de marcadores moleculares SSRs

c) Diseñar un protocolo de multiplicación, enraizamiento *in vitro* y de adaptación *ex vitro* de *G. pumila*

Hipótesis

a) La mayor variabilidad genética se encuentre dentro de las poblaciones

b) La capacidad morfogénica de *G. pumila* puede ser explotada a través de técnicas de multiplicación *in vitro*
Development and characterization of microsatellite loci in *Gaultheria pumila* Lf. (Ericaceae)

Abstract

Polymorphic microsatellite markers were developed for *Gaultheria pumila* (Ericaceae) to evaluate genetic diversity and population structure within its native range in Chile. This is one of the most important Ericaceae species endemic to Chile. It has a large commercial potential and can be a very interesting target for breeding programs since it is very resistant to different abiotic conditions. Ten polymorphic Simple Sequence Repeat (SSR) loci were isolated from *Gaultheria pumila* using new-generation 454 FLX Titanium pyrosequencing technology. The mean number of alleles per locus ranged from 2 to 4. Observed and expected heterozygosity ranged from 0.00 to 1.0 and 0.00 to 0.64, respectively. From 10 SSR markers developed for *G. pumila*, nine markers are promising candidates for analyzing genetic variation within and between natural populations of *G. pumila* and other species from the same genus.

**Key words**: molecular marker, pyrosequencing, Chilean berry.
Introduction

The *Gaultheria* genus belongs to the Ericaceae family and it is adapted to tropical and temperate conditions. All of these species are commonly known as “*chaura*” or “*murta*” (Teillier and Escobar 2013). *Gaultheria pumila* is one of the most important species in terms of population size; colonizing plant (Luteyn 2002) and has a symbiotic mycorrhiza effect (Medina et al. 2009) even a botanic variety has been identified (*Gaultheria pumila* var. leucocarpa). It is an interesting species for domestication as has shown a high level of phenotypic variability and high levels of polyphenols and anthocyanins (Middleton 1992, Villagra et al. 2014). However, the lack of basic knowledge of agronomically important traits as well about the organization of its genetic variability provides serious limitations to the domestication process and its conversion to new crop. For this reason, the aim of this study was to develop a set of polymorphic microsatellite markers can be used as a tool to assess the diversity and genetic structure of *Gaultheria* genus.

Methodology

1.1.1 Samples collection and DNA extraction

In order to carry out complete sequencing, four samples of *G. pumila* were collected in two places, 1) Villarrica volcano in Región de La Araucanía Chile, S: 39°21’570, W: 71°57’865 (El Playón sector), and 2) near the Puyehue volcano, in the Región de Los Lagos, Chile, S: 40°41’484, W: 72°32’719 (Orilla de Rio). The genomic libraries were built selecting samples based on fruit color (red-pink and white). Total genomic DNA of *G. pumila* was extracted from young leaves with a modified CTAB (cetyltrimethylammonium bromide) method (Murray and Thompson 1980). The quality and quantity of isolated DNA was determined using a Nanodrop and agarose gel electrophoresis for 30 minutes.
1.1.2 DNA sequencing and microsatellite identification
The library preparation and shotgun pyrosequencing of 5 μg DNA aliquot on a 454 GS-FLX Titanium instrument (Roche Applied Science, MACROGEN, Ltd. Seoul, South Korea) was prepared with the kit of Titanium Pico Titer Plate (Roche Diagnostics) following the manufacturer’s protocols. A total of 164 sequences were read, with an average length of 417 pb, which were generated by three independent runs. The sequences were assembled using the software GS De Novo Assembler (V2.9). The MSATCOMANDER (http://code.google.com/p/msatcommander/) program was used for locating microsatellites.

1.1.3 SSR validation
The PCR fragment amplification and validation of selected SSRs was performed following the method developed by Schuelke (2000) which uses three primers: a forward primer with an M13 (-21) tail at its 5' end, a normal reverse primer and the universal M13 (-21) primer labeled with either 6-FAM, VIC, PET or NED fluorochromes. The microsatellite information and GenBank accession numbers are listed in Table 1. PCR amplifications were performed in a 20 μl reaction mixture with 10 ng templates DNA, 0.15 mM of each dNTP; 1 X Taq polymerase reaction buffer; 1.5 mM MgCl₂; 0.025 μM forward primer; 0.1 μM reverse primer; 0.1 μM M13 primer and 0.35 U Taq DNA polymerase. PCR amplifications were performed in an Applied Biosystems Veriti (Life Technologies) under the following conditions: initial denaturation at 94 °C for 5 min; 30 cycles of 30s at 94 °C, annealing temperature specific to each primer pair for 45 s, extension at 72 °C for 45 s, followed by 8 cycles of 30 s at 94 °C, annealing at 53 °C for 45 s, extension at 72 °C for 45 s and a final extension at 72 °C for 30 min. The annealing temperature (°C) requirements of primers are specified in Table 1. PCR products were resolved as following: 2 μL of PCR
products were mixed with 10 μL HiDi formamide (Applied Biosystems) and 0.2 μL GeneScan 500LIZ size standard (Life Technologies, Foster City, CA) and separated by capillary electrophoresis on an ABI 3130xl Prism Genetic Analyzer with POP-7 polymer (Life Technologies, Foster City, CA) in the Genetic Resources Unit, La Platina-INIA, Chile. Allele sizes were automatically calculated with GeneMapper software v4.0 and manually checked.

1.1.4 Analysis of data
Three populations of *G. pumila* were selected to evaluate the variability in the isolated loci: Región de La Araucanía, (Villarrica volcano, n= 10); S 39°34’463, W 71°28´509, Región de Los Lagos, (Puyehue volcano, n= 10), S 40°40’538 W 72°07´103, Región de Magallanes (Punta Arenas, n=10) S 53°9’413 W 71°1´437. Genetic parameters, such as observed number of alleles (*Na*) and observed and expected heterozygosity (*Ho-He*), were estimated with PopGene (Yeh et al. 1997). The inbreeding coefficient (*FIS*) (Weir and Cockerham 1984) was determined using Genetix (Belkhir et al. 2003). Deviation from the Hardy-Weinberg equilibrium was determined with GENEPOP v 4.2 (Raymond and Rousset 1995). The presence of null alleles was checked using MICRO-CHECKER version 2.2.3 (Van Oosterhout et al. 2004). In addition, tested cross-amplification with two species, *G. mucronata* and *G. caespitosa* collected in the same population as *G. pumila* were carried out.

Results and Discussion

1.1.5 Selection of polymorphic microsatellite
Primers were considered successful when one clear band was detected on 2% agarose gel. The mononucleotide repeat lengths were 10, the dinucleotide were six and four for the tri - tetra - penta and hexanucleotides. The sequences that contained more repetition were the mononucleotides averaging 59%, while the dinucleotides were 23%
trinucleotides 12% tetranucleotides 3% and pentanucleotide 2%. A total of 17 loci microsatellite were tested, which included 4 mono, 5 di, 4 tri, 3 tetra, and 1 pentanucleotide. The first test consisted of selecting the best SSRs that showed amplification, and were visualized in 2% agarose gel. Ten microsatellites were selected for further development and analysis (Table 1.1). From these microsatellite loci, nine were variable and polymorphic between populations. The microsatellite locus GP.7 was inconsistent in different tested conditions, and it was not included further.

Table 1.1 Characteristics of ten microsatellite loci and primer pair’s development for *G. pumila*. For each locus, the name, primer sequence, products size, repeat motif, allele size range (bp), Fluorescent dye, annealing temperature (Ta) and GenBank accession number are indicated

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sequences (5’-3’)</th>
<th>Repeat motif</th>
<th>Allele size (bp)</th>
<th>Dye</th>
<th>Ta(°c)</th>
<th>GenBank</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP.7</td>
<td>F:CGCATTCACTCAACCTCTCA</td>
<td>(ACTC)^4</td>
<td>360</td>
<td>FAM</td>
<td>52.1</td>
<td>KX719822</td>
</tr>
<tr>
<td></td>
<td>R:TGGTGTTGAAGGCTTTGGA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP.9</td>
<td>F:ACCCGCTCTAGATCCTCTCT</td>
<td>(T)^10</td>
<td>378-380</td>
<td>FAM</td>
<td>60.5</td>
<td>KX719823</td>
</tr>
<tr>
<td></td>
<td>R:AGGAGGGAATGCAAACCTCTCT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP.10</td>
<td>F:GGGTACGCGTAGCTGGAAT</td>
<td>(AT)^8</td>
<td>323-327</td>
<td>VIC</td>
<td>60.5</td>
<td>KX719824</td>
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<tr>
<td></td>
<td>R:TCGTAACAAAACGCTTCAAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP.12</td>
<td>F:ACGATTATAGAGGACGCCTGGA</td>
<td>(CTT)^4</td>
<td>198-201</td>
<td>NED</td>
<td>52.1</td>
<td>KX719825</td>
</tr>
<tr>
<td></td>
<td>R:CAGAAGACGAAATCGGAGCCG</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP.13</td>
<td>F:AGAGTAAGAGCTCTTTCCGA</td>
<td>(ATT)^4</td>
<td>161-278</td>
<td>NED</td>
<td>52.1</td>
<td>KX719826</td>
</tr>
<tr>
<td></td>
<td>R:GCAGACTGAATCCGAGCTGA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP.14</td>
<td>F:GCATAGCCGCGTTGCAAC</td>
<td>(ACGC)^4</td>
<td>196-228</td>
<td>PET</td>
<td>52.1</td>
<td>KX719827</td>
</tr>
<tr>
<td></td>
<td>R:ACCGAAGATCCGACCACGTG</td>
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<tr>
<td>GP.15</td>
<td>F:GGGCTGCTGCTCAAATCAAT</td>
<td>(T)^10</td>
<td>347-351</td>
<td>VIC</td>
<td>52.1</td>
<td>KX719828</td>
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<tr>
<td></td>
<td>R:ACCGCTTCAATCGTAGATGA</td>
<td></td>
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<tr>
<td>GP.16</td>
<td>F:GCTATTTCTAGGCCTGGGACC</td>
<td>(AT)^6</td>
<td>281-283</td>
<td>FAM</td>
<td>52.1</td>
<td>KX719829</td>
</tr>
<tr>
<td></td>
<td>R:GCACATACATAGATGTCTGGA</td>
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</tr>
<tr>
<td>GP.17</td>
<td>F:GAGAGAATCCACCACCAGGCA</td>
<td>(T)^10</td>
<td>202-205</td>
<td>VIC</td>
<td>60.5</td>
<td>KX719830</td>
</tr>
<tr>
<td></td>
<td>R:CAAGCGAAGTGATGATCAGA</td>
<td></td>
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</tr>
<tr>
<td>GP.18</td>
<td>F:ACCGAAAGATCCGACCACATG</td>
<td>(GCGT)^4</td>
<td>192-228</td>
<td>PET</td>
<td>52.1</td>
<td>KX719831</td>
</tr>
<tr>
<td></td>
<td>R:GCATAGCCGGTTGCAAAC</td>
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</tbody>
</table>
1.1.6 Genetic diversity analysis

The mean number of alleles per locus ranged from 1 to 4, with an average of 1.96. Observed and expected heterozygosity ranged from 0.00 to 1.0 and 0.00 to 0.67, respectively (Table 1.2). In addition, three microsatellite loci: GP.12, GP.13 and GP.17 exhibited significant deviation from Hardy-Weinberg Equilibrium (HWE), for the Puyehue population. In the Villarrica population the locus GP.14 was monomorphic, while the loci GP.9, GP.10, GP.12, GP.13 and GP.18 had no significant deviations. For the Punta Arenas samples, four loci did not show significant deviations from HWE, which were GP.9, GP.10, GP.12 and GP.15. Such deviations can be due to the small sample size, selfing, or to the substructure of the populations. Additionally, out of ten SSR loci, five were reproducible in *G. mucronata* and six were reproducible in *G. caespitosa*, which suggests that the markers could be useful in will carrying out studies of genetic diversity in other *Gaultheria* species.

Table 1.2 Estimated genetic parameters *Na*, *Ho*, *He*, *Fis* and Hardy-Weinberg test for ten microsatellite loci in three wild populations of *G. pumila*, Puyehue, Villarrica and Punta Arenas

<table>
<thead>
<tr>
<th>Locus</th>
<th>Puyehue (n=10)</th>
<th>Villarrica (n=10)</th>
<th>P. Arenas (n=10)</th>
<th>Cross-amplification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Na</em></td>
<td><em>Ho</em></td>
<td><em>He</em></td>
<td><em>Fis</em></td>
</tr>
<tr>
<td>GP.7</td>
<td>1</td>
<td>0.00</td>
<td>0.00</td>
<td>NA</td>
</tr>
<tr>
<td>GP.9</td>
<td>1</td>
<td>0.00</td>
<td>0.00</td>
<td>-0.24</td>
</tr>
<tr>
<td>GP.10</td>
<td>2</td>
<td>0.30</td>
<td>0.27</td>
<td>-0.27</td>
</tr>
<tr>
<td>GP.12</td>
<td>2</td>
<td>0.00</td>
<td>0.18*</td>
<td>1.00</td>
</tr>
<tr>
<td>GP.13</td>
<td>2</td>
<td>1.00</td>
<td>0.53*</td>
<td>-0.46</td>
</tr>
<tr>
<td>GP.14</td>
<td>2</td>
<td>0.10</td>
<td>0.10</td>
<td>-0.00</td>
</tr>
<tr>
<td>GP.15</td>
<td>3</td>
<td>0.70</td>
<td>0.67</td>
<td>0.45</td>
</tr>
<tr>
<td>GP.16</td>
<td>1</td>
<td>0.00</td>
<td>0.00</td>
<td>1</td>
</tr>
<tr>
<td>GP.17</td>
<td>2</td>
<td>1.00</td>
<td>0.53*</td>
<td>0.70</td>
</tr>
<tr>
<td>GP.18</td>
<td>2</td>
<td>0.10</td>
<td>0.10</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Note: + = Successful PCR amplification; - = unsuccessful PCR amplification; *Na*= number of alleles; *Ho*= observed heterozygosity; *He*= expected heterozygosity; *Fis*= fixation index. * Significant deviation from Hardy-Weinberg Equilibrium (p = <0.05).
Conclusions

For _G. pumila_, ten microsatellite loci were identified; however, only nine proved to be suitable markers to be used in population genetics, for studying genetic structure and genetic variability. These findings provide a basis for starting a domestication program for this species, which potentially could include other relatives in the Ericaceae family. Further research should be done to find out new SSR microsatellite markers and to test them among _G. pumila_ and other species of this genus.
Literature cited


Pico-Mendoza et al. – Gaultheria microsatellite

DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE LOCI IN GAULTHERIA PUMILA LF. (ERICACEAE)¹

Documento enviado como Prime Note a la revista *Applications in Plant Sciences*
2 Capítulo II

Genetic diversity and population structure of *Gaultheria pumila* Lf. (Ericaceae)

Abstract

*Gaultheria pumila* Lf. is a berry native from Chile, with high contents of polyphenols and anthocyanins. This species has a high potential for consumption due to its organoleptic qualities and morphometric characteristics. There are few reports on its utility and potential uses. Sampling was carried out in 11 populations from four Regions of Chile. A total of 160 individuals were collected and analysed using a set of six microsatellite markers. The neutral test did not show selective force acting on the analysed SSR loci. The genetic diversity per locus, showed a mean observed heterozygosity of $H_o=0.53$ while the expected heterozygosity was $H_e=0.54$. The polymorphic information content was 0.47. The average Shannon diversity index was moderate to high with a value of 0.99. The fixation index showed an average of -0.16. The average level of polymorphic loci in all populations was 96.97. The analysis of molecular variance indicated that the species has a low level of genetic fragmentation showing 94% of the genetic diversity within populations while there was only 6 % between populations. Significant correlations were found between genetic and geographic distance. The multivariate and Bayesian analysis showed that the genetic structure is conformed of two genetic groups. The species appears to be highly connected, probably due to the activity of pollinators and small animals that feed on its fruits and subsequently spread the seeds. These observations on the genetic diversity in the natural populations of *G. pumila*, will allow the design of efficient strategies for carrying out selection of suitable types for exploitation and developing a breeding program.
Key words: Allelic frequency, heterozygosity, inbreeding coefficient, AMOVA

Introduction

The genus *Gaultheria* belongs to the Ericaceae family and it is adapted to tropical and temperate climates. This genus is important in terms of functional human nutrition as many of its species contain high levels of flavonoids and simple phenols (Middleton 1992, 1993, Villagra et al. 2014). It is also important because it contains different compounds with anti-rheumatic effects (Liu et al. 2013). Many of these species are diploids, but some of them are tetraploid or hexaploid (Mukhopadhyay et al. 2016). The distribution of this genus in the American continent has been documented from México to Argentina (Luteyn 2002, Hermann and Cambi 2006). In Chile, twelve species of this genus have been reported, including *Gaultheria pumila* (Teillier and Escobar 2013). The genus is found from the Región Metropolitana to the Región de Magallanes. The species *G. pumila* is a dioecious evergreen shrub, commonly known as “*chaura or mutilla del zorro*” depending on the site where it is found. It has been observed that *G. pumila* has a high degree of adaptability to extreme conditions, and can be found either under snow in the winter or exposed to high temperatures and high UV radiation during the summer. This species is found in the understory of the native Araucano forests, and is present as a colonizing plant, being one of the first to appear in areas affected by disasters (Luteyn 2002). It has symbiotic mycorrhizal associations (Medina et al. 2009) that introduce a high ecological value in ecosystem restoration processes. *G. pumila* is used by the native people of the Austral Patagonia, where it has been used for food and medicines (Dominguez 2010). In natural populations the species shows a huge phenotypic variation. Fruits have different forms (globose or ovoid) with sizes between 6 to 12 mm in diameter, and colours (white, pink or red). Additionally, the fruits are very aromatic and tasty, flattened at the apex, fleshy, have intense flavour and contain
different phytochemical (Villagra et al. 2014). The red fruits had average values of monomeric anthocyanins of $5.94 \pm 0.42$ g per 100 g of sample, while pink and white fruits had lower values of $3.85 \pm 0.19$ g and $0.626 \pm 0.41$ g (Villagra et al. 2014). Morphological differences could easily be detected between individuals within the natural populations of *G. pumila*. All attributes of *G. pumila* previously mentioned, make this species an interesting target for domestication, regarding its potential use as functional food. Unfortunately, little is known about the propagation of the species out of its natural habitat, cultural techniques, nutritional requirements and even less about its genetic composition. Thus, this species remains as unexploited genetic resource. To take advantage of this resource, it is necessary to develop basic studies concerning the composition of its genetic diversity and how the populations are associated (Gepts 2004).

Molecular markers, such as microsatellites (SSRs), offer a powerful tool for population genetic studies in *G. pumila*. These markers are highly polymorphic, inherited in a Mendelian, codominant manner, are abundant in the genome, highly reproducible and selectively neutral (Vendramin et al. 1996). For these reasons, microsatellites have been widely used in various studies, such as molecular characterization of different species, as well as for analyzing the diversity and genetic relationships within or among species (Gostimsky et al. 2005). In the Ericaceae family these markers have been used to perform molecular studies for example in *Rhododendron* genus: *R. decorum* (Wang et al. 2009), *R. delavayi* (Wang et al. 2010), *R. simsii* (Wang et al. 2016) and *R. ferrugineum* (Delmas et al. 2011). In the *Vaccinium* genus, these markers have been useful for population genetic studies as well as cultivar identification (Boches et al. 2005, 2006, Garriga et al. 2013). At the same time studying phenotypes and genetic diversity in natural populations and landrace collections is important for germplasm
conservation, selection and breeding (Ahmad et al. 2010). This study aims to determine the genetic diversity and population structure of *G. pumila* in its range of distribution in order to generate scientific information that would underlie any domestication programs and for the selection of potential genotypes in breeding programs. The final objective is to develop sustainable exploitation of these underutilized genetic resources.

Methodology

2.1.1 Plant material

Leaves of 160 accessions of *G. pumila* were collected from 11 sectors distributed in four of Chile’s administrative Regions: Maule, La Araucanía, Los Lagos and Magallanes (Table 2.1). The sampled sites were georeferenced through the Global Positioning System. Samples were collected from March to August 2013. Once collected, leaf samples were placed in plastic bags and immediately conserved in ice; then they were transported to the lab where they were placed at -80 ºC until they were processed for DNA extraction.

Table 2.1 Description of sampled populations and subpopulation for *G. pumila* within its range in Chile

<table>
<thead>
<tr>
<th>Regions</th>
<th>Pop</th>
<th>Samples number</th>
<th>Latitude S</th>
<th>Longitude W</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maule</strong></td>
<td>Reserva Radar Siete Tazas</td>
<td>Parque Inglés (PI)</td>
<td>10</td>
<td>35º29'420</td>
</tr>
<tr>
<td><strong>La Araucanía</strong></td>
<td>Villarrica</td>
<td>Parque Araucaria (PT)</td>
<td>15</td>
<td>39º21'570</td>
</tr>
<tr>
<td></td>
<td>Playón (PZ)</td>
<td>7</td>
<td>39º34'463</td>
<td>71º28'509</td>
</tr>
<tr>
<td></td>
<td>El Mirador (EM)</td>
<td>22</td>
<td>39º23'09</td>
<td>71º58'006</td>
</tr>
<tr>
<td></td>
<td>Centro Sky (CK)</td>
<td>15</td>
<td>39º22'915</td>
<td>71º55'565</td>
</tr>
<tr>
<td></td>
<td>Conguillio (CN)</td>
<td>15</td>
<td>38º34'400</td>
<td>71º46'973</td>
</tr>
<tr>
<td><strong>Los Lagos</strong></td>
<td>Puyehue</td>
<td>Parque Nacional (PN)</td>
<td>9</td>
<td>40º40'538</td>
</tr>
<tr>
<td></td>
<td>Camino Puyehue (CH)</td>
<td>19</td>
<td>40º40'599</td>
<td>72º34'501</td>
</tr>
<tr>
<td><strong>Magallanes</strong></td>
<td>Punta Arenas</td>
<td>Punta Arenas (PA)</td>
<td>20</td>
<td>53º9'413</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reserva Magallanes (RM)</td>
<td>11</td>
<td>53º10'490</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reserva Laguna Parrillar (PL)</td>
<td>17</td>
<td>53º24'140</td>
</tr>
</tbody>
</table>
2.1.2 DNA extraction, PCR amplification, and microsatellite analysis

Two hundred milligrams of young leaves of *G. pumila* were placed in 2.0 ml tubes (LabCon, Petaluna, California, USA) and cooled in liquid nitrogen. Leaf samples were smashed with the help of Precellis 24 and Cryollys systems (Bertin Technologies, France) at 6000 rpm with three cycles of 30 seconds each. Total DNA extraction of all samples was performed using a modified CTAB (cetyltrimethylammonium bromide) protocol (Doyle 1990, Carrasco et al. 2007). After that, all samples were precipitated with 90% ethanol. The concentration of DNA was estimated using a NanoDrop spectrophotometer (ND-1000 Peqlab, Erlangen, Germany). The DNA concentration was of 200 ng/μl, the absorbance relationship 260/280 and 260/230 were 1.70 and 0.63, respectively. The DNA integrity was checked through electrophoresis in agarose gel at 1% during 30 minutes at 100 volts. The Polymerase Chain Reaction (PCR) was carried out with an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing temperature specific to each primer pair for 45 s, (Table 2.2) and extension at 72 °C for 45 s, followed by 8 cycles of 30 s at 94 °C, annealing at 53 °C for 45 s, and extension at 72 °C for 45 s and a final extension at 72°C for 30 min. Each PCR reaction had 20 μl reaction mixture with 10 ng templates DNA, 0.15 mM of each dNTP; 1 X Taq polymerase reaction buffer; 1.5 mM MgCl2; 0.025 μM forward primers; 0.1 μM reverse primer; 0.1 μM 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, CA). Allele sizes were automatically calculated with GeneMapper software v4.0.

Table 2.2 Characterization of six microsatellite loci and primer pair’s development for *G. pumila*

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer sequences (5’-3’)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Repeat motif</th>
<th>Allele size range (bp)</th>
<th>Fluorescent dye</th>
<th>Ta(°C)</th>
<th>GenBank accession nº</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP.10</td>
<td>F GGGTACGCGTAGTGTTAAT</td>
<td>(AT)&lt;sup&gt;8&lt;/sup&gt;</td>
<td>323-327</td>
<td>VIC</td>
<td>60.5</td>
<td>KX719824</td>
</tr>
<tr>
<td></td>
<td>R TCGTACAAACCGCCCTCAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP.12</td>
<td>F AGGATTATAGAGACGAGCTGGA</td>
<td>(CTT)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>198-201</td>
<td>NED</td>
<td>52.1</td>
<td>KX719825</td>
</tr>
<tr>
<td></td>
<td>R CAGAGACGGAAATCGAAACGG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP.13</td>
<td>F AGAGTTAGACGCTCTTCTCCGA</td>
<td>(ATT)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>161-278</td>
<td>NED</td>
<td>52.1</td>
<td>KX719826</td>
</tr>
<tr>
<td></td>
<td>R GCAGACTCGAATCGCGCAGTA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP.14</td>
<td>F GCATAGCCCCGTTGTCAAAC</td>
<td>(AGC)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>196-228</td>
<td>PET</td>
<td>52.1</td>
<td>KX719827</td>
</tr>
<tr>
<td></td>
<td>R ACCGAAGATCCCGACATCG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP.16</td>
<td>F GCATTTCGCGCCCGGACC</td>
<td>(AT)&lt;sup&gt;6&lt;/sup&gt;</td>
<td>281-283</td>
<td>FAM</td>
<td>52.1</td>
<td>KX719829</td>
</tr>
<tr>
<td></td>
<td>R GCACAATACATAGATGTTGATCGA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GP.18</td>
<td>F ACCGGAGATCCCGACCACG</td>
<td>(GCGT)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>192-228</td>
<td>PET</td>
<td>52.1</td>
<td>KX719831</td>
</tr>
<tr>
<td></td>
<td>R GCATAGCCCCCTGCGCAAAC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Ta (°C) = annealing temperature

2.1.3 Statistical analysis

Genetic diversity index was estimated for each microsatellite locus and each population. POPGENE v 1.31 (Yeh et al. 1999) was used to calculate the observed number of alleles (*Na*), effective number of alleles (*Ne*), gene flow (*Nm*), Shannon’s Information index (*I*), percentage of polymorphic loci (*PPL*), observed heterozygosity (*Ho*) and the expected heterozygosity (*He*). Also, the Test of Neutral Evolution of Ewens-Watterson (Ewens 1972) was performed for all microsatellite loci. The inbreeding coefficient for each locus (*F<sub>IT</sub> F<sub>ST</sub> F<sub>IS</sub>* (Weir and Cockerham 1984) was estimated by resampling using the Jack-knife method with FSTAT v 2.9.3 (Goudet 1994), while the Factorial Analysis of Correspondence (*FCA*) was determined using Genetix (Belkhir et al. 2003).

Polymorphic information content (*PIC*) and null allele (*F Null*) estimation were calculated using CERVUS (Kalinowski et al. 2007). The null allele was calculated using an iterative algorithm based on the observed and expected frequencies of various
genotypes (Summers and Amos 1997). Deviation from the Hardy-Weinberg equilibrium (EHW) and Linkage disequilibrium (LD) were determined with GenePop v 4.2 (Raymond and Rousset 1995) with the sequential Bonferroni correction carried out for all SSR’s. The molecular analysis of variance (AMOVA) was applied to estimate the variance components of individuals among and within the populations using GenAlex software (Peakall and Smouse 2006). The Mantel Test (Mantel 1967) 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 v. 2.3.3 software (Evanno et al. 2005). For each analysis different population genetic clusters (K=1-10) were evaluated with 10 runs per K value. For each run, the initial burn-in period was set to 100,000 with 1,000,000 MCMC iterations, under the admixture model. The ∆K measure obtained with STRUCTURE HARVESTER (Earl and vonHoldt 2012) was used as an ad hoc quantity to detect the rate of change in the log probability of data between successive K value and the corresponding variance of log probabilities.

Results

2.1.4 Genetic diversity analysis for each locus SSR
Six polymorphic microsatellites markers were applied to 160 samples of *G. pumila* from the different geographical Regions (Table 2.3). The average null allele frequency was very low for all loci (mean of 0.10). The neutral test for all loci showed no evidence of positive selection. Two SSR markers were in Hardy-Weinberg equilibrium (GP14 and GP18). In the analysis of the 330 possible binary combinations, none showed significant linkage disequilibrium (LD) after applying Bonferroni correction (p<0.00003046). The observed number of alleles (Na) was different for all the markers with an average of 5.67, where the highest value in loci GP18 (10) and the lowest value in the loci GP12
and GP16 (3). The mean of observed heterozygosity ($Ho$) and expected heterozygosity ($He$) was 0.53 and 0.54, respectively. The range of observed heterozygosity moved from 0.03 (GP12) to 0.98 (GP10). Meanwhile the expected heterozygosity ranged from 0.34 (GP14) to 0.74 (GP13). Overall, Shannon index ($I$) for genetic diversity was 0.99, fluctuating from 0.743 (GP12) to 1.46 (GP13). In addition, the polymorphism information content ($PIC$) for all loci ranged from 0.32 (GP14) to 0.70 (GP13) with an average of 0.47. The $F$-statistics analysis for all loci showed negative values for the loci GP10, GP13, GP14, GP18 and two positive values for GP12 and GP 16. The average $Fis$ values were -0.16 which was significantly different from zero ($P=0.0007$). The $Fst$ value ranged from 0.04 (GP10) to 0.39 (GP16). This result evidenced the high genetic cohesion of all populations. The overall gene flow ($Nm$) between all populations was 2.05 providing an estimate of the average number of migrations between the populations. GP10 was the locus that showed the highest value of migration (4.71) and GP16 showed the lowest values (0.31) (Table 2.3).

Table 2.3  Estimates of genetic diversity (per locus) in $G. pumila$ based on six SSR loci. $Na$, observed number of alleles; $Ne$, effective allele; $I$, Shannon index of genetic diversity; $Ho$, observed heterozygosity; $He$, expected heterozygosity; $Fis$, inbreeding coefficient; $Fit$, inbreeding coefficient at the total samples level; $Fst$, proportion of differentiation among populations; $Nm$, gene flow; $PIC$, Polymorphism information content; $HW$, Hardy-Weinberg equilibrium; $F(Null)$ null allele.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Na</th>
<th>Ne</th>
<th>I</th>
<th>Ho</th>
<th>He</th>
<th>Fis</th>
<th>Fit</th>
<th>Fst</th>
<th>Nm</th>
<th>PIC</th>
<th>HW</th>
<th>$F(Null)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GP10</td>
<td>4</td>
<td>2.34</td>
<td>0.97</td>
<td>0.98</td>
<td>0.57</td>
<td>-0.77</td>
<td>-0.69</td>
<td>0.04</td>
<td>4.71</td>
<td>0.48</td>
<td>*</td>
<td>-0.28</td>
</tr>
<tr>
<td>GP12</td>
<td>3</td>
<td>2.04</td>
<td>0.74</td>
<td>0.03</td>
<td>0.51</td>
<td>0.93</td>
<td>0.93</td>
<td>0.11</td>
<td>1.15</td>
<td>0.39</td>
<td>*</td>
<td>0.88</td>
</tr>
<tr>
<td>GP13</td>
<td>6</td>
<td>3.92</td>
<td>1.47</td>
<td>0.94</td>
<td>0.74</td>
<td>-0.44</td>
<td>-0.25</td>
<td>0.13</td>
<td>1.34</td>
<td>0.70</td>
<td>*</td>
<td>-0.13</td>
</tr>
<tr>
<td>GP14</td>
<td>8</td>
<td>1.52</td>
<td>0.76</td>
<td>0.39</td>
<td>0.34</td>
<td>-0.29</td>
<td>-0.14</td>
<td>0.11</td>
<td>1.70</td>
<td>0.32</td>
<td>n/s</td>
<td>-0.1</td>
</tr>
<tr>
<td>GP16</td>
<td>3</td>
<td>2.17</td>
<td>0.84</td>
<td>0.25</td>
<td>0.54</td>
<td>0.24</td>
<td>0.54</td>
<td>0.39</td>
<td>0.31</td>
<td>0.43</td>
<td>*</td>
<td>0.34</td>
</tr>
<tr>
<td>GP18</td>
<td>10</td>
<td>2.12</td>
<td>1.18</td>
<td>0.61</td>
<td>0.53</td>
<td>-0.21</td>
<td>-0.15</td>
<td>0.04</td>
<td>3.12</td>
<td>0.50</td>
<td>n/s</td>
<td>-0.09</td>
</tr>
<tr>
<td>Mean</td>
<td>5.67</td>
<td>2.35</td>
<td>0.99</td>
<td>0.53</td>
<td>0.54</td>
<td>-0.16</td>
<td>0.01</td>
<td>0.14</td>
<td>2.05</td>
<td>0.47</td>
<td>n/s</td>
<td>0.10</td>
</tr>
<tr>
<td>SD</td>
<td>2.875</td>
<td>0.817</td>
<td>0.282</td>
<td>0.380</td>
<td>0.129</td>
<td>0.258</td>
<td>0.246</td>
<td>0.055</td>
<td>0.650</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.1.5 Genetic diversity analysis for each populations

The genetic analysis showed an average of 96.97 % of polymorphic loci, with almost all the populations having 100% of polymorphism. The lowest percentage of polymorphic loci (83%) was found in the Parque Inglés (PI) populations in the Región del Maule, and Parque de Araucarias (PT) in the Región de la Araucanía (Table 2.4). The observed heterozygosity ranged from 0.46 in Parque de Araucarias (PT) from Región de La Araucanía, to 0.66 in the Reserva de Magallanes (RM) from Región de Magallanes and Antártica. The lowest expected heterozygosity ($He$) value was found in the population of Conguillio (CN) 0.37 and the highest in the population of Camino Puyehue (CH) 0.56, from Región de Los Lagos.

The mean values of observed heterozygosity ($Ho=0.54$) was higher than the expected heterozygosity ($He=0.47$). The average Shannon diversity index ($I$) varied between populations, showing an average of 0.75, and ranging from 0.57 (CN) to 0.91 (CH). The highest level of genetic diversity was found in the Puyehue populations (CH) from the Región de Los Lagos ($He=0.56$, $I=0.91$). The lowest was found in Conguillio (CN) and Parque Araucaria (PT) from the Región La Araucanía ($He= 0.37$, $I=0.57$; $He=0.38$, $I=0.60$) respectively. The inbreeding coefficient ($Fis$) was positive only for two populations PN (0.03) and CH (0.16) respectively, with other populations showing negative values. These ranged from -0.39 in the Playón site (PZ) of the Region La Araucanía, to 0.16 in the site Camino Puyehue (CH) from the Región Los Lagos with an average of -0.16.

The genetic diversity was estimated for each population, based on the amplified polymorphic alleles by microsatellites markers. Over all populations 96.97% of the loci showed polymorphism. The lowest percentage of polymorphic loci (83%) was shown in the sector of Parque Ingles (PI) and Parque de Araucaria (PT) that belong to Maule and
La Araucania Regions, respectively. While in the remaining populations, the average of polymorphic loci was 100% (Table 2.4).

Table 2.4 Descriptive statistics over all loci for each population of G. pumila. Pop; populations where samples were taken; Na, number of alleles; Ne; number of effective alleles; I, Shannon index; Ho, observed heterozygotes; He, expected heterozygotes; Fis, Inbreeding coefficient; PPL, percentage of polymorphic loci.

<table>
<thead>
<tr>
<th>Regions</th>
<th>Pop</th>
<th>Na</th>
<th>Ne</th>
<th>I</th>
<th>Ho</th>
<th>He</th>
<th>Fis</th>
<th>PPL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maule</td>
<td>PI</td>
<td>3.16</td>
<td>2.07</td>
<td>0.77</td>
<td>0.62</td>
<td>0.45</td>
<td>-0.39</td>
<td>83%</td>
</tr>
<tr>
<td>Araucanía</td>
<td>PT</td>
<td>2.50</td>
<td>1.75</td>
<td>0.60</td>
<td>0.46</td>
<td>0.38</td>
<td>-0.21</td>
<td>83%</td>
</tr>
<tr>
<td></td>
<td>PZ</td>
<td>2.33</td>
<td>1.80</td>
<td>0.65</td>
<td>0.61</td>
<td>0.45</td>
<td>-0.39</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>EM</td>
<td>3.66</td>
<td>2.02</td>
<td>0.80</td>
<td>0.50</td>
<td>0.47</td>
<td>-0.06</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>CK</td>
<td>3.50</td>
<td>2.36</td>
<td>0.89</td>
<td>0.53</td>
<td>0.53</td>
<td>-0.008</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>CN</td>
<td>2.33</td>
<td>1.69</td>
<td>0.57</td>
<td>0.48</td>
<td>0.37</td>
<td>-0.32</td>
<td>100%</td>
</tr>
<tr>
<td>Los Lagos</td>
<td>PN</td>
<td>2.16</td>
<td>1.90</td>
<td>0.68</td>
<td>0.54</td>
<td>0.52</td>
<td>0.03</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>CH</td>
<td>3.00</td>
<td>2.49</td>
<td>0.91</td>
<td>0.47</td>
<td>0.56</td>
<td>0.16</td>
<td>100%</td>
</tr>
<tr>
<td>Magallanes</td>
<td>PA</td>
<td>2.83</td>
<td>1.89</td>
<td>0.72</td>
<td>0.49</td>
<td>0.45</td>
<td>-0.10</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>RM</td>
<td>2.83</td>
<td>2.14</td>
<td>0.79</td>
<td>0.66</td>
<td>0.50</td>
<td>-0.33</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>PL</td>
<td>3.16</td>
<td>2.08</td>
<td>0.84</td>
<td>0.58</td>
<td>0.51</td>
<td>-0.12</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>MEAN</td>
<td>2.86</td>
<td>2.02</td>
<td>0.75</td>
<td>0.54</td>
<td>0.47</td>
<td>-0.16</td>
<td>96.97%</td>
</tr>
</tbody>
</table>

2.1.6 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 genetic variation between populations (Table 2.5).

Table 2.5 Analyses of Molecular Variance (AMOVA) in different natural populations of G. pumila, Chile

<table>
<thead>
<tr>
<th>Sources of Variation</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Estimated variance</th>
<th>Percentage variation</th>
<th>Fixation index</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among Population</td>
<td>10</td>
<td>18.836</td>
<td>0.062</td>
<td>6%</td>
<td>Fst= 0.058</td>
<td>p=0.001</td>
</tr>
<tr>
<td>Within Population</td>
<td>149</td>
<td>148.562</td>
<td>0.997</td>
<td>94%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>159</td>
<td>167.398</td>
<td>1.059</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The Mantel Test showed a significant positive correlation between genetic distance and geographical distance (correlation coefficient Rxy= 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 revealed that the maximum value (0.55) was detected between the populations of CN and RM, and the minimum genetic distance (0.017) was between the populations of CN and PZ. The populations belonging to the regions of La Araucanía to Magallanes, showed a high degree of admixture, based on results obtained from Bayesian model-based Structure analysis of 160 samples of G. pumila. Application of this model indicated that there are two genetic groups (Fig. 2.2).

![Genetic groups estimated by Bayesian analysis. 1) 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. 2) Estimated population structure and genetic cluster of the eleven populations of G. pumila and 160 individuals with K=2 to 6. Both groups were found in all collecting points except in PI in the Región del Maule. While maximal ΔK was obtained at K=2, as the best number of clusters. The K= 3, 4, 5 and 6 were complex admixture, that did not fit with the criteria to be considered as genetic groups. In order to further investigate the genetic distribution situation, a factorial analysis of correspondence (AFC) between populations was used to generate a scattergram. The first three factors explained 71.4 % of the variation. These analyses again gave two genetic groups, as shown in Fig 2.3, with most of the individuals]
belonging to one genetic group, except in the case of Parque Inglés (PI) where most of the individuals clustered in the second group.

Figure 2.3 Factorial analysis of correspondence between eleven populations of *G. pumila* of four regions in Chile. The axe 1 (component 1) axe 2 (component 2) axe 3 (component 3) refer to 30.88%, 28.55% and 11.99% of the total variation, respectively.

These results can be explained considering that it is the northern population sampled and to the influence of geographic distance on the genetic relations between all the populations. The scattergram showed clearly the differentiate of all populations. The cluster one composed the largest number of genotypes from all regions and was homogenous in Magallanes, La Araucanía and Los Lagos. The Cluster two has genotype only from the Región del Maule.
Discussion

2.1.7 Genetic diversity of Gaultheria pumila

Microsatellites markers have been widely used to detect genetic variability in the distinct species of Ericaceae family, especially in the Vaccinium genus (Boches et al. 2006, Hirai et al. 2010, Česoniene et al. 2013). However, this is the first time that this kind of study, using SSR markers, has been carried out in any wild species from the Gaultheria genus. Genetic diversity per locus in G. pumila showed that the observed heterozygosity (Ho) ranged from 0.25 to 0.98 and expected heterozygosity ranged from 0.343 to 0.748. These results are similar to those reported in other Ericaceae, such as Rhododendron simsii (Wang et al. 2016), R. ferrugineum (Delmas et al. 2011) and Erica coccinea (Segarra-Moragues et al. 2009) where Ho and He showed large range of variation.

In this study, the descriptive statistics for diversity genetic over all loci showed moderate to high levels of genetic diversity for G. pumila (Na = 5.67, Ho= 0.538, He= 0.542, I= 0.993). This moderate value of genetic diversity could be due to factors such as: number of individuals sampled and isolation by distance, or influential factors from forest fragmentation (Kramer et al. 2008). These levels of variability are similar to those reported in species with high economic and ornamental values such as in Prunus pseudocerasus Lindl (Chen et al. 2015).

In Gaultheria fragantissima, Bantawa et al. (2011) found a high level of polymorphism (68.52%) using RAPDS. This level of polymorphism in this related species was later confirmed using ISSR markers (82.82%) (Apte et al. 2006).

In Gaultheria shallon (Wilkin et al. 2005), the percentage of polymorphic loci detected with AFLP markers reached 89%. These similarities between G. pumila and its relatives from the genus could be explained by the fact that they are all open pollinated species,
normally inhabiting extreme ecological conditions (Luteyn, 2002) and this forced them to retain a large genetic background in order to adapt to these conditions.

It is also important to mention that some species of Ericaceae, which also had high genetic diversity in the wild, have already been cultivated and represent very important commercial plants, such as in *Vaccinium macrocarpon* (Fajardo et al. 2013), or with promising characteristic as *V. meridionale* (Ligarreto et al. 2011), as well in other species such as grapevine (*Vitis sp.*) (Emanuelli et al. 2013), *Prunus pseudocerasus* (Chen et al. 2015), *P. salicina* (Carrasco et al. 2012), *Fragaria chiloensis* (Carrasco et al. 2007, 2013) and *Vasconcellea chilensis* (Carrasco et al. 2014). However, it is important to note that all these species have been affected by a selection process and improvement, highlighting certain characteristic features in each of them.

The inbreeding coefficient (*Fis*) shown in this study had an average negative value and showed had an excess of heterozygosity that could be due to the immigration process or absence of selfing (Balloux 2004). These negative values would imply that the excess of heterozygosity is probably caused by the reproductive biology of the species, since it is open pollinated. Also, genetic flow is due to the mobility of wild animals that eat fruit, and then disperse their seeds, causing a permanent distribution. Through the four years of this study, it was possible to observe visits of pollinator insects especially of genus *Bombus spp.* (Montalva et al. 2011). The insects were seen around the plants, as well as wild animals, such as rabbits (Camus et al. 2008), birds, lizards and foxes feeding on the fruits. Precisely because this plant is commonly a food source of the foxes in the wild, *G. pumila* is usually named in rural areas as “*Mutilla del Zorro*”.

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2.1.8 Population structure

The evaluated populations were sampled from four regions of the country, covering the whole latitudinal distribution range of the species. The northernmost population was collected from the Región del Maule while the southernmost population was from the Región de Magallanes, the limits of the species distribution. The analysis of molecular variance AMOVA showed a very high level of variation within population (94%), indicating that most of the genetic variation is explained by the genetic exchange among individuals, and not because of the genetic differentiation of the geographically isolated groups. These results coincide with previously reported observations that found a high level of phenotypic variation in natural populations of the species (Villagra et al. 2014). This pattern of distribution of the genetic variability is very similar to that detected for “maqui” Aristotelia chilensis (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 population and 5% between populations; which is remarkably similar to G. pumila in Chile. Bayesian based analysis of genetic grouping detected only two groups in G. pumila. The first group contains the individuals from the populations belonging to La Araucanía, Los Lagos, and Magallanes, where a consistent mixture of alleles suggested a constant dynamism which does not allow the development of differentiation (Hartl and Clark 1998). The second group comprises only one population Parque Inglés (PI) in the Región del Maule, the population from the most northern limit of the species distribution. This can be explained because population density here is lower and also the distribution is more fragmented than in the southern populations, making this a different situation to the more southern population and maybe the extreme edge of the species distribution. Also, the positive correlation between distance and genetic flow, detected by the Mantel Test, suggest a lower genetic connectivity of this small population to the others, which are
more closely packed in the southern Region sampled. These results show that the populations that belong to extreme Regions share few alleles, where they tend to differentiate. Consequently, the isolation of these populations can be affected by factors such as pollen dispersal, plant longevity, life forms, ranges and geographic variations (Hamrick and Godt 1996, Ramanatha Rao and Hodgkin 2002). This reduces their abundance and frequency, affecting the genetic connectivity (Hamrick and Godt 1996). In this case, each region that was sampled differs in the characteristics such as latitude, altitude and temperature. These results are in agreement with the positive relation that the Mantel Test presented, the results shown that there is isolation by distance, and precisely those populations represent one of the limits of its distribution. From these results it is postulated that the genetic diversity contained in G. pumila is uniformly distributed through most to the distribution range in Chile. This organization of the genetic variability allows the identification of a strategy to select those genotypes that have desirable agronomic traits of interest, and that can be considered in future breeding work aimed at domestication.
Conclusion

Microsatellite markers detected genetic diversity in *G. pumila* from eleven wild populations along its distribution range in Chile. The species shows a moderate to high level of genetic diversity and a very low level of genetic fragmentation, indicating a high degree of genetic connectivity and gene exchange throughout its geographical distribution. These results can provide useful information to continue the domestication programs already initiated in the species, in order to support the selection of individuals from most genetically rich populations.
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GENETIC DIVERSITY AND POPULATION STRUCTURE OF GAULTHERIA PUMILA L.F. (ERICACEAE)

Documento enviado a la revista *Genetic Resources and Crop Evolution*
3 Capítulo III

In vitro propagation of Gaultheria pumila (Ericaceae), a Chilean native berry with commercial potential

Abstract
A micropropagation protocol for multiplication of G. pumila Lf (Ericaceae), a wild Chilean native berry, was developed. Young shoots collected during the growing season (October to December) belong to a wild population on the Villarrica Volcano, in Región de la Araucanía, Chile. Nodal segments were used for in vitro establishment after testing several disinfection treatments with different concentrations of sodium hypochlorite. In order to evaluate the best regeneration media during in vitro establishment, disinfected explants were placed onto WPM100 supplemented with a range of concentrations of 2-iP. Disinfection with 1% of sodium hypochlorite for 40 minutes, followed by a second disinfection round with 2% of sodium hypochlorite for 25 minutes and cultivation on MS medium supplemented with 2.0 mgL⁻¹ of 2-iP gave the highest efficiency of disinfected plants. In the propagation stage, the highest multiplication rates were obtained when 1mgL⁻¹ Zeatin was added to the basal WPM100 medium. In vitro rooting and pre-acclimation were better when elongated plants were cultivated on WPM100 medium supplemented with 3 mgL⁻¹ of naphthalene acetic acid. This general protocol will be useful to propagate genotypes of this native species, being also an important tool towards its domestication and commercial use.

Keywords: In vitro culture, native berries, multiplication, rooting
**Introduction**

*Gaultheria pumila*, belong to the family Ericaceae, is a wild berry species native from Chile. It is commonly known as chaura or mutilla del zorro, because its fleshy, attractive fruits are eaten by small mammalians and other animals. In addition, *G. pumila* has several attributes, such as high diversity of fruits, morphometric traits, adaptability to several ecological conditions and high content of polyphenols (Middleton 1992, Villagra et al. 2014).

All of these properties make this species very attractive for cultivation as a commercial plant, particularly taking advantages of its nutritional potentials as a functional food (Lasekan 2014) as well as its ecological plasticity. As many other Chilean native fruits, *G. pumila* has a rich and diversified composition of bioactive compounds with potential health benefits (Schreckinger et al. 2010, Ruiz et al. 2013).

Chaura has been used as a food and a medicinal plant by the indigenous people in Chile like the Aónikenk, Selk’nam, Kawésqar, Yagan and Haush people, from Southern Chilean Patagonia (Dominguez 2010). In species of the genus *Gaultheria*, several attractive compounds have been identified such as polyphenols (Middleton 1992), anthocyanins (Villagra et al. 2014) and essential oils (Bantawa et al. 2011). Also, it has been shown that species from this genus can show anti-inflammatory, anti-oxidative, antibacterial and analgesic activities (Liu et al. 2013).

However, propagation of the *Gaultheria* genus outside the wild has been hard to achieve. In *G. fragrantissima* Wall, a species with high content of methyl salicylate rich essentials oils, in which there is an increasing commercial interest, they had to develop efficient propagation technologies before using it on a commercial scale (Ranyaphi et al. 2012). Regarding *G. pumila*, the species is mainly reproduced by seeds in the wild.
The seeds are small and each fruit contains around of 50 seed per fruit. However, preliminarily we have determined that the germination potential of seed seems low.

The species can also be propagated asexually by underground stems forming shoots during the warmer seasons, thus producing new plant colonies around the original plant. However, no information has been previously generated for asexual propagation of *G. pumila* under nursery conditions.

*In vitro* culture techniques are very suitable for propagating high volumes of plants in a limited period. Tissue culture may be defined as the aseptic culture and isolation of cells, tissues, organs or whole plants under controlled nutritional and environmental conditions (Höxtermann 1997, Thorpe 2007).

Plant tissue culture has been successfully used to obtain disease free plants, germplasm conservation, massive propagation of selected genotypes, genetic transformation, embryo rescue and some others important applications (García-Gonzáles et al. 2010, González-Benito and Martín 2010). The micropropagation of plants through *in vitro* culture has five fundamental stages: Stage 0: preparation of donor plant, Stage I: introduction and establishment, Stage II: propagation of plants, Stage III: rooting and explant preparation for the *ex vitro* conditions and Stage IV: *ex vitro* adaptation or plant acclimatization (García-Gonzáles et al. 2010).

Considering the efficiency of *in vitro* propagation techniques, their application in *G. pumila* would be ideal for providing a fast and efficient propagation while maintaining a high health status. One of the more relevant applications of tissue culture in plants is the support of domestication projects of non-cultivated plants (Gepts 2004a, 2004b) since it is necessary to produce enough plants from selected genotypes in a limited period in order to evaluate the agronomic performance under cultivated conditions.
Micropropagation is also needed to develop disease free germplasm from the studied species and to rapidly scale-up for commercial exploitation (García-Gonzáles et al. 2010). This study aimed to develop a general protocol for micropropagation of *G. pumila*, addressing the different steps of the tissue culture process.

**Methodology**

3.1.1  **Plant material**

Plants of *G. pumila* were selected from the sector of Parque Nacional Villarrica, Región La Araucanía S: 39°34’463, W: 71°28’509. The plants were transported with soil in a humid cooler box to the nursery of the Universidad Católica del Maule, Chile. Once in the greenhouse, the plants were planted in five pounds pots containing soil brought from the sampling location. The mother plants were watered daily with 200 ml water per plant. No fertilizer or pesticides were used during preparation of the plants. Light pruning was done to eliminate older branches in order to stimulate the formation of new shoots. After shoot production, healthy, 10 cm long explants were collected and prepared in 1.0 cm pieces containing two to three vegetative buds.

3.1.2  **Experimental design and statistical analysis**

For the disinfection step six treatments were tested (Table 3.1). Each treatment had three repetitions with seven nodal segments per replication. Only one nodal segment was planted per flask. The effects of each treatment on the number of contaminated explants and the number of regenerated plants was evaluated five weeks after planting to choose the best disinfection protocol.

To successfully establish the plant tissue cultures the effects of the basal medium, cytokynin type and concentration on plantlet recovery were studied. Twenty four treatments with three replicates and fifteen explants per replication were assayed. Five nodal segments were planted per plant in this experiment (Annex 1).
For the *in vitro* multiplication step, twelve treatments combining the interaction between auxins and cytokinins to obtain the best multiplication rate were tested (Annex 2). For this study, each treatment was replicated four times with five nodal explants per replication. Only one nodal segment was planted per culture flask. The effect of the different treatments was considered in order to choose the best micropropagation condition.

For *in vitro* rooting and plantlet preparation before acclimatization, the effect of different auxins at different concentrations on plant development and *in vitro* rooting was evaluated. Twelve treatments were evaluated, and each treatment included three replications and ten explants per replication (Annex 3).

Statistical analysis were performed using the InfoStat statistic software (Balzarini et al. 2008). Normality of the data was determined by the Q-Q plot graphic and corroborated with Shapiro-Wilks test of residues. For detecting the best treatment an analysis of variance (ANOVA) and Tukey HSD test with 95% confidence level was done in the case of the date had a normal distribution of the variance. For those data, not complying with the normality assumptions a Kruskal-Wallis test with a level of significance of 95% was performed.

### 3.1.3 General environmental conditions

For all experiments in this study the basal media was supplemented with 30 g L\(^{-1}\) of sucrose and 8 gL\(^{-1}\) of Agar. The pH of the media was adjusted to 5.7 before sterilization by autoclaving for 20 minutes at 1 kg cm\(^{-2}\) and 121°C. For all the experiments described above, the cultures were transferred into a culture room at 24±1°C and 12 hours light 12 hours dark photoperiod under cool white fluorescent lamps (60 μmol m\(^{-2}\) s\(^{-1}\)).
3.1.4 Influence of the disinfection protocol and plant growth regulators on explant decontamination and plant development during *in vitro* establishment

3.1.5 Disinfection and establishment

Explants of 1.0 cm length containing two to three vegetative buds were used. A previous wash of the selected explants was done with distilled water and common detergent for twenty minutes, followed with a deep wash in sterile distilled water (500 ml) with three drops of Tween 20 for 20 min. After that the explants were three times washed with sterile distilled water to eliminate any detergent residues under a laminar flow cabinet. Once washed, the disinfection treatments were applied as follows: A first wash with 1% (v/v) sodium hypochlorite for 30 or 40 min, depending on the disinfection treatment; a second wash with 2% sodium hypochlorite for 25 min. After disinfection, the explants were washed three times with sterile distilled water. Oxidized tissues were removed from the explants before planting on semisolid Murashige and Skoog (MS) medium (Murashige and Skoog 1962), supplemented with different concentrations of 2-iP (0.5, 1.0 and 2.0 mgL⁻¹). The general designs of the experiments are shown in table 3.1. The Final evaluation of the experiment was done five weeks after planting in each treatment.

Table 3.1 Treatments applied for disinfection and *in vitro* establishment of *G. pumila* explants

<table>
<thead>
<tr>
<th>Treatments</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-iP (mg/L⁻¹) + Murashige and Skoog (MS) medium</td>
<td>0.5</td>
<td>1.0</td>
<td>2.0</td>
<td>0.5</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Concentration of sodium hypochlorite (%v/v) and disinfection time (minutes)</td>
<td>1% : 40</td>
<td>1% : 40</td>
<td>1% : 40</td>
<td>1% : 30</td>
<td>1% : 30</td>
<td>1% : 30</td>
</tr>
<tr>
<td></td>
<td>2% : 25</td>
<td>2% : 25</td>
<td>2% : 25</td>
<td>2% : 25</td>
<td>2% : 25</td>
<td>2% : 25</td>
</tr>
</tbody>
</table>

3.1.6 Plant formation and stabilization of *in vitro* plantlets

After selecting the best disinfection and *in vitro* establishment condition, it was necessary to stabilize the behaviour of the established explants. The type of cytokinin (2 isopentenil adenine, 2-iP; 6-Bencil aminopurine, BAP; Zeatin, Zea) as plant growth
regulator and their concentration (0.5, 1.0 or 2.0 mgL$^{-1}$) on the morphogenic response of established explants were evaluated.

Each growth regulator in different concentration was added to four different basal media: full strength MS (MS100), half strength MS medium (MS50), full strength WPM (WPM100) and half strength WPM medium (WPM50). Only explants coming from the selected disinfection and in vitro establishment protocol were used in this experiment. Media preparation and sterilization were developed as described above. All treatments were grown under the environmental conditions previously described (Section 3.1.3). For selecting the best treatment, the following morphogenic responses were evaluated, such as: number of shoot, percentage of shoot, shoots formation per explant, average shoot length (mm) and number of leaves.

3.1.7 Effect of plant growth regulator interaction on the in vitro multiplication of G. pumila

For the multiplication step, all in vitro plants were grown in the best stabilization medium for two cycles of four weeks each. Plants coming from this condition were selected and prepared as nodal explants harbouring at least one bud. The explants were then planted on the WPM100 medium with different concentrations of IBA combined with Zeatin or 2-iP, to obtain the best multiplication rate. The environmental conditions for this experiment were as indicated above (Section 3.1.3). After six weeks of culture the effect of the different treatments on shoot number per explant, shoot length, were evaluated for selecting the best multiplication conditions.

3.1.8 Root development in vitro

In vitro rooting and preparation of the individual plantlet is critical to obtain high rates of plant survival during the ex vitro phase. The effect of the auxins such as Indolbutiric acid (IBA), Indol-3-acetic acid (IAA) and Naftalenacetic acid (NAA), added at different
concentrations to the basal WPM medium, on rooting and plant development was tested. All treatments were kept under the same environmental conditions described for the previous experiments (Section 3.1.3) for 40 days before evaluation. The percentages of roots, roots per explant and calli formation, were evaluated for selecting the best in vitro rooting conditions.

**Results**

**3.1.9 Effect of the disinfection protocol and plant growth regulators on explant decontamination and plantlet development during the in vitro establishment**

**3.1.10 Disinfection and establishment**

The highest disinfection efficiency ($p=0.01$) was obtained when the explants were exposed to disinfection treatment T3 (disinfection with 1% sodium hypochlorite for 40 minutes, followed by a second wash with 2% sodium hypochlorite for 25 minutes and cultivation on Murashige and Skoog medium (MS) supplemented with 2.0 mgL$^{-1}$ of 2-iP). Using this treatment only 5% of the explant was contaminated (Fig. 1). Fungi and bacteria were the main contaminants observed both associated to the explants or the medium. However, the highest morphogenic response was seen in treatment two, where the 2-iP was reduced to 1 mgL$^{-1}$ compared to treatment three, but the disinfection protocol was the same. Using this 2-iP concentration shoot production per explant increased up to 56%, which was statistically different to the rest of the treatments ($p=<0.01$).
Figure 3.1 *In vitro* establishment of *G. pumila*. Efficiency of the different protocols tested during *in vitro* establishment on disinfection protocols (Percentage of contaminated explants) and morphogenic response (percentage of shoot formation per treatment) in *G. pumila*. Below, behavior of explants during *in vitro* disinfection and establishment; A: shoot formation from a decontaminated explant; B: oxidized explant seven days after disinfection and culture on the regeneration medium; C: Explant contaminated with fungi.

### 3.1.11 Plantlet formation and stabilization *in vitro*

The stabilization of the established explants after disinfection was necessary to homogenize their morphogenic response. It was found that cultivation of the disinfected explants on treatment 18 (WPM100 + 1.0 mgL⁻¹ Zeatin) produced more shoots than the rest of the treatments (*p*=0.01), this was followed by treatment 24 (WPM50 + 1.0 mgL⁻¹ Zeatin). In general, shoots developed very well in these two treatments, had expanded leaves at each node. Shoots also grew more in T18 (8.3±4.67 mm of shoot length) with more leaves per shoots (4.2±2.2 leaves per shoot) (Table 3.2).
Table 3.2 Effect of culture media and plant growth regulators on plant morphogenesis of *G. pumila*. Only treatments with the most explants with shoot formation are shown. Different letters in the same column mean significant differences between treatments according to the Kruskal-Wallis test (*p* ≤0.05).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Number of shoot evaluated</th>
<th>% Shoot</th>
<th>Shoots formation per explant</th>
<th>Average shoot length (mm)</th>
<th>Number of leaves/shoot</th>
</tr>
</thead>
<tbody>
<tr>
<td>T13 (WPM100%+2-iP 1.0 mg L(^{-1}))</td>
<td>2</td>
<td>4</td>
<td>2.0±1.0a</td>
<td>7.5±6.3b</td>
<td>4.5±6.3b</td>
</tr>
<tr>
<td>T14 (WPM100%+2-iP 2.0 mg L(^{-1}))</td>
<td>7</td>
<td>16</td>
<td>2.1±1.4a</td>
<td>13.8±4.4b</td>
<td>6.7±1.9ab</td>
</tr>
<tr>
<td>T16 (WPM100%+BAP2.0 mg L(^{-1}))</td>
<td>3</td>
<td>7</td>
<td>1.0±0.0a</td>
<td>24.3±10a</td>
<td>11.0±6.5a</td>
</tr>
<tr>
<td>T18 (WPM100%+Zeatin 1.0 mg L(^{-1}))</td>
<td>21</td>
<td>47</td>
<td>2.1±1.0a</td>
<td>8.3±2.9b</td>
<td>4.2±2.2b</td>
</tr>
<tr>
<td>T23 (WPM50%+Zeatin 0.5 mg L(^{-1}))</td>
<td>7</td>
<td>16</td>
<td>1.1±0.3a</td>
<td>9.7±3.3b</td>
<td>1.7±1.7b</td>
</tr>
<tr>
<td>T24 (WPM50%+Zeatin 1.0 mg L(^{-1}))</td>
<td>13</td>
<td>29</td>
<td>2.5±1.0a</td>
<td>11.5±3.9b</td>
<td>6.0±2.6ab</td>
</tr>
</tbody>
</table>

*p* values

>0.05  <0.01  <0.01

3.1.12 Effects of the interaction of auxin and cytokinin on the morphogenic response during *in vitro* multiplication

Micropropagation was significantly influenced by the different treatments tested (*p* = <0.05). When placed on treatment T1 (WPM100 + 1.0 mgL\(^{-1}\) Zeatin), an average of six shoots per explant was obtained with an average shoot length of 4.8 cm. Treatment 2 (WPM100 2.0 mgL\(^{-1}\) Zeatin) produced an average of 3.3 cm of shoot length per explant, with 4.3 shoots per explant (Table 3.3). After six weeks of culture, the regenerated shoots showed expanded leaves and grew vigorously (Fig. 3.2). Oxidation of the explant was very low in all the experiment with no statistical differences between treatments (*p* = >0.05).
Table 3.3 Morphogenic response of *G. pumila* in different culture media, concentration and combination of plant growth regulators. Different letters in the same column refer to significant differences between treatments. Tukey HSD test (p≤0.05).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Average of shoot length (cm)</th>
<th>Number of new shoots per explant</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1(WPM100+1mgL$^{-1}$ Zeatin)</td>
<td>4.8±1.0 a</td>
<td>6.0±0.8 a</td>
</tr>
<tr>
<td>T2(WPM100+2mgL$^{-1}$ Zeatin)</td>
<td>3.3±1.0 b</td>
<td>4.3±0.5 bcd</td>
</tr>
<tr>
<td>T3(WPM100+1mgL$^{-1}$ Zeatin +0.25 mg L$^{-1}$ IBA)</td>
<td>2.3±0.5 cd</td>
<td>3.3±0.5 de</td>
</tr>
<tr>
<td>T4(WPM100+2mgL$^{-1}$ Zeatin +0.25 mg L$^{-1}$ IBA)</td>
<td>1.5±0.6 de</td>
<td>2.8±1.0 e</td>
</tr>
<tr>
<td>T5(WPM100+1mgL$^{-1}$ Zeatin +0.5 mg L$^{-1}$ IBA)</td>
<td>2.5±0.6 bc</td>
<td>3.0±0.0 e</td>
</tr>
<tr>
<td>T6(WPM100+2mgL$^{-1}$ 2-iP)</td>
<td>2.5±0.6 bc</td>
<td>3.5±1.3 cde</td>
</tr>
<tr>
<td>T7(WPM100+2mgL$^{-1}$ 2-iP)</td>
<td>3.0±0.8 bc</td>
<td>4.5±0.6 bc</td>
</tr>
<tr>
<td>T8(WPM100+3mgL$^{-1}$ 2-iP)</td>
<td>2.8±0.6 bc</td>
<td>4.8±1.0 b</td>
</tr>
<tr>
<td>T9(WPM100+2mgL$^{-1}$ 2-iP + 0.25 mg L$^{-1}$ IBA)</td>
<td>1.3±1.0 e</td>
<td>3.3±0.5 de</td>
</tr>
<tr>
<td>T10(WPM100+3mgL$^{-1}$ 2-iP + 0.25 mg L$^{-1}$ IBA)</td>
<td>1.0±0.0 e</td>
<td>2.8±0.5 e</td>
</tr>
<tr>
<td>T11(WPM100+2mgL$^{-1}$ 2-iP +0.5 mg L$^{-1}$ IBA)</td>
<td>0.8±0.5 e</td>
<td>2.5±0.6 e</td>
</tr>
<tr>
<td>T12(WPM100+3mgL$^{-1}$ 2-iP +0.5 mg L$^{-1}$ IBA)</td>
<td>0.8±0.5 e</td>
<td>2.8±0.5 e</td>
</tr>
</tbody>
</table>

p value          <0.01          <0.01

Figure 3.2 Response of nodal segments to different multiplication treatments in *G. pumila*.

3.1.13 Evaluation of auxins on the *in vitro* rooting and pre-acclimatization of individualized plantlets

Addition of auxins to the basal medium increased significantly in the root formation. At the same time, addition of 3 mgL$^{-1}$ of NAA induced the highest rooting efficiency (47% rooted explants, *p*=0.01), followed by the addition of 4 mgL$^{-1}$ of NAA that produced 40% of explants rooting, with a mean of 1.23 and 1.13 root per explant in the treatments 11(WPM +3 mgL$^{-1}$ NAA) and 12 (WPM +4 mgL$^{-1}$ NAA) respectively (Table 3.4). However, the treatments T1, T2, T3, T4, T5, T6 and T8, did not produce roots. Callus formation was induced when media were supplemented with NAA and IBA.
Table 3.4 Root formation in *G. pumila* induced by different media and plant growth regulators. sd: without response. Different letters within of same column indicate significant differences between treatments. Kruskal-Wallis test (p≤0.05).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Percentage of roots</th>
<th>Roots per explant</th>
<th>Calli formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 (WPM +1 mgL^{-1} IBA)</td>
<td>sd</td>
<td>sd</td>
<td>0.40±0.50bc</td>
</tr>
<tr>
<td>T2 (WPM +2 mgL^{-1} IBA)</td>
<td>sd</td>
<td>sd</td>
<td>0.17±0.38c</td>
</tr>
<tr>
<td>T3 (WPM +3 mgL^{-1} IBA)</td>
<td>sd</td>
<td>sd</td>
<td>0.47±0.51b</td>
</tr>
<tr>
<td>T4 (WPM +4 mgL^{-1} IBA)</td>
<td>sd</td>
<td>sd</td>
<td>0.40±0.50bc</td>
</tr>
<tr>
<td>T5 (WPM +1 mgL^{-1} IAA)</td>
<td>sd</td>
<td>sd</td>
<td>sd</td>
</tr>
<tr>
<td>T6 (WPM +2 mgL^{-1} IAA)</td>
<td>(sd)</td>
<td>(sd)</td>
<td>(sd)</td>
</tr>
<tr>
<td>T7 (WPM +3 mgL^{-1} IAA)</td>
<td>3</td>
<td>0.03±0.18b</td>
<td>sd</td>
</tr>
<tr>
<td>T8 (WPM +4 mgL^{-1} IAA)</td>
<td>(sd)</td>
<td>(sd)</td>
<td>(sd)</td>
</tr>
<tr>
<td>T9 (WPM +1 mgL^{-1} NAA)</td>
<td>23</td>
<td>0.43±0.9ab</td>
<td>0.83±0.38a</td>
</tr>
<tr>
<td>T10 (WPM +2 mgL^{-1} NAA)</td>
<td>27</td>
<td>1.0±1.86ab</td>
<td>0.93±0.25a</td>
</tr>
<tr>
<td>T11 (WPM +3 mgL^{-1} NAA)</td>
<td>47</td>
<td>1.23±1.5a</td>
<td>0.97±0.18a</td>
</tr>
<tr>
<td>T12 (WPM +4 mgL^{-1} NAA)</td>
<td>40</td>
<td>1.13±1.96a</td>
<td>1.00±0.00a</td>
</tr>
</tbody>
</table>
Discussion

Any plant explant to be introduced in vitro needs a surface disinfection process that have to be accurate and efficient (García-Gonzáles et al. 2010). This phase is key and one of the most important for the in vitro establishment of plant tissues, suggesting the combination of bactericide and fungicide products. The selection of disinfection products will depends on the type of explants that will be introduced.

Among the most commonly used during the disinfection step sodium hypochlorite (Tilkat et al. 2009), calcium hypochlorite (Garcia et al. 1999) and ethanol (Singh and Gurung 2009) are listed. However, some tissues with a high content of lignin or cellulose, such as woody plants and organ tissues developed in the soil require more drastic disinfection treatments, as short immersion in mercury chloride (HgCl₂) (Husain and Anis 2009).

In this work, two cycles of disinfection using 1% of sodium hipoclorite for 40 minutes and a 2% of sodium hipoclorite for 25 minutes gave the best results with 95% of explant disinfection. Previous studies carried out in Rhododendron ponticum L., a related species from the Ericaceae family, with commercial bleach 25% (v/v) (5% of sodium hypochlorite) for 20 minutes, produced 43% of contaminated explants (Almeida et al. 2005).

The combination of sterilization products is often used to improve explant disinfection efficiency. In Vaccinium arctostaphylos L and V. myrtillus L., surface sterilization with 70% ethanol for 1 min followed with a disinfection for 15 min with 3% of sodium hypochlorite gave positive disinfection results (Çüce et al. 2013, Çüce and Sökmen 2015).
Despite the degree of toxicity of mercury chloride (HgCl$_2$), its utility is very frequent in the disinfection process of difficult species, presenting over 80% of efficiency on decontamination of species in Ericaceae family during disinfection and in vitro establishment (Georgieva et al. 2016. Alekseevna Erst et al. 2015)

Some related species from the Ericaceae genus have demonstrated to be very hard to be disinfected and established in vitro, forcing the combination of different chemicals to increase the disinfection efficiency. In *Rhododendron ledebourii*, a first wash with 70% of ethanol for 30 seconds followed by a 15 min wash in a 0.15% (w/v) solution of HgCl$_2$ produced only 40% and 20% of disinfected explants in two different genotypes (Erst et al. 2014). Similar combinations of Ethanol and HgCl$_2$ have given different results in other berries such as *Fragaria vesca* L., *Rubus idaeus* L., *Vaccinium myrtillus* L., *Vaccinium vitis-idaea* L. (Georgieva et al. 2016).

The differences between regarding disinfection efficiency and morphogenic response of the established explants obtained in *G. pumila*, when compared with other species from the same family, might be based on the use of decreasing concentrations of sodium hypochlorite, in two separate washes, avoiding explant toxicity but eliminating most of the contaminating microorganisms.

However, disinfection procedure is not the only factor affecting the in vitro establishment of any explant. During this step it is also critical to induce the morphogenic responses for obtaining new plants and/or new organs. The best morphogenic responses in *G. pumila* established explants were obtained when disinfected explants were cultivated on WPM100 medium, supplemented with Zeatin at 1.0 mgL$^{-1}$, giving 47% of regenerated explants.
Despite there is not previous results of *in vitro* culture in *G. pumila*, the results reported here are similar to those obtained for other Ericaceae species. In *Vaccinium arctostaphylos*, explants cultivated on WPM medium supplemented with 1.0 mgL\(^{-1}\) Zeatin and 0.1 mgL\(^{-1}\) of IBA induced shoot formation in 74% of the cultivated explants (Cüce et al. 2013). Also, using Zeatin as cytokinin source very effectively stimulated the shoot induction, leaf formation, and the shoot development and growth in other species of the *Vaccinium* genus (Gajdosová et al. 2006, Cüce et al. 2013).

In the multiplication stage, the addition of cytokinins to the WPM100 medium produced higher propagation efficiencies since shoot formation as well as shoot elongation were significantly higher in these conditions. On the other hand, supplementing the media with auxin did not have any significant effect on plants morphogenesis.

Despite of it has been demonstrated that the interaction between auxins and cytokinins can play a key role in plant development (Su et al. 2011), it has been found that in *G. pumila* this interaction did not significantly influenced on the multiplication efficiency. According to the results previously showed the best results for plant morphogenesis was obtained when only cytokinins were present in the basal medium.

These results are similar to those informed for *Vaccinium macrocarpon*, where the the single effect of 2-iP as cytoquinin source produced the best plant general development the multiplication of the species (Debnath and McRae 2001). Also, it has been demonstrated that the morphogenic response to cytokinin could depend strongly on genotype, as shown in *Arbutus unedo* L., (Gomes et al. 2010) and some raspberry genotypes (Gajdošová et al. 2006).
On the contrary, in *Fragaria chiloensis*, a wild berry plant native from Chile the interaction between auxins and cytokinins induced the highest efficiency in shoots and leaves formation (Quiroz 2014). Also, this combination of grow regulator induces to the formation of roots in specie as *Eucomis* (Aremu et al. 2016).

Finally, as in many Ericaceae species, *in vitro* rooting in *G. pumila* is very hard to obtain. Addition of auxins, in general, produced some roots, but the efficiency is still very low (46% in medium supplemented with NAA). However, the general plant development is very good and will probably help to obtain high survival efficiencies during the *ex vitro* step.

Contrasting to these results in the related species *Vaccinium corymbosum* L. cv. ‘Elliot’, the addition of BAP produced more than 90% of rooted explantes (Vescan et al. 2012). Also, in other blueberry cultivars addition of IBA induced 75% of rooted plants (Guang-Jie et al. 2008).

At this stage, in addition to the production of roots, the induction of calli and shoots are stimulated. In *Casuarina cunninghamiana* Miq., first the callus is formed and later the adventitious roots are formed from the callus (Shen et al. 2010). The formation of new organs in the rooting stage, allows the survival of the explant, giving opportunity for the formation of roots in the longer term, especially in species difficult to root, as in the Ericaceae family.
Conclusion

For the first time a micropropagation technology for the *in vitro* multiplication of *G. pumila* was developed. This technology will allow the massive propagation of this species to assist its domestication and commercial uses. However, further studies must be developed to extend this protocol to other species from the genus in Chile, as well as to standardize the technology among the different *G. pumila* selected genotypes. Also, it is still necessary to assess the behavior of the *in vitro* produced plants during *ex vitro* adaptation.
Literature cited


4 Annex

Annex 1. Treatments for the establishment of G. pumila in different medium and different growth regulators

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Medium</th>
<th>Dilution of culture medium</th>
<th>Grown regulator</th>
<th>Concentration of grown regulators</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>MS</td>
<td>100%</td>
<td>2-iP</td>
<td>1.0 mg L⁻¹</td>
</tr>
<tr>
<td>T2</td>
<td>MS</td>
<td>100%</td>
<td>2-iP</td>
<td>2.0 mg L⁻¹</td>
</tr>
<tr>
<td>T3</td>
<td>MS</td>
<td>100%</td>
<td>BAP</td>
<td>1.0 mg L⁻¹</td>
</tr>
<tr>
<td>T4</td>
<td>MS</td>
<td>100%</td>
<td>BAP</td>
<td>2.0 mg L⁻¹</td>
</tr>
<tr>
<td>T5</td>
<td>MS</td>
<td>100%</td>
<td>Zeatin</td>
<td>0.5 mg L⁻¹</td>
</tr>
<tr>
<td>T6</td>
<td>MS</td>
<td>100%</td>
<td>Zeatin</td>
<td>1.0 mg L⁻¹</td>
</tr>
<tr>
<td>T7</td>
<td>MS</td>
<td>50%</td>
<td>2-iP</td>
<td>1.0 mg L⁻¹</td>
</tr>
<tr>
<td>T8</td>
<td>MS</td>
<td>50%</td>
<td>2-iP</td>
<td>2.0 mg L⁻¹</td>
</tr>
<tr>
<td>T9</td>
<td>MS</td>
<td>50%</td>
<td>BAP</td>
<td>1.0 mg L⁻¹</td>
</tr>
<tr>
<td>T10</td>
<td>MS</td>
<td>50%</td>
<td>BAP</td>
<td>2.0 mg L⁻¹</td>
</tr>
<tr>
<td>T11</td>
<td>MS</td>
<td>50%</td>
<td>Zeatin</td>
<td>0.5 mg L⁻¹</td>
</tr>
<tr>
<td>T12</td>
<td>MS</td>
<td>50%</td>
<td>Zeatin</td>
<td>1.0 mg L⁻¹</td>
</tr>
<tr>
<td>T13</td>
<td>WP</td>
<td>100%</td>
<td>2-iP</td>
<td>1.0 mg L⁻¹</td>
</tr>
<tr>
<td>T14</td>
<td>WP</td>
<td>100%</td>
<td>2-iP</td>
<td>2.0 mg L⁻¹</td>
</tr>
<tr>
<td>T15</td>
<td>WP</td>
<td>100%</td>
<td>BAP</td>
<td>1.0 mg L⁻¹</td>
</tr>
<tr>
<td>T16</td>
<td>WP</td>
<td>100%</td>
<td>BAP</td>
<td>2.0 mg L⁻¹</td>
</tr>
<tr>
<td>T17</td>
<td>WP</td>
<td>100%</td>
<td>Zeatin</td>
<td>0.5 mg L⁻¹</td>
</tr>
<tr>
<td>T18</td>
<td>WP</td>
<td>100%</td>
<td>Zeatin</td>
<td>1.0 mg L⁻¹</td>
</tr>
<tr>
<td>T19</td>
<td>WP</td>
<td>50%</td>
<td>2-iP</td>
<td>1.0 mg L⁻¹</td>
</tr>
<tr>
<td>T20</td>
<td>WP</td>
<td>50%</td>
<td>2-iP</td>
<td>2.0 mg L⁻¹</td>
</tr>
<tr>
<td>T21</td>
<td>WP</td>
<td>50%</td>
<td>BAP</td>
<td>1.0 mg L⁻¹</td>
</tr>
<tr>
<td>T22</td>
<td>WP</td>
<td>50%</td>
<td>BAP</td>
<td>2.0 mg L⁻¹</td>
</tr>
<tr>
<td>T23</td>
<td>WP</td>
<td>50%</td>
<td>Zeatin</td>
<td>0.5 mg L⁻¹</td>
</tr>
<tr>
<td>T24</td>
<td>WP</td>
<td>50%</td>
<td>Zeatin</td>
<td>1.0 mg L⁻¹</td>
</tr>
</tbody>
</table>

Annex 2. Treatments performed in the multiplication stage of G. pumila, with different concentrations of growth regulators and their interactions

<table>
<thead>
<tr>
<th>IBA</th>
<th>Zeatin 1.0 mg L⁻¹</th>
<th>Zeatin 2.0 mg L⁻¹</th>
<th>2-iP 2.0 mg L⁻¹</th>
<th>2-iP 3.0 mg L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>T1</td>
<td>T2</td>
<td>T7</td>
<td>T8</td>
</tr>
<tr>
<td>0.25 mg L⁻¹</td>
<td>T3</td>
<td>T4</td>
<td>T9</td>
<td>T10</td>
</tr>
<tr>
<td>0.5 mg L⁻¹</td>
<td>T5</td>
<td>T6</td>
<td>T11</td>
<td>T12</td>
</tr>
</tbody>
</table>
Annex 3. Treatments made in the rooting stage of *G. pumila*, with different concentrations of auxins

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Concentration of grow regulators</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>-</td>
</tr>
<tr>
<td>T1</td>
<td>WPM +1 mgL⁻¹ IBA</td>
</tr>
<tr>
<td>T2</td>
<td>WPM +2 mgL⁻¹ IBA</td>
</tr>
<tr>
<td>T3</td>
<td>WPM +3 mgL⁻¹ IBA</td>
</tr>
<tr>
<td>T4</td>
<td>WPM +4 mgL⁻¹ IBA</td>
</tr>
<tr>
<td>T5</td>
<td>WPM +1 mgL⁻¹ IAA</td>
</tr>
<tr>
<td>T6</td>
<td>WPM +2 mgL⁻¹ IAA</td>
</tr>
<tr>
<td>T7</td>
<td>WPM +3 mgL⁻¹ IAA</td>
</tr>
<tr>
<td>T8</td>
<td>WPM +4 mgL⁻¹ IAA</td>
</tr>
<tr>
<td>T9</td>
<td>WPM +1 mgL⁻¹ NAA</td>
</tr>
<tr>
<td>T10</td>
<td>WPM +2 mgL⁻¹ NAA</td>
</tr>
<tr>
<td>T11</td>
<td>WPM +3 mgL⁻¹ NAA</td>
</tr>
<tr>
<td>T12</td>
<td>WPM +4 mgL⁻¹ NAA</td>
</tr>
</tbody>
</table>
5 Conclusiones generales
El propósito de este trabajo, radicó en sentar las bases para la domesticación de *Gaultheria pumila*, a través de dos ejes de investigación: el primero, determinar la diversidad genética contenida en la especie a través de marcadores moleculares microsatélites (SSR); y segundo, buscar los mecanismos idóneos de propagación a través de técnicas de cultivo *in vitro*.

Para el primer caso, se desarrollaron marcadores moleculares microsatélites específicos de la especie, seleccionados a partir de cuatro librerías genómicas. Estas librerías fueron obtenidas a través de técnicas de secuenciación masiva 454 FLX Titanium pyrosequencing technology. La secuencia con mayor repetición fueron las mononucleótidas, con un promedio del 59%, seguido por los dinucleótidos 23%, trinucleótidos 12%, tetranucleótidos 3% y pentanucleótido 2%. La validación de los SSR, se realizó utilizando tres cebadores: un cebador directo con una cola M13 (-21) en su extremo 5', un cebador inverso normal, y el cebador universal M13 (-21) marcado con fluorocromos de 6-FAM, VIC, PET o NED, separados por electroforesis por capilaridad. La variabilidad de los loci microsatélites fueron probados en 30 individuos pertenecientes a tres regiones (La Araucanía, Los Lagos y Magallanes), 10 individuos por Región. De estos marcadores, seis fueron polimórficos, tres fueron mononucleótidos, y un marcador no amplificó. Los marcadores desarrollados presentaron amplificación en otras especies del genero *Gaultheria*, siendo reproducibles en especies emparentadas. Las secuencias constan en el banco de genes GENEBANK.

Los marcadores microsatélites desarrollados, fueron aplicados en 160 individuos, de once sitios, pertenecientes a cuatro regiones dentro del rango de distribución en Chile. Se realizó el test de neutralidad de los marcadores microsatélites, donde no mostraron
evidencias de selección positiva. Cuatro loci SSR presentaron desequilibrio de Hardy-Weinberg. En el análisis de combinaciones binarias, no mostraron desequilibrio de ligamiento, por lo que cada partidor segrega de forma independiente. Los loci SSR mostraron un alto polimorfismo en todas las poblaciones, con una media de 97.5%. Los valores detectados sugieren un nivel moderado a alto de diversidad genética. Referente a los índices de fijación \((F\text{-statics})\), el valor promedio de \(Fis\) muestra valores negativos, lo que sugiere un exceso de heterocigotos observados. Estos valores se relacionan con la biología reproductiva de la especie y sobre todo con el número de migrantes, cuyo flujo génico mantiene en constante dinamismo a las poblaciones. Los valores de \(Fst\), muestran una alta cohesión entre poblaciones, es decir, posee poca estructura, lo que nos lleva a concluir que las poblaciones de \(G. pumila\) tienen una distribución continua, sobre todo en las poblaciones de las regiones céntricas (La Araucanía y Los Lagos y Magallanes) y apenas diferenciándose con las poblaciones límites de su distribución, en este caso Maule. Referente al análisis de la varianza molecular (AMOVA), la mayor variabilidad genética se encontró dentro de las poblaciones, representando el 94% de la variabilidad y entre poblaciones apenas 6%. El análisis bayesiano, nos mostró dos clúster genéticos. El primer clúster, agrupa las poblaciones de las regiones de La Araucanía, Los Lagos y Magallanes, y el segundo clúster, los individuos de la Región del Maule. Este agrupamiento tiene varias connotaciones, entre ellas, que Maule corresponde a una Región límite en su distribución, donde la frecuencia y abundancia de individuos es menor que en las otras regiones. Además, existe una relación positiva entre distancias genéticas y geográficas, corroborado con en el test de Mantel.

Paralelamente al estudio molecular, se trabajaba en el segundo eje, que consistía en buscar los mecanismos idóneos para la propagación de \(G. pumila\) a través de técnicas de cultivo \textit{in vitro}. En este proceso tecnológico, pudimos desarrollar tres etapas
(desinfección- establecimiento, multiplicación y enraizamiento in vitro) con protocolos eficientes en cada una de ellas. Partiendo con la selección del material a introducir, deben provenir de una planta donante bien formada, con brotes nuevos y sobre todo sin problemas de agentes contaminantes. Limpieza superficial con abúndate agua y detergentes común, más desinfecciones con hipoclorito de sodio en dos concentraciones (1 y 2%) y en dos tiempos (40 y 25 min), mostraron un alto porcentaje de asepsia. Además, los explantes se establecieron bien en un medio bajo en sales como el de Murashige and Skoog (MS) suplementado con 2mg L⁻¹ de 2-iP. Esta etapa es muy importante, de aquí depende que el material esté libre de contaminantes y pueda garantizar las etapas venideras dentro del proceso de multiplicación. En la etapa de multiplicación, pudimos darnos cuenta que la interacción de auxinas y citoquininas no presentó mayor incidencia en la formación y altura de brotes. Los mejores resultados fueron originados en los tratamientos que fueron suplementados solo con zeatina en concentraciones de 1 mgL⁻¹. En la etapa de enraizamiento in vitro, se utilizó explantes que provenían de un medio WPM suplementados con zeatina. En esta etapa, los explantes estimulados con NAA, presentaron el mayor número de raíces, donde el 46% de los explantes presentaron al menos una raíz.

Los resultados obtenidos en esta investigación, se utilizarán para continuar con las pretensiones de domesticar a G. pumila, partiendo desde la caracterización de genotipos de interés hasta su multiplicación masiva, de tal modo que sus atributos puedan ser aprovechados de forma sostenible.