Online Resources

Isolation and cross-amplification of the first set of polymorphic microsatellite markers for two high-Andean cushion plants

Running title: First polymorphic microsatellites for two cushion plants

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Introduction

Almost all ecological restoration programs imply the introduction of individuals into disturbed sites. However, individual candidates for repopulation often come from diverse sources, and the success of such programs therefore depends strongly on knowledge of key natural processes, which can be inferred from genetic individual-based information (Houde et al. 2015). Regarding this, some fundamental aspects of natural populations linked to restoration success can be assessed using a population genetics approach. These include, but are not limited to, endogamy levels, individual relatedness or adaptability among potential sources of translocated individuals (Houde et al. 2015). In this context, the use of specific genetic markers is a powerful tool since it can help to identify important ecological and evolutionary processes operating in local wild populations (Hughes et al. 2008). For this reason, we developed the first set of microsatellite markers for *Azorella madreporica* and its close relative *Laretia acaulis*, two foundational Apiaceae cushion plants native to the Southern Andes (Cavieres *et al.* 2000). This was made to improve current knowledge of important ecological features of this key species of high-Andean ecosystems, and assist restoration and conservation programs in a region particularly vulnerable to global change trends.

Among high-mountain flora, cushion-plants are recognized as pivotal ecological elements in almost all mountainous formations (Kikvidze et al. 2015). They typically occur in harsh environments, and in recent decades have been recognized as one of the main structural elements of several functional dynamics in their respective ecosystems (Reid

and Lortie 2012). However, high-mountain ecosystems are increasingly threatened worldwide by natural (climate change) pressures (Elsen and Tingley 2015), and by anthropogenic (mining) activities, particularly in South America (Oyarzun and Oyarzun 2011).

In the Southern Andean steppe ecoregion of South America (27º - 39°S), cushion plant species like *A*. *madreporica* and *L. acaulis* are among the main structural elements of the high-Andes flora (Cavieres et al. 2000). Commonly known as *yaretas*, they form a well-established altitudinal vegetation belt along the lower Andean zone (Cavieres et al. 2000); therefore, they represent key foundation species for restoration programs in this ecoregion. However, no specific genetic tools are available today to enhance the success of conservation-related strategies for both species in these fragile ecosystems, for which this crucial aspect is still not taking into account.

Individual-based genetic data have previously been used to elucidate a wide range of biological and ecological processes, such as ancient and contemporary migration routes, reproductive isolation and even selection in wild species (meta) populations (Orsini et al. 2013; Mandák et al. 2016), all of which are key processes affecting the success of restoration programs. Hence, in order to provide the genetic tools that are needed to gather these kind of ecological information, we developed and cross-amplified 28 specifically designed microsatellite markers (14 in *A. madreporica* and 14 in *L. acaulis*), and also tested the cross amplification of 25 markers from the related species *Azorella selago*. The set of new polymorphic microsatellite loci will be used in the study of the altitudinal and spatial distribution of genetic diversity in both species.

Methods

Fresh leaves from 24 individuals of each species were collected along a gradient of environmental conditions (3500-4200 m) in the high-Andean Río Estrecho basin in the Chilean Atacama region (Fig. 1, 70°06' W - 29°27' S), and stored with silica-gel until dry. 100 mg of green leaves from each sample was ground to a fine powder using stainless steel beads with a Mini BeadBeater-16 (BioSpec, USA). About 15 mg of the ground material was used for DNA extraction, applying the cetyl-trimethyl-ammonium bromide (CTAB) method (Doyle and Doyle 1987), following the protocol described in Cota-Sánchez et al. (2006). Genomic DNA was quantified with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA), and its integrity checked by electrophoresis in 1% agarose gels. Genomic libraries enriched with AC and AG motifs were constructed by Genetic Marker Services (www.geneticmarkerservices.com). 37 positive clones were isolated (*A. madreporica* = 21, *L. acaulis* = 16), and 14 primer pairs in each species were designed with Primer[3] (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and optimized (Rozen and Skaletsky 2000). These specific primer pairs were first cross-tested in eight individuals from each species. In addition, we tested the cross-amplification of 25 primer pairs from the sub-Antarctic cushion *Azorella selago,* the closest taxonomic species to either species in this study, using a wide gradient of annealing temperatures (48 - 62 °C), following the protocol described by Cerfonteyn et al. (2011). Eight of these markers are published (Molecular Ecology Resources Primer Development Consortium 2010), while the remaining 17 (unpublished) were kindly provided by Dr. C. Born.

The successfully optimized microsatellite markers were then amplified by PCR in 24 samples using a Veriti thermal cycler (Life Technologies, USA), with a fluorescently-

labeled forward primer (6FAM, HEX, NED or VIC; see Table 1), in 10 µL reactions composed of 20 ng of genomic DNA, 1.5 mM $MgCl_2$, 1 µL of *AmpliTaq* Gold reaction buffer (10X, Life Technologies), 200 µM each dNTP, 0.2 µM of each primer and 0.25 U of *AmpliTaq* Gold polymerase (Life Technologies). Thermal cycling conditions were: 10 min at 94°C for DNA denaturation and polymerase activation, followed by 30 cycles of 30 s at 94°C, 30 s at specific annealing temperature (Table 1) and 30 s at 72 °C, with a final elongation step of 15 minutes at 72 °C. Successful PCR products, as visualized in a 1.2% agarose gel, were sent to the sequencing unit of the Ecology Department at the Pontificia Universidad Católica de Chile for amplicon separation by capillary electrophoresis using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, USA). Allele size was determined using the GeneMarker software (Softgenetics, USA), based on comparison with the migration of the GeneScan-500 Size Standard (Applied Biosystems, Chile). Polymorphic Information Content (PIC) was obtained for each locus in both species with the PIC function from the *polysat* R-package (Clark and Jasieniuk 2011). Observed and expected heterozygosity were calculated using the *adegenet* R-package (Jombart 2008), while departure from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between all loci pairs were tested in each species using GENEPOP v.4.2 (Raymond and Rousset 1995). The presence of null alleles at each locus was also evaluated with the software MICRO-CHECKER (van Oosterhout 2004).

Results

Among the 53 primer pairs tested in each species, only 11 yielded clear and reproducible amplification products, all of them derived from the new enriched libraries and predominantly from the *Azorella madreporica* library (see Table 1 and 2). For the 11

successfully optimized polymorphic microsatellite markers, eight microsatellite loci were polymorphic in *A. madreporica* and six in *L. acaulis* (Table 1). Although some of the 25 primer pairs from *Azorella selago* also yielded positive amplification in *A. madreporica* (9) and *L. acaulis* (11), the referred markers could not be optimized since all of them consistently showed a multiband pattern (data not shown).

The average number of alleles per locus was 5.1 for *A. madreporica*, ranging from 3 (loci *Azm*2 and *Azm*12) to 8 (locus *Azm*11), and 5.8 for *L. acaulis*, ranging from 3 (locus *Azm*4) to 10 (locus *Azm*6) (Table 2). The levels of observed heterozygosity were relatively high and similar between species, ranging between 0.166 and 0.772 in *A. madreporica,* and between 0.565 and 0.875 in *L. acaulis*. Similarly, PIC values were found between 0.334 - 0.748 and 0.494 - 0.799 respectively, and in both species the lower value corresponded to those loci with less alleles (Table 2). Only the microsatellite marker *Azm*7 showed genotype frequencies that deviate significantly from Hardy-Weinberg proportions in *A. madreporica* ($p = 0.007$). This specific marker was also the only one that showed significant evidence for the presence of null alleles in this species ($p < 0.05$). However, it is important to note that genotype frequencies at *Azm*7 did not deviate significantly from Hardy-Weinberg equilibrium in *L. acaulis*. No significant linkage disequilibrium was found between any pair of loci in either species after sequential Bonferroni correction. One monomorphic loci (*Azm*14) was also reported in Table 1 because it might be polymorphic in other populations, especially accounting for the fact that surveyed individuals in this study belong to the northern distribution limit for both species.

Discussion

The polymorphic microsatellite loci reported in this study for *A. madreporica* and *L. acaulis* harbored high levels of genetic diversity, as well as a wide range of allelic richness, making them useful for individual-based genetic analyses. In addition, although it was impossible to successfully amplify the *A. selago* primer pairs in either of the studied species, cross-amplification between *A. madreporica* and *L. acaulis* was in fact successful. Thus, the use of these microsatellite markers in other taxa of this group remains a possibility. Nevertheless, despite we choose to work with dinucleotide microsatellite markers instead trinucleitide ones due to their great variability within the plant individual genome (Scotti et al. 2002), greater efforts are required in the case of *L. acaulis,* for which the development of polymorphic primer pairs was found to be more complex.

Despite their wide variability, if compared with other studies of genetic diversity involving alpine plant species, the obtained H_e values for all loci in *A. madreporica* and *L. acaulis* appear to be higher than most mean H_e estimations previously reported (Stöcklin et al. 2009). Furthermore, if the observed H_e values are compared specifically with those from other cushion plants (Cerfonteyn et al. 2011; Mortimer et al. 2008) they are still in average higher. This could be in part the result of the designed sampling along the complete altitudinal gradient of both species, in which strong ecologic changes are expected, with potential influences over the distribution of individual genotypes (Ohsawa and Ide 2008). In a similar way, average PIC values in both *A. madreporica* and *L. acaulis* loci were found to be similar, or even higher, to those usually showed by most plant species (Varshney et al. 2008).

Climate change is driving an upward range displacement of plant communities in mountain ecosystems globally, and high-mountain plants are particularly affected (Elsen

and Tingley, 2015). Located above the upper altitudinal limit of trees, *A. madreporica* and *L. acaulis* are typically restricted to extreme elevations (Cavieres *et al.* 2000). These landscapes are both highly fragmented and of limited spatial extent, offering few opportunities for colonization (Elsen and Tingley, 2015). Furthermore, in addition to climate change, these ecosystems are also impacted in northern Chile by important anthropogenic threats linked to large-scale mining activities (Oyarzún and Oyarzún 2011). These activities usually cause intense, localized habitat disturbances, and a common compensatory measure in restoration and conservation programs includes propagating and/or translocating individuals of the most vulnerable plant species (Batson *et al.* 2015).

Unfortunately, for most wild species the lack of basic biological and ecological knowledge has resulted in very low reintroduction success (Worthely *et al.* 2013). Nevertheless, since the combination of ecological, genetic and spatial data now permits analysis and monitoring of complex ecological processes (Hughes *et al.* 2008), the use of genetic data in restoration programs could significantly improve the efficiency of these initiatives (Mijangos *et al.* 2015). In this context, the potential threats faced by high-mountain ecosystems under the current global trends provide a strong argument for genetic characterization and future monitoring of high-Andean plant species. Therefore, the new set of microsatellite markers developed in this study will be particularly useful to assess spatial genetic structure in both cushion plant species, in order to assist restoration programs in one of the most active mining regions of the world.

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TABLE 1 Primer sequences and characteristics of the microsatellite loci designed for *Azorella madreporica* an
The corresponding GeneBank accession number for each marker is provided below their respective code.

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TABLE 1 Primer sequences and characteristics of the microsatellite loci designed for *Azorella madreporica* and *Laretia acaulis*. The corresponding GeneBank accession number for each marker is provided below their respective code.

Locus	Primer sequences (5´- 3´)	Repeat motif	Fluorescent dye	Size range (bp) *A. madreporica*
Azm2 **(MG675637)**	**F**: GCAGAGATTCTCTTGGTCACG **R**: ACCACACTACTGCATGCATG	$(GT)_{14}(AT)_{8}$	VIC	170 - 174
Azm3 **(MG675638)**	**F**: TTCGACTTTGTTTTCATGATTTGC **R**: AAATCGCTATCTAGTTCATTGTAGC	$(TA)_{7}(CA)_{17}...(CA)_{5}$	NED	128 - 156
Azm4 **(MG675639)**	**F**: TGTGAACCGTGTTTGTGTTTG **R**: GCAGCTCCAGTCAGCATTTC	$(CA)_{4}...(CA)_{8}...(CA)_{7}...(CA)_{3}$	PET	-
Azm5 **(MG675640)**	**F**: GCAGCCAAATGCAACTCATC **R**: TCCCATATTACAACTGCCTAC	$(CA)_{14}$	PET	270 - 292
Azm6 **(MG675641)**	**F**: AACAAGCAGTTGCAGTAGCG **R**: CACACACACAAAAGCGCAAAC	$(CA)_{21}$	NED	-
Azm7 **(MG675642)**	**F**: AGCATCGAACTCGGATCTGC **R**: AGGATTTGGACCCGCTATGG	$(CA)_{12}$	NED	237 - 243
Azm8 **(MG675643)**	**F**: TGGAACAAACGATCTGAATTGC **R**: TGAGTGTTTGCTGCACTTCC	$(TA)_{3}(CA)_{12}$	6FAM	99 - 129
Azm11 **(MG675644)**	**F**: CAAATAATTTGTGGGACTTTGTTG **R**: ACACAACCAATCCAAGATACCAC	$(CA)_{12}$	PET	81 - 105
Azm12 **(MG675645)**	**F**: TTCACATGAAGGACGACTATG **R**: TTCTCTTGGTCACGGACTTC	$(TA)_{7}(CA)_{10}$	VIC	121 - 125
Azm13 **(MG675646)**	**F**: TCGGTTTTTGTGGTTTTACG **R**: ACGGAGGAAATAATTGTGGGAC	$(CA)_{11}$	PET	174 - 180
Azm14 **(MG675647)**	**F**: AACTAGTTATGTCTTCACCCATCC **R**: GAATGATCCACAATCTGGCTGC	$(CA)_{12}$	NED	172
Lar3 **(MG675636)**	**F**: ACAAGGTCCCTCTTGCAAGG **R**: TGTCTTTTACAATACCAGAATGAAGT	$(GA)_{22}$	6FAM	-

TABLE 2 Genetic diversity (number of alleles by locus [***A***], polymorphic information content (**PIC**), observed [H_o] and expected [H_e] heterozygosity), and annealing temperature in °C (**Ta**) for the polymorphic microsatellites isolated in *Azorella madreporica* (8) and *Laretia acaulis* (6). Significant departures from Hardy-Weinberg equilibrium ($p < 0.01$) are indicated with *.

Locus	*Azorella madreporica*					*Laretia acaulis*				
	A	**PIC**	H_o	H_e	**Ta**	***A***	**PIC**	H_o	H_e	**Ta**
***Azm*2**	3	0.334	0.166	0.288	56	1	-	-	-	-
***Azm*3**	6	0.658	0.751	0.756	60	1	-	-	-	-
***Azm*4**	-	-	-	-	-	3	0.494	0.791	0.766	54
***Azm*5**	7	0.743	0.772	0.766	63	5	0.658	0.833	0.784	60
***Azm*6**	-	-	-	-	-	10	0.799	0.875	0.868	60
***Azm*7**	4	0.609	0.375 *	0.672	59	4	0.680	0.580	0.806	55
***Azm*8**	6	0.758	0.739	0.773	63	1	-	-	-	-
***Azm*11**	8	0.744	0.737	0.771	65	-	-	-	-	-
***Azm*12**	3	0.386	0.238	0.291	58	1	-	-	-	-
***Azm*13**	4	0.479	0.541	0.510	60	4	0.511	0.565	0.554	54
***Lar*3**	-	-	-	-	-	9	0.795	0.806	0.899	62

FIGURE 1 Overview of the Estrecho River valley (**a**) and detail of the studied species populations: *Laretia acaulis* (**b**) and *Azorella madreporica* (**c**).