SCIENTIFIC NOTE

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Genetic variation within three populations of *Phycella australis* (Phil.) Ravenna from Biobío Region, Chile, evaluated using ISSR markers

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Phycella australis (Phil.) Ravenna is a Chilean plant with high ornamental potential; however, the intensive extraction as a cut flower might be detrimental for the conservational state by ignoring the state of genetic variation. The objective of this investigation was to assess genetic variability within and between three populations of *P. australis* in the Biobío Region using inter-simple sequence repeat (ISSR) markers. The evaluated areas correspond to three locations in the province of Concepción, Biobío Region: Desembocadura (36º48' S, 73º10' W), Santa Juana (36º58' S, 72º58' W), and Lipinhue (37º00' S, 72º58' W). Six ISSR primers were used obtaining 51 fragments, from which 72.5% were polymorphic. From the three evaluated sites Santa Juana showed a higher percentage of polymorphic loci (76.47%). From this variability, 83% belong to within population variability and only 17% belong to variability between populations. The dendrogram generated using the unweighted pair group method with arithmetic mean (UPGMA) method, grouped Lipinhue and Santa Juana sites together, which agrees with the geographic locations. This investigation proved that *P. australis* has high genetic variability despite the exploitation for economic purposes.

Key words: Amaryllidaceae, genetic variability, molecular marker, ornamental plant.

INTRODUCTION

Chile's position in the international market for cut flowers has had an upward trend over the past 15 yr, but at the end of this period show signs of stagnation (Reyes and Barrera, 2009). However, Chile still has a great level of exportation of cut flowers to Netherland, USA, Japan, Argentina, among others; reaching close to US$4 million annually (Reyes and Barrera, 2009). Nevertheless, still is a weak competitor in relation with the production of traditional flowers. An economic strategy to cope with this problem is to offer species such as *Phycella australis* (Phil.) Ravenna, a species that is now beginning to be marketed in the Biobío Region, Chile, which due that is an endemic flower would not have any competitors abroad.

The *Phycella* genus (Amaryllidaceae) is endemic from Chile and Argentina, includes six different species (Arroyo-Leuenberger and Dutilh, 2008) distributed along the central zone of Chile, between Coquimbo and Biobío Regions. *Phycella* plants are perennial, bulbous with flowers arranged in umbels with 2-3 flowers, red and yellowish in the base tepals (Baeza et al., 2007a), flowers are 3 to 5 cm in size, being *P. australis* the most austral distributed plant in the country (Baeza et al., 2007a).

Phycella australis is a species with high ornamental potential (Muñoz et al., 2011), due to their desirable characteristics as a cut flowers which includes long stem, big flowers, and bright colors. However, the massive extraction of these flowers may become a risk to the permanence of this plant in their natural environment, especially if the present level of genetic diversity is unknown, including the genetic variation rates and population structures.

There is limited genetic information about P. australis, however, the chromosomic number has been determined, indicating that is a species with $2n = 2x = 16$ chromosomes, just like *P. bicolor* Ruiz *et* Pav. (Baeza et al., 2007a; 2007b). Besides, the karyotype was also described, indicating that *P. australis* has an asymmetric karyotype with haploid form 2m + 4sm + 2st. On the other hand, Schiappacasse et al. (2005) reported different types of sexual and asexual propagation in different geophytes plants from Chile, including *P. australis*. With respect to genetic variation, no studies have been informed in this species.

As noted in field, there is an incipient commercial exploitation of the species in the Biobío Region that consists in the flower extraction from their natural habitat to be commercialized in local flower markets. The extraction or overexploitation of flowers from populations

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with low genetic diversity might cause damage in the evolutionary response of population to adapt to changes in the environment, affecting the conservational state (Lande and Shannon, 1996; Lynch, 1996). Therefore the lack of information on population genetics of *P. australis* makes necessary the generation of research that allow the development of knowledge about the current conservational state and how this can be affected by the exhaustive flower extraction for economic purposes.

In this regard, molecular markers represent a highly accurate tool for evaluating genetic diversity (Arnao et al., 2008). They allow the recognition of polymorphic regions within the genome excluding the influence of environmental effects or the developmental state of the individual (Jiménez and Collada, 2000; Semagn et al., 2006). Specifically inter simple sequence repeats (ISSR) are dominant markers that do not need previous knowledge of the DNA sequence (Lara et al., 2003). They are fast, simple to perform, and unlike random amplified polymorphic DNA (RAPDs) are more reproducible due to the longest primer sequences, and able to generate highly polymorphic patterns, being use frequently in studies involved in genetic diversity in plants (Parab and Krishnan, 2008). Works such as Fuentes et al. (2007) in *Rhodophiala* Presl., Lara et al. (2003) in *Psychotria acuminata* Benth, and Deng et al. (2006a) in *Lycoris longituba* Y. Xu & G.J. Fan, among others, are examples of the use of ISSR markers, and often in conjunction with RAPDs marker to assess the genetic variation in plants.

In this scientific note, the genetic variation between and among population in the endemic plant *Phycella australis* in three populations of the Biobío Region was estimated using ISSR markers.

MATERIALS AND METHODS

Plant material

Thirty-six *P. australis* (Figure 1) individuals were collected randomly from three populations (12 each) located in the Biobío Region (Figure 2), leaves samples were taken. Populations were named: Desembocadura (36º48' S, 73º10' W), Santa Juana (36º58' S, 72º58' W), and Lipinhue (37º00' S, 72º58' W). Samples were immediately submerged in liquid nitrogen, transported to the laboratory and saved at -80 ºC until its analysis.

Total DNA extraction

Genomic DNA was isolated from 150 mg of powdered leaves using liquid nitrogen and used the 2X CTAB (cetyltrimethylammonium bromide) protocols of Doyle and Doyle (1987). The extraction buffer consisted of 2% CTAB, 2 M NaCl, 50 mM EDTA, 100 mM Tris-HCl pH 8.0, 2% PVP, 8 mM ascorbic acid, and 5 mM DIECA. The suspension was mixed, incubated at 60 °C for 45 min, followed by chlorophorm:isoamyl alcohol (24:1) extraction and precipitation with 1.5 volume of

Figure 1. Photograph of *Phycella australis* taken in the field.

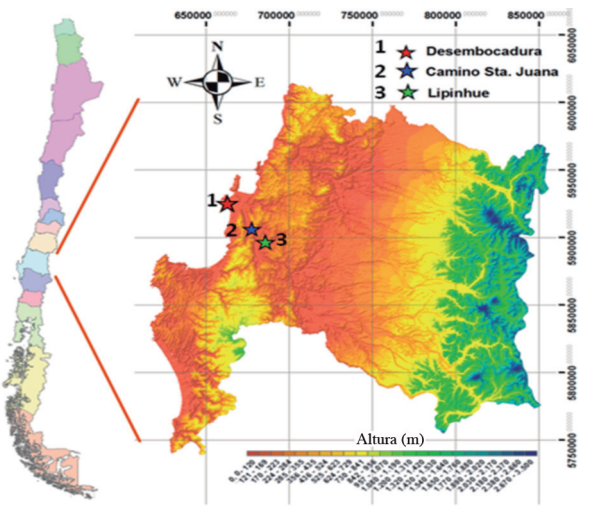

Figure 2. Reference map of the studied populations: 1) Desembocadura, 2) Santa Juana, and 3) Lipinhue.

isopropanol al -20 °C for 1 h. The DNA was pelleted down by centrifugation at 14 000 rpm for 10 min and then was suspended in Ultra Pure water. The concentration was estimated through spectrophotometry (Picodrop, Hinxton, UK) and visualized in agarose gel electrophoresis.

ISSR amplification

Six ISSR primers (Table 1) previously tested in *Rhodophiala* Presl. and *Lycoris longituba* (Deng et al., 2006b; Fuentes et al., 2007) were used. The amplification was performed in a 50 μL reaction volume containing 200 ng of genomic DNA, 0.5 μL of Tag polymerase (Promega®), 0.2 μM of each primer, 0.25 mM of each dNTP and 3 mM $MgCl_2$. Initial denaturation was 3 min at 94 °C, followed by 38 cycles of 30 s at 94 °C, 30 s at 50 °C, 90 s at 72 °C. An additional cycle of 7 min

at 72 °C was used for primer extension; the polymerase chain reaction (PCR) was performed in a thermal cycler (Swift maxi, ESCO, Singapore).

The PCR products were analyzed on 2% agarose gels and stained with 2 μL GelRed (Biotium, Hayward, California, USA) photographed under ultraviolet light by using gel documentation system. Molecular weight was estimated by using 1 kb DNA Ladder (Promega, Madison, Wisconsin, USA).

Data analysis

According to band patterns, a binary matrix was constructed by assigning a binary number to each DNA sample: 1 for presence of the band; 0 for absence. This matrix was analyzed using GenAlEx version 6.41 software (Peakall and Smouse, 2006). The number of polymorphic loci, percentage of polymorphic loci, Shannon diversity index (I) (Shannon and Weaver, 1949), expected heterozygosity (He) and degree of population differentiation (G_{st}) (Nei, 1978) were calculated to estimate genetic diversity on these three *P. australis* populations. Besides, a Nei (1978) genetic distances dendrogram was constructed using the UPGMA method (Sneath and Sokal, 1973) through the POPGENE software (Yeh et al., 1997). Finally an analysis of molecular variance (AMOVA) was performed using GenA1Ex software (Excoffier, 1993). To verify the relation between the genetic distance and geographic distance a correlation analysis was performed using a Mantel Test (Mantel, 1967).

RESULTS AND DISCUSSION

According to the results it is possible to state that selected primers were adequate to detect genetic diversity on this species. The six ISSR primers chosen were able to amplify 51 fragments (Table 1); an average of 8.5 bands was obtained per primer. In this study, a higher number of band per primers were obtained, in relation to another Amaryllidaceae species *Lycoris longituba* (Deng et al., 2006a) where only 7.5 bands per primer were found. This indicates, that *P. australis* has a genome with more complementary zones for primers used than *P. longituba*. Furthermore, this can be compared with other ISSR primers used in *Tigridia pavonia* (L.f.) Redouté (Piña et al., 2010) and *Phaseolus vulgaris* L. (Galván et al., 2003) where average 4.2 and 8 bands were obtained, respectively.

Table 1. ISSR primers selected and the average number of bands (Nb) by a primer in *Phycella australis* in the province of Concepción.

Primer	Primer sequence	Nb
ISSR-03	5'ACACACACACACACACTT 3'	7
ISSR-04	5'ACACACACACACACACAG 3'	9
ISSR-23	5'ACACACACACACACACTA 3'	8
ISSR-44	5'ACACACACACACACACGA 3'	9
ISSR-47	5'ACACACACACACACACGT 3'	9
ISSR-59	5'AGAGAGAGAGAGAGAGGC 3'	9
	Total	51
	Rates	8.5

The genetic variability rates obtained from the binary matrix are available on Table 2. These three populations showed an average of 37 polymorphic loci corresponding to 72.55%. The polymorphism percentage detected in *P. australis* is within the range previously founded for other Amaryllidaceae species such as *Narcissus poeticus* L. and *N. radiiflorus* Salisb. (88%, Tucci et al., 2004) and in *L. longituba* (65.96%, Deng et al., 2006b), despite of the fact that this studies have been performed with others types of molecular markers (AFLP and RAPD, respectively).

Hamrick and Godt (1996) agree that plants with different reproductive methods, seed dispersion mechanisms, geographic distribution, and life forms tend to maintain different levels of genetic variability within populations. According to the values of genetic variability from Nybom (2004) using RAPD, *P. australis* has a within population variability (He: 0.239) that agrees with other endemic species (He: 0.20), with an expected heterozygosity of He: 0.239 in average.

Besides, according to the AMOVA genetic variability of the species is explained by within population variation with an 83% ($p < 0.01$) (Table 3). According to the results, genetic variability is higher within populations, and this percentage is quite similar to that obtained in *L. longituba*, with 71.75% variation within studied populations (Deng et al., 2006b).

The Nei's average genetic identity (Nei, 1973) was obtained (Gst: 0.171), with a value very similar to the ones reported by Nybom (2004) for endemic species (Gst: 0.18). The genetic similarity relatedness between the three populations in the dendrogram of Nei's genetic identities (Figure 3) showed greater genetic identity between Santa Juana and Lipinhue populations. This correlation agrees which Parab and Krishnan (2008), indicating that geographic distances often explain genetic similarities.

Mantel test indicated that there is strong correlation between geographic and genetic distance (R: 0.620;

Table 2. Analyzed genetic diversity rates in three populations of *Phycella australis* in the province of Concepción based on inter-simple sequence repeat (ISSR).

Population	LP	P (%)	I	He
Desembocadura	36	70.59	0.4132	0.219
Santa Juana	39	76.47	0.4407	0.280
Lipinhue	36	70.59	0.3916	0.220
Average	37	72.55	-	0.239

LP: numbers polymorphic loci; P: percentage of polymorphic loci; I: Shannon rate; He: expected heterozygosity.

Table 3. Analysis of molecular variance (AMOVA) among and within populations of *Phycella australis* based on ISSR in the province of Concepción.

Variability source	gl	SC	CM	CV	V (%)
Among populations	2	53.667	26.833	1.581	17
Within populations	33	259.417	7.861	7.861	83
Total	35	313.083	-	9.442	

gl: degrees of freedom; SC: sum of squares; CM: mean squares, CV: coefficient of variance; V: percentage of variance with $p < 0.01$ level of significance.

P: 0.496) (Table 4). According to results, Santa Juana and Lipinhue are generically similar, indicating lower differences between them. But, among these two populations, Lipinhue is the one that is more genetically similar to Desembocadura.

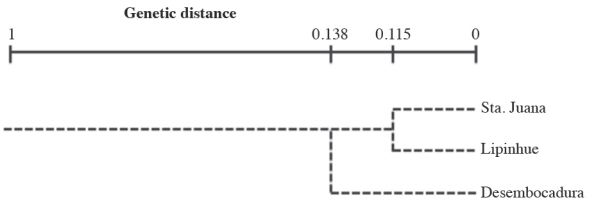

Figure 3. Dendrogram (unweighted pair group method with arithmetic mean, UPGMA) based on Nei's genetic distance matrix for three populations of *Phycella australis* analyzed.

Table 4. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) in three populations of *Phycella australis* in the province of Concepción.

Population	Desembocadura	Santa Juana	Lipinhue
Desembocadura	-	0.8587	0.8834
Santa Juana	0.1523	-	0.8914
Lipinhue	0.1240	0.1150	-

CONCLUSIONS

From the conservation perspective for this species, the results indicate that the genetic variability is enough to ensure the maintenance of the natural populations, probably due to commercial extraction is incipient yet. The genetic variability between populations indicates that genetic flow exists among studied populations. This result is important to ensure a proper genetic variability for maintenance of the species considering the actual climate change scenario. Due to the economic potential value of *P. australis* as ornamental plant, is important to consider these genetic results for the development of conservation strategies.

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