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

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Abstract. In the southern Andes mountains (27–39°S) *Azorella madreporica* and *Laretia acaulis*, two Apiaceae cushion plant species commonly known as *yaretas*, conform a well-established altitudinal vegetation belt along the lower Andean zone. These species have been considered as fundamental components of several ecological dynamics within their communities; however, high-mountain ecosystems are increasingly threatened worldwide by natural and anthropogenic pressures and the southern Andes are not the exception. Recognizing that genetic information is crucial for the success of any conservation or restoration initiative in wild populations, 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*. In a region which is particularly vulnerable to global change trends, this new polymorphic microsatellite loci will be useful in the study of the genetic diversity of these high-mountain cushion plants, which are pivotal in the structuring of their native ecosystems.

Keywords. cushion plants; high-Andes; microsatellite markers; *Azorella madreporica*; *Laretia acaulis*.

Introduction

Almost all ecological restoration programmes imply the introduction of individuals into disturbed sites. However, individual candidates for repopulation often come from diverse sources, and the success of such programmes, therefore, depends strongly on the 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 in restoration and conservation programmes 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 they 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

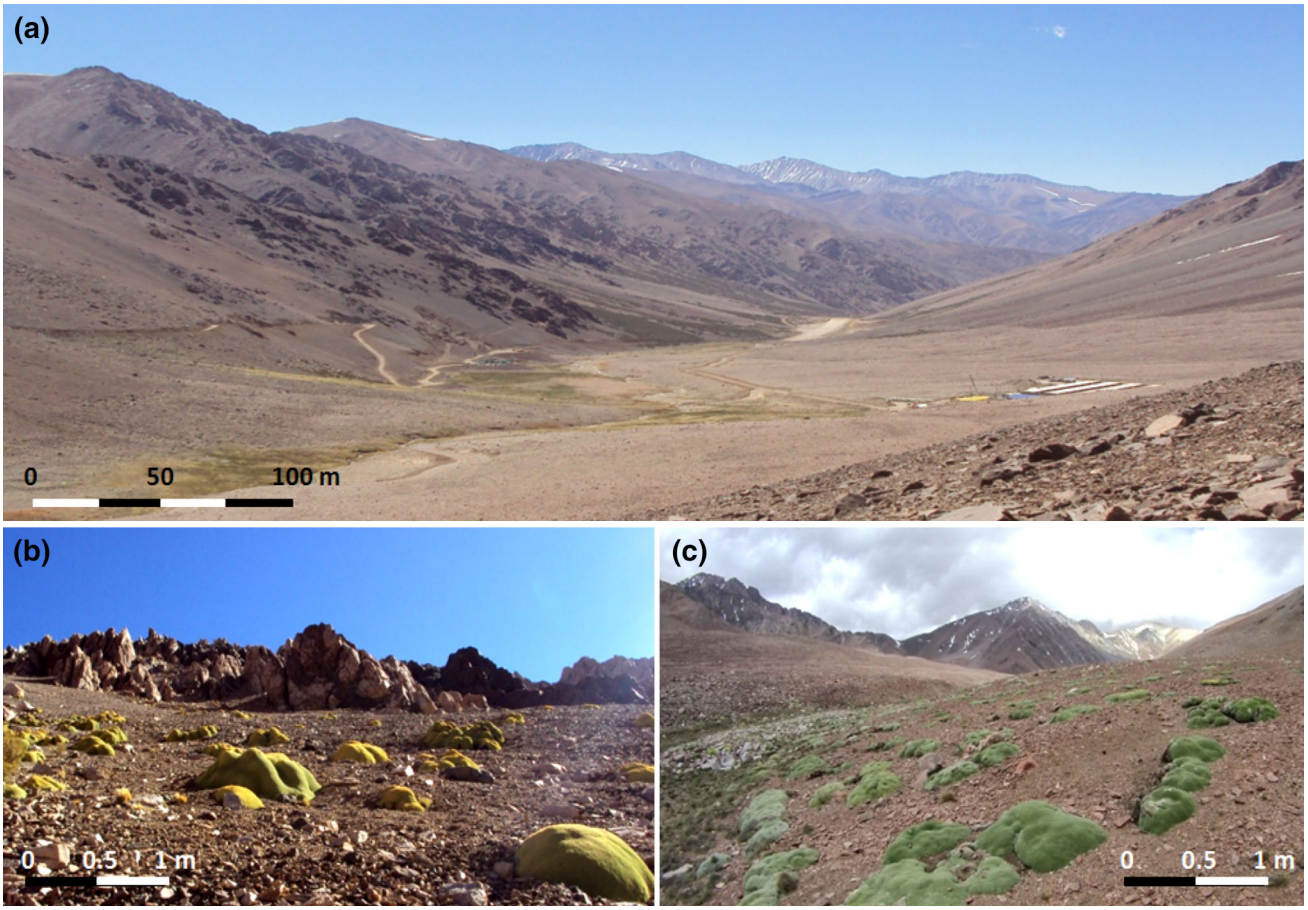

Figure 1. (a) Overview of the Estrecho river valley and details of the studied species populations: (b) *L. acaulis* and (c) *A. madreporica*.

(Elsen and Tingley 2015), and by anthropogenic (mining) activities, particularly in South America (Oyarzún and Oyarzún 2011).

In the southern Andean steppe ecoregion of South America (27−39°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 programmes in this ecoregion. However, till today no specific genetic tools are available to enhance the success of conservation-related strategies for both species in these fragile ecosystems, for which this crucial aspect is still not taken 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 programmes. Hence, to provide the genetic tools that are needed to gather this 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*. A set of new polymorphic microsatellite loci will be used in this study of the altitudinal and spatial distributions 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 (70°06′W−29°27′S) (figure 1), 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 was checked by electrophoresis in 1% agarose gel. Genomic libraries enriched with AC and AG motifs were constructed by Genetic Marker Services (www.geneticmarkerservices.com). Thirty-seven positive clones were isolated (*A. madreporica* = 21, *L. acaulis* = 16), and 14 primer pairs in each species were designed with Primer3 (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 *A. 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 polymerase chain reaction (PCR) in 24 samples using a Veriti thermal cycler (Life Technologies, USA), with a fluorescently labelled 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 (10×, 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 min at 72°C. Successful PCR products, as visualized in 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 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 heterozygosities were calculated using the *adegenet* R-package (Jombart 2008), while departure from the 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 MICRO-CHECKER software (van Oosterhout *et al.* 2004).

Results

Among the 53 primer pairs tested in each species, only 11 yielded clear and reproducible amplification products, all of them were derived from the newly enriched libraries, predominantly from the *A. madreporica* library (see tables 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 *A. 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 three (loci *Azm*2 and *Azm*12) to eight (locus *Azm*11) and 5.8 for *L. acaulis*, ranging from three (locus *Azm*4) to 10 (locus *Azm*6) (table 2). The levels of observed heterozygosity were relatively high and similar between the 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 and 0.748 and 0.494 and 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 for *Azm*7 did not deviate significantly from the HWE in *L. acaulis*. No significant LD was found between any pair of loci in either species after sequential Bonferroni correction. One monomorphic locus (*Azm*14) is also reported in table 1 because it might be polymorphic in other populations, especially accounting for the fact that the 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* harboured 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 chose to work with dinucleotide microsatellite markers instead of trinucleotide markers because of their great variability within the plant individual genome (Scotti *et al.* 2002),

Table 1. Primer sequences and characteristics of the microsatellite loci designed for *A. madreporica* and *L. acaulis*. The corresponding GenBank 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*	Size range (bp) *L. acaulis*
*Azm*2 (MG675637)	F: GCAGAGATTCTCTTGGTCACG R: ACCACACTACTGCATGCATG	$(GT)_{14}(AT)_8$	VIC	170–174	166
*Azm*3 (MG675638)	F: TTCGACTTTGTTTTCATGATTTGC R: AAATCGCTATCTAGTTCATTGTAGC	$(TA)_7(CA)_{17}\ldots(CA)_5$	NED	128–156	101
*Azm*4 (MG675639)	F: TGTGAACCGTGTTTGTGTTTG R: GCAGCTCCAGTCAGCATTTC	$(CA)_4\ldots(CA)_8\ldots(CA)_7\ldots(CA)_3$	PET	–	118–140
*Azm*5 (MG675640)	F: GCAGCCAAATGCAACTCATC R: TCCCATATTACAACTGCCTAC	$(CA)_{14}$	PET	270–292	81–95
*Azm*6 (MG675641)	F: AACAAGCAGTTGCAGTAGCG R: CACACACACAAAAGCGCAAAC	$(CA)_{21}$	NED	–	186–192
*Azm*7 (MG675642)	F: AGCATCGAACTCGGATCTGC R: AGGATTTGGACCCGCTATGG	$(CA)_{12}$	NED	237–243	235–241
*Azm*8 (MG675643)	F: TGGAACAAACGATCTGAATTGC R: TGAGTGTTTGCTGCACTTCC	$(TA)_3(CA)_{12}$	6FAM	99–129	99
*Azm*11 (MG675644)	F: CAAATAATTTGTGGGACTTTGTTG R: ACACAACCAATCCAAGATACCAC	$(CA)_{12}$	PET	81–105	–
*Azm*12 (MG675645)	F: TTCACATGAAGGACGACTATG R: TTCTCTTGGTCACGGACTTC	$(TA)_7(CA)_{10}$	VIC	121–125	111
*Azm*13 (MG675646)	F: TCGGTTTTTGTGGTTTTACG R: ACGGAGGAAATAATTGTGGGAC	$(CA)_{11}$	PET	174–180	186–192
*Azm*14 (MG675647)	F: AACTAGTTATGTCTTCACCCATCC R: GAATGATCCACAATCTGGCTGC	$(CA)_{12}$	NED	172	–
*Lar*3 (MG675636)	F: ACAAGGTCCCTCTTGCAAGG R: TGTCTTTTACAATACCAGAATGAAGT	$(GA)_{22}$	6FAM	–	281–307

Table 2. Genetic diversity.

Locus	*A. madreporica**					*L. acaulis**				
	A	PIC	H_o	H_e	T_a	*A*	PIC	H_o	H_e	T_a
*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

A, number of alleles by locus; PIC, polymorphic information content; H_o, observed; H_e, expected heterozygosity; T_a, annealing temperature in °C for the polymorphic microsatellites isolated in *A. madreporica* (eight) and *L. acaulis* (six). *Significant departures from the HWE ($P<0.01$)

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). Further, if the estimated H_e values are compared specifically with those from other cushion plants (Mortimer *et al.* 2008; Cerfonteyn *et al.* 2011) they are still higher in average. This could be in part the result of the designed sampling along the complete altitudinal gradient of both species, in which strong ecological 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.* 2005).

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). Further, 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 programmes 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 (Wortley *et al.* 2013). Nevertheless, since now the combination of ecological, genetic and spatial data permits analysis and monitoring of complex ecological processes (Hughes *et al.* 2008), the use of genetic data in restoration programmes 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 particularly be useful to assess spatial genetic structure in both cushion-plant species, to assist restoration programmes in one of the most active mining regions of the world.

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