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Isolation and cross-amplification of fifteen microsatellites for oil-rewarding Calceolaria species (Calceolariaceae)

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ABSTRACT. Calceolaria is a diversified American genus whose species are mainly visited by oil-collecting bees, representing a highly specialized plant-pollinator system. However, although this is a unique plant system, different aspects of its ecology and evolution remain unexplored. In this study we characterized fifteen polymorphic microsatellites for four Andean Calceolaria species using next-generation sequencing. The number of alleles per locus (Na) for the study species ranged from 2 to 11, and the observed (Ho) and expected (He) heterozygosity ranged from 0 to 0.85 and 0.3 to 0.87, respectively, while FIS ranged from 0.03 to 1.0. Among the four species, C. cana showed the greatest genetic diversity, followed by C. filicaulis. C. arachnoidea and C. lanigera showed similar, lower genetic diversity. These makers are a useful tool to conduct futures studies on population structure and gene flow. Specifically, this set of markers will be helpful to estimate selfing rates as a first approximation to the understanding of the role of specialization in plant mating system evolution

KEY WORDS: Calceolaria, Chile, Microsatellites, Oil-rewarding flower.

INTRODUCTION

Oil-flowering species have been described in only eleven families of Angiosperms inhabiting mostly the tropical and subtropical regions (Ehrhart, 2000). Most of them maintain a mix of obligate and facultative dependence interaction with oil-collecting bees and together represent a highly specialized plant-pollinator interaction (Michener, 2007). Calceolariaceae is a diversified American oil-flowering family, composed of 250 species distributed from Mexico to Tierra del Fuego, mostly associated with rivers in the Andean region and coastal areas of the Pacific and the southern Atlantic coast of Argentina (Molau, 1988; Sérsic, 2004). They are characterized by zygomorphic bilabiate flowers of different colors and shapes, whose lower lip contains a trichome gland called elaiophore (Ehrhart, 2000; Sérsic, 2004). Most of the species are visited by oil-collecting bees of *Centris* and *Chalepogenus* genera, which actively collect oil from the elaiophore for larval feeding and nest sealing (Vogel, 1974; Rasmussen and Olesen, 2000; Murúa and Espíndola, 2015). Despite most of the species depend strictly on insects for pollination, a wide diversity of reproductive strategies has also been observed, ranging from autonomous self-compatible to fully or partially self-incompatible (Sérsic, 2004; Murúa et al., 2014).

Although *Calceolaria* flowers are an interesting and unique plant system, different aspects of its ecology and evolution remain unexplored. This is unfortunate, since *Calceolaria* species represent an excellent model to study how specialization arises and is maintained, and the ecological and genetic consequences of this on population structure, gene flow and migration rates. However, to study these topics it is necessary to develop highly variable molecular markers, and according to our knowledge to date no markers with high levels of polymorphism have been developed for these plant species. Here, we study four *Calceolaria* species occupying the Andean region in Chile (Figure 1A, B, C and D). The species differ in their morphology (*e.g*. closed or open corolla), color, elaiophore size (*i.e.* some have a reduced gland) and distribution (Figure 1E). These characteristics make them interesting subjects of study since they represent the great variability that exists within the genus. Several aspects of their ecology are known for some species (*e.g*. pollination ecology and reproduction; Murúa et al., 2014; Murúa et al., 2017), however nothing is known about their genetic variability and how this is related to the ecological aspects. Accordantly, here we isolated for the first time a set of polymorphic microsatellites from four *Calceolaria* species inhabiting the Andes in Chile using next generation sequencing.

MATERIALS AND METHODS

Study sites, DNA isolation and sequencing

Samples of *Calceolaria arachnoidea, C. cana, C. filicaulis and C. lanigera* were collected in four different locations along the Andes in Chile (Figure 1E). During the spring-summer season of 2015-16 in each site fifteen plants were randomly chosen, their leaves collected and immediately stored and dried in paper bags with silica gel.

Genomic DNA was extracted from ~10-20 mg of dry leaf of one individual per species using a modified cetyltrimethyl ammonium bromide (CTAB) protocol (Doyle and Doyle, 1987). The DNA quantity was measured using a nanodrop spectrophotometer (Thermo Fisher) and quality was checked with a Qubit 2.0 Fluorometer (Life Technologies). Then the DNA was sent to OMICS Solutions (Centro Nacional de Genómica y Bioinformática, Santiago, Chile; http://omics-solutions.cl) to perform pyrosequencing in an Ion torrent PGM 318 chip (Thermo Scientific) and one library was constructed per species.

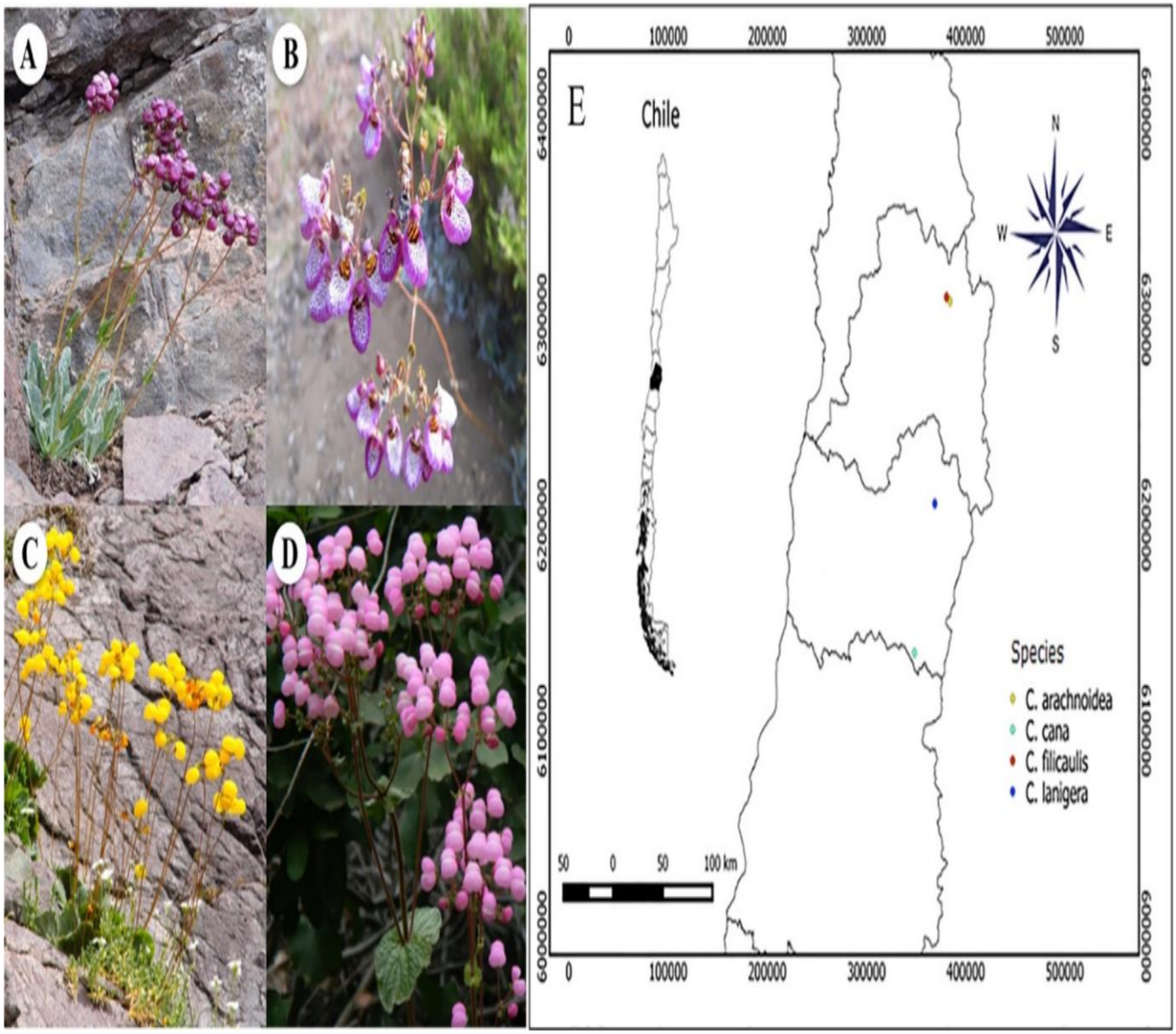

Figure 1. A-D) Plant species and E) geographic locations of the four *Calceolaria* species studied. A) *C. arachnoidea*, B) *C. cana*, C) *C. filicaulis* and, D) *C. lanigera*.

Totals of 188,750, 263,757, 280,905 and 291,788 non-redundant readings were obtained for *C. arachnoidea*, *C. cana*, *C. filicaulis* and *C. lanigera*, respectively. QDD software 2.1 version (Meglécz et al., 2010) was used to detect mostly di and tetra-nucleotide repeat motifs to design the primers using the following criteria: a) ten dinucleotide repeats, b) primers situated within at most 20 bp of the microsatellite region, c) G+C content of 30-70%, d) PCR product longer than 150 bp and e) melting temperature of 60 ºC. Finally, ten primer pairs per species (forty pairs in total) were synthesized by Macrogen Inc. (South Korea).

Primer amplification was tested preliminarily on three samples per species. Ten microsatellites per species were tested and cross-amplified to the rest of the study species. The PCR were performed using a mixture of 1 µL PCR buffer (10x), 2 µL $MgCl_2$ (5 mM), 1 µL dNTP (2.5 mM; Invitrogen), 0.2 µL M13-tailed forward primer (0.2 mM), 0.2 µL reverse primer (0.4 mM), 0.15 µL fluorescently labelled M13 universal primer (2 ng), 1 µL GoTaq G2 (Invitrogen, ThermoFisher Scientific, Waltham, MA, USA), 1µL BSA, 1 µL template DNA (20-30 ng/µL) and H_2O. PCR cycling conditions started with 5 minutes of denaturation at 95 ºC, followed by 30 cycles of 1 minute at 95 ºC, 1 minute of annealing at 5 8ºC, 1-minute extension at 72 ºC and 10 minutes of final extension at 72 ºC. PCR product amplification was checked in 2% agarose gels and genotyped on a 3130xl Genetic Analyzer (Applied Biosystems, Life Technologies, ThermoFisher Scientific, Waltham, MA, USA) at the Pontificia Universidad

Católica de Chile. The primers that showed amplification were tested on fifteen individuals per species and genotyped to perform the primer characterization (Table 1).

Table 1. Characteristics of primers developed for the four *Calceolaria* species.

Locus	Primer sequence	Repeat Motif	T_a (ºC)	Size (bp)	GenBank Accession number
Cal-1	F: TTTCCAGTTCGAACAAACCC	(AC)8	65-55	133-147	MF574816
	R: GTCGAGTATCTTGAAGCCCG				
Cal-2	F: AATAACTCGTGCTGTCCTCTAGTT	(AG)14	65-55	233-235	MF574817
	R: CAATAGCTTTGAGCGGTCATC				
Cal-3	F: CATCCTTTAAATTGTTGGAACG	(AC)18	65-55	365-403	MF574818
	R: TGAACGACTCATGGATCTTACG				
Cal-4	R: CAATTCAGAATATTTCCATTGCTT	AAAT (8)	65-55	217-235	MF574819
	F: AAACCACTATTGCGGTGTCA				
Cal-5	F: TGGAGTGGAAATGAATCTAATCAA	(AC)10	65-55	217-257	MF574820
	R: AGAATCGTAACCATCAATGTATGC				
Cal-6	F: TTTGCACTAGGTCTGAATTCCAT	AC (18)	65-55	148-182	MF574821
	R: TTTGGAAGGACCCTTGGCT				
Cal-7	F: CACTTCAAAGCGTCGATTTCT	(AC)9	65-55	192-218	MF574822
	R: GCTTTGATGAAATTGGAAACC				
Cal-8	F: TTGGGACAGAAGAAAGTTCAAA	(AG)7	65-55	172-216	MF574823
	R: TTCGAGTTTATCCAAGGCAA				
Cal-9	F: TTCATGTTGTCCTCTCAGCAA	(AAAG)9	65-55	261-269	MF574824
	R: TGGAAGAGCATGAAATTGAGG				
Cal-10	F: AAGGATATCAACGCTCTCATACG	(AG)7	65-55	199-223	MF574825
	R: GAGATGATATGCGCGACTGA				
Cal-11	F: CTCTTGTATAACTTACAAACTTGCCC	(AC)11	65-55	163-177	MF574826
	R: CAAACAGTTGGCCAATCCAC				
Cal-12	F: TTTCTCAAACCAGGAGGGAA	(AG)11	65-55	170-190	MF574827
	R: AATGGCGATGGAAGTTCAAA				
Cal-13	F: GAGAATTTGCAACCTCTGGG	(AG)10	65-55	309-331	MF574828
	R: TCCATCTTATGTATACTGAGCATTCC				
Cal-14	F: CGGCACAAGGACAAGAGTTC	(AC)14	65-55	126-156	MF574829
	R: GCAAAGTCAATAGCTTATTATCAACG				
Cal-15	F: TCAAAGGCAATGTTTCACGA	(AC)14	65-55	210-236	MF574830
	R: AAAGTTGGAAATAGCAACGACA				

T_a = annealing temperature.

Data analysis

Genotypes were analyzed with GeneMapper 5 (Applied Biosystems); possible genotyping mistakes and the presence of null alleles in each microsatellite were examined in Microchecker software 2.2.3 version (van Oosterhout et al., 2004). For each of the fifteen polymorphic loci the number of alleles per locus (*Na*), observed (*Ho*) and expected (*He*) heterozygosity, departures from Hardy-Weinberg equilibrium (HWE) and the fixation index F_{IS} were estimated using GeneAlex (Peakall and Smouse, 2012).

RESULTS

Thirty-three of the 40 primers tested on fifteen samples per species amplified, of which thirteen were monomorphic, five showed unreliable amplification and fifteen were polymorphic (Table 2). Microsatellite characteristics of those polymorphic loci for the four-study species are shown in Table 2. The number of alleles per locus (*Na*) ranged from 2 to 11 in the four species. The observed heterozygosity (*Ho*) ranged from 0 to 0.85 and the expected heterozygosity (*He*) showed values from 0.3 to 0.87. The F_{IS} values ranged from 0.03 to 1.0 (Table 2). Departures from Hardy-Weinberg equilibrium (HWE) were observed for several loci, which in most cases may be explained by the presence of null alleles (Table 2).

Calceolaria cana was the species with the highest genetic diversity (mean ± standard error; *Na*= 6.73±0.56; *Ho*= 0.5±0.05; *He*= 0.72± 0.03, F_{IS}= 0.29±0.11; Table 2), followed by *C. filicaulis*, which showed an average *Na* of 6.07±0.65 and mean *Ho* and *He* of 0.28±0.07 and 0.61±0.04 SE, respectively. The average F_{IS} was 0.5±0.08. Finally, *C. arachnoidea* and *C. lanigera* showed similar genetic diversity estimations (Table 2). The number of alleles per locus (*Na*) was on average 5.53±0.6 and 5.93±0.45, respectively. The observed and expected heterozigosity was almost the same for *C. arachnoidea* (*Ho*=0.42±0.09; *He*= 0.59±0.06; Table 2) and *C. lanigera* (*Ho*=0.4±0.07; *He*= 0.64±0.04; Table 2), as well the fixation index (*C. arachnoidea*= 0.34±0.11; *C. lanigera*= 0.36±0.11).

Table 2. Genetic parameters for each locus of the four *Calceolaria* species.

	C. arachnoidea (n=15)				*C. filicaulis (n=15)*				*C. cana (n=15)*				*C. lanigera (n=15)*			
Locus	***Na***	***Ho***	***He***	***Fis***	***Na***	***Ho***	***He***	***Fis***	***Na***	***Ho***	***He***	***Fis***	***Na***	***Ho***	***He***	***Fis***
Cal-1	6	0.86	0.8	-0.08*	5	0.31	0.52	0.4*	5	0.33	0.72	0.54*	2	0.85	0.49	-0.73*
Cal-2	2	0	0.12	1.00*	4	0.2	0.34	0.42*	9	0.71	0.77	0.08*	7	0.5	0.52	0.03
Cal-3	10	0.53	0.85	0.37*	6	0.4	0.69	0.42*	4	0.29	0.87	0.58*	8	0.33	0.82	0.6*
Cal-4	5	0.2	0.44	0.54*	7	0.31	0.55	0.44*	11	0.39	0.87	0.56*	7	0.21	0.8	0.73*
Cal-5	6	0.64	0.74	0.13	9	0.67	0.82	0.19	7	0.6	0.75	0.2*	6	0.29	0.52	0.45*
Cal-6	6	0.79	0.67	-0.18*	4	0.13	0.55	0.76*	4	0.46	0.57	0.19*	3	0.1	0.52	0.81*
Cal-7	3	0.08	0.39	0.79*	5	0.07	0.69	0.9*	6	0.53	0.76	0.29	5	0.08	0.64	0.88*
Cal-8	3	0	0.43	1.00*	4	0.13	0.74	0.82*	8	0.87	0.73	0.48	3	0	0.58	1.0*
Cal-9	3	0.08	0.39	0.13	5	0.14	0.67	0.78*	7	0.71	0.75	0.05	7	0.7	0.79	0.12
Cal-10	3	0.07	0.49	0.85*	11	0.73	0.82	0.1	4	0.27	0.42	0.37	3	0.36	0.52	0.32*
Cal-11	5	0.29	0.32	0.1	11	0.27	0.83	0.68*	9	0.6	0.75	0.2*	6	0.25	0.48	0.48*
Cal-12	7	0.75	0.79	0.05*	7	0.3	0.55	-0.23*	7	0.71	0.81	0.12	8	0.4	0.84	0.52*
Cal-13	7	1	0.78	-0.28*	4	0.17	0.52	0.68*	6	0.4	0.77	-0.19*	6	0.53	0.55	0.03*
Cal-14	9	0.33	0.82	0.6*	6	0.36	0.6	0.41*	9	0.33	0.75	0.56*	10	0.73	0.81	0.09
Cal-15	8	0.73	0.77	0.05*	3	0.07	0.3	0.77*	5	0.31	0.48	0.36*	8	0.71	0.78	0.08
Mean	**5.53**	**0.42**	**0.59**	**0.34**	**6.07**	**0.28**	**0.61**	**0.50**	**6.73**	**0.50**	**0.72**	**0.29**	**5.93**	**0.40**	**0.64**	**0.36**
SE	**0.60**	**0.09**	**0.06**	**0.11**	**0.65**	**0.07**	**0.04**	**0.08**	**0.56**	**0.05**	**0.03**	**0.11**	**0.45**	**0.07**	**0.04**	**0.11**

Note: Na= number of alleles, *He* = expected heterozygosis, *Ho* = observed heterozygosis, F_{IS}= fixation index, *n* = sampled size per population. Departures from Hardy-Weinberg equilibrium are indicated by an asterisk ($p<0.05$). Mean and standard error (SE) for each parameter are shown. Geographic coordinates: *C. arachnoidea* = Valle Nevado (33º21´14.2´´S, 70º 14´55.5´´W); *C. filicaulis* = La Parva (33º20´09.1´´S, 70º 17´13.1´´W); *C. cana* = Alto Huemul (34º53´32.5´´S, 70º 39´36.9´´W); C. lanigera = Pangal (34º14´09.1´´S, 70º 28´56´´W).

DISCUSSION

Although *Calceolaria* species and their pollinators have been described as a highly specialized plant-pollinator system (Sérsic, 2004), this genus exhibits different level of specialization according to pollinator availability (Sérsic, 2004; Murúa et al., 2014; Murúa and Espíndola, 2015; Murúa et al., 2017), which may determine the pollen exchange with different consequences for genetic diversity. For example, *C. cana* has been described as species that has lost the specialized interaction with the oil-collecting bees (Murúa and Espíndola, 2015). However, a recent study reported that the species is mainly pollinated by pollen-collecting bees (Murúa and Espíndola, 2015), which may maintain a high level of pollen exchange and consequently increase within-population genetic diversity. *C. filicaulis* is a species that inhabits high elevations (i.e. 2500 m) where is exclusively pollinated by *Centris nigerrima* (unpublished data). It is possible that in periods where *C. nigerrima* is absent or present in lower abundance the opportunity for cross-pollination and gene exchange may be reduced, affecting this population negatively. Both *C. arachnoidea* and *C. lanigera* share the ability to produce seeds without pollinator assistance (*C. arachnoidea* = 1.8 seed per fruit (Murúa et al., 2014); *C. lanigera* = 12.65 seed per fruit; unpublished data), which can contribute negatively to the global genetic diversity reported for these species.

Here we developed for the first time a set of fifteen polymorphic microsatellites able to cross-amplify among the studied *Calceolaria* species. These markers are a useful tool to conduct future studies on population structure and gene flow, especially in those species of limited distribution (*e.g. Calceolaria lanigera*). Also, this first set of markers will be helpful to estimate selfing rates as a first approximation to the understanding of the role of specialization in plant mating system evolution.

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