Isolation and Characterization of 12 Microsatellite Loci in Soapbark, Quillaja saponaria (Quillajaceae)

Authors: Letelier, Luis, Harvey, Nick, Valderrama, Aly, Stoll, Alexandra, and González-Rodríguez, Antonio

Source: Applications in Plant Sciences, 3(5)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1500024

Applications in Plant Sciences 2015 3(5): 1500024

PRIMER NOTE

ISOLATION AND CHARACTERIZATION OF 12 MICROSATELLITE LOCI IN SOAPBARK, *QUILLAJA SAPONARIA* (QUILLAJACEAE)[1]

LUIS LETELIER[2,6], NICK HARVEY[3], ALY VALDERRAMA[2,4], ALEXANDRA STOLL[5], AND ANTONIO GONZÁLEZ-RODRÍGUEZ[2]

[2]Centro de Investigaciones en Ecosistemas, Universidad Nacional Autónoma de México, Antigua Carretera a Pátzcuaro No. 8701, Col. Ex-Hacienda de San José de la Huerta, 58190 Morelia, México; [3]Genetic Marker Services, Brighton, United Kingdom; [4]Universidad Bernardo O'Higgins, Centro de Investigación en Recursos Naturales y Sustentabilidad, General Gana 1702, Santiago, Chile; and [5]Centro de Estudios Avanzados en Zonas Áridas, Campus Andrés Bello, Universidad de La Serena, La Serena, Chile

- *Premise of the study:* Microsatellite primers were developed for the endemic Chilean tree *Quillaja saponaria* (Quillajaceae), a common member of the sclerophyllous Mediterranean forest, to investigate intraspecific patterns of genetic diversity and structure.
- *Methods and Results:* Using an enriched library, 12 polymorphic microsatellite loci were developed in *Q. saponaria*. All loci consisted of dinucleotide repeats. The average number of alleles per locus was 5.3 (2–13), with a total of 64 alleles recorded in 39 individuals from three populations.
- *Conclusions:* The microsatellite markers described here are the first characterized for *Q. saponaria*. The polymorphic loci will be useful in studies of genetic diversity and genetic population differentiation in natural populations of this species.

Key words: Chile; microsatellites; *Quillaja saponaria*; Quillajaceae; soapbark.

Quillaja saponaria Molina is an endemic Chilean tree, known as soapbark, soap bark tree, or quillay (as it is known in Chile). It is an evergreen tree of the family Quillajaceae (Kubitzki, 2007), found from the Coquimbo Region to Arauco Province in the Bío-Bío Region, approximately between 31° and 38° south (García and Ormazabal, 2008). It grows from sea level to 1600 m a.s.l., preferably in dry areas that are poor in nutrients. The family is monotypic with a single genus and two species from warm-temperate South America (Chile, Brazil, and northern Argentina). *Quillaja saponaria* is considered one of the most important and representative species of the sclerophyllous forest from central Chile, and these communities are part of a biodiversity hotspot called the Chilean Winter Rainfall–Valdivian Forest (Mittermeier et al., 1998; Myers et al., 2000). Moreover, *Q. saponaria* is a timber species (García and Ormazabal, 2008), its flowers are melliferous (Montenegro et al., 2009), and the bark is rich in saponins and medicinal adjuvants (Kensil et al., 1991; San Martín and Briones, 1999).

[1]Manuscript received 9 March 2015; revision accepted 23 March 2015.

The authors thank Chile Abroad Grants program (Doctorate level, Call 2010), Comisión Nacional de Investigación Científica y Tecnológica (CONICYT, Chile), II Concurso Fondo de Investigación del Bosque Nativo (Project no. 041-2011), Corporación Nacional Forestal (CONAF, Chile), and the Postgraduate Program in Biological Sciences of Universidad Nacional Autónoma de México, fulfilling the requirement for the degree of Doctor in Sciences of the Biological Sciences postgraduate program.

[6]Author for correspondence: lletelier@cieco.unam.mx

doi:10.3732/apps.1500024

Our purpose is to evaluate the effects of anthropogenic fragmentation on the patterns of genetic variation and connectivity in populations of *Q. saponaria*. For this reason, we isolated and characterized 12 nuclear microsatellite loci that are being successfully applied to describe spatial patterns of genetic structure. These are the first microsatellite markers developed for a *Quillaja* Molina species.

METHODS AND RESULTS

Microsatellite isolation was performed by the simple sequence repeat (SSR) development company Genetic Marker Services (Brighton, United Kingdom; www.geneticmarkerservices.com). Genomic DNA was extracted from a single *Q. saponaria* (Qsa) individual collected in the locality of Coya, near the city of Rancagua, O'Higgins Region, Chile (locality 5 in Appendix 1), using a modified cetyltrimethylammonium bromide (CTAB) protocol described by Doyle and Doyle (1990), and used to develop an enriched library to isolate microsatellite (SSR)–containing loci. Enrichment involved incubating adapter-ligated restricted DNA with filter-bonded synthetic repeat motifs: $(AG)_{17}$, $(AC)_{17}$, $(AAC)_{10}$, $(CCG)_{10}$, $(CTG)_{10}$, and $(AAT)_{10}$. Thirty-two motif-positive *Escherichia coli* JM109 clones were detected and sequenced, of which 23 contained exploitable repeat motifs with sufficient flanking regions to design forward/reverse primer pairs. The online primer design software Primer3 (Rozen and Skaletsky, 1999) was used to develop primer pairs amplifying fragments ranging in size from 100 to 250 bp, to help minimize later multiloading overlap ambiguities during sequencer genotyping. The primers were then tested for successful amplification on one individual from each of seven populations, chosen to represent the whole latitudinal range of the species' distribution (Cuesta el Espino, Cuesta Los Cristales, Santa Marta, Fundo La Rosa, La Gatera, San Fabián de Alico, and Santa Bárbara; Appendix 1), using a touchdown PCR protocol. PCR amplifications were performed in a 25-μL final volume containing 7 pmol of each primer, 1.5 mM of $MgCl_2$,

0.2 mM of each dNTP, 1× PCR buffer, 0.8 µg/µL bovine serum albumin (BSA), 0.5 units *Taq* polymerase (Promega Corporation, Madison, Wisconsin, USA), and 1.5 µL of DNA diluted 20-fold. For all loci, the same touchdown PCR program was used, consisting of 32 cycles with denaturation at 95°C for 60 s; annealing for 60 s with temperature decreasing 1°C every two cycles from 64°C to 59°C (12 cycles), then 10 cycles at 58°C, and 10 cycles at 57°C; elongation at 72°C for 60 s; and a final extension at 72°C for 5 min. Products were checked for specificity and polymorphism on a cooled high-resolution agarose gel, consisting of 4% MetaPhor (Lonza, Basel, Switzerland) agarose in TAE, run in a cold room at 10°C. Sixteen loci showed clear and specific bands with size variation among the seven individuals assayed (Table 1). Of these, the 12 with the highest degree of apparent polymorphism were chosen for fluorescent labeling (Table 1).

To determine the number of alleles, expected and observed heterozygosity, and to test for deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium, we tested the 12 fluorescent-labeled primer pairs on 39 individuals from three different populations, situated in the northern and southern parts of the species' range (i.e., Angostura, Astillero, and Cuesta Batuco; localities 1, 8, and 9 in Appendix 1). The PCR amplifications were performed with the Platinum Multiplex PCR Master Mix (Applied Biosystems, Carlsbad, California, USA) using three mixes, each with four fluorescent-labeled primers (Applied Biosystems, Warrington, United Kingdom; Table 1): Mix 1 (Qsa7, Qsa11, Qsa13, Qsa24), Mix 2 (Qsa16, Qsa17, Qsa18, Qsa70), and Mix 3 (Qsa19, Qsa26, Qsa28, Qsa66). The PCR amplifications were performed in a 5-µL final volume containing final concentrations of 1× Platinum Multiplex PCR Master Mix, 2 mM of additional $MgCl_2$, 0.10 µM of primer mix, and 20 ng of template DNA, with the following cycling conditions in all cases: an initial heat activation at 94°C for 5 min; followed by a touchdown PCR consisting of 32 cycles with denaturation at 95°C for 60 s; annealing for 60 s with temperature decreasing 1°C every two cycles from 64°C to 59°C (12 cycles), then 10 cycles at 58°C and 10 cycles at 57°C; elongation at 72°C for 60 s; and a final extension at 72°C for 5 min. The PCR products (2 µL) were mixed with 10 µL of Hi-Di formamide and 0.3 µL of GeneScan 600 LIZ Size Standard before analysis on an automatic ABI-PRISM 3100-Avant sequencer (Applied Biosystems, Carlsbad, California, USA). Fragment analysis and final sizing were performed using Peak Scanner software (version 1.0; Applied Biosystems, Carlsbad, California, USA).

The number of alleles, expected and observed heterozygosity, and the significance of the deviations from HWE and linkage equilibrium were estimated with Arlequin 3.5.1.2 (Excoffier et al., 2005). The average of the total number of alleles per locus was 5.3, with a range between two (Qsa24) and 13 (Qsa19). Significant deviations from expectations under HWE after a Bonferroni correction for multiple comparisons were found in three loci from population Astillero, and in two loci from Cuesta Batuco, probably due to the small sample sizes or to the presence of null alleles, particularly in the case of the heterozygote deficits observed at loci Qsa26 and Qsa66 in population Astillero and at Qsa66 in Cuesta Batuco (Table 2). No instances of significant linkage disequilibrium were found among pairs of loci after Bonferroni correction.

CONCLUSIONS

The 12 microsatellite loci characterized here for *Q. saponaria* are the first developed for this species and genus. These markers will be useful in studies of the population genetic diversity and structure of the species for the purpose of conservation under a scenario of land use and climate change.

TABLE 1. Characteristics of 16 microsatellite loci developed in *Quillaja saponaria*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	Fluorescent dye	GenBank accession no.
Qsa7	F: TGAAAGAACGTGTGTGTGAGAA R: AATTCCAAAATGCCAGCAGA	$(AG)_{10}$	116–124	6-FAM	KP775627
Qsa11	F: TCTCTACCAGGTCCTTGCTCA R: AAACACTTATGCCTTCGCAGA	$(TG)_8$	148–154	NED	KP775628
Qsa13	F: CGCAGACATGCTTCTAGTTC R: CAAAAAGCTTAGCGACAAAC	$(GA)_{14}$	158–166	VIC	KP775629
Qsa16	F: AACCGTCTCAACCACATACA R: GGAAGAGGGTGAAAATGAAA	$(CT)_{15}$	205–221	NED	KP775630
Qsa17	F: TTGGGGTATGAAAACATTGA R: AAAGGCATTCCCACATAAAG	$(AG)_{12}$	136–144	6-FAM	KP775631
Qsa18	F: TGGAGCTAGGTTACTCGTTGG R: AAGCCACGATTCATACATTTCA	$(TG)_6$	112–122	PET	KP775632
Qsa19	F: AGAATTGGGGTGTGAGGACT R: TAGGAAAATCCCAGCAAGCA	$(AG)_{15}$	120–150	VIC	KP775633
Qsa24	F: TTCTTTGGTTTTGTTTTGCTC R: GCAAAGGTCGCTACAGTTTT	$(TG)_9$	133–135	PET	KP775634
Qsa26	F: AACTCACAGTGACGACTTTCTCA R: GGAGTCTGTGCTGGTAGTGG	$(TC)_9$	93–97	NED	KP775635
Qsa28	F: GTACTACTAGCTAGAACTCCCC R: GACCTAGTCCTTCTTTGTGT	$(TC)_{10}$	160–174	NED	KP775636
Qsa66	F: TTGGGGATATGTCGGGTAGA R: CACCAACGCAATCAAACATT	$(AG)_{11}$	151–163	PET	KP775637
Qsa70	F: TTTGGAGCTGGTGATGTGAA R: AATACCGGACAACCATGAGG	$(TTC)_8$	126–130	NED	KP775638
*Qsa29	F: CAAGTGCGACAGTCAATCTG R: TGCTGCCAGAAATTCCTAGAT	$(AG)_{16}$	200		KP775639
*Qsa30	F: GATTGCCAGAACAAAGTGAA R: AATTCCATTCACATTCACAAAA	$(TC)_{13}$	199		KP775640
*Qsa31	F: GATTTTGGGCGATTGAAAAA R: TGTCACCGTCATTCGCTAAA	$(AG)_9$	122		KP775641
*Qsa32	F: ATTGATCAGATGTGCGGTTG R: TCTCCTCTCCCTCACTTGGA	$(AG)_{11}$	160		KP775642

* Untested for polymorphism.

TABLE 2. Genetic properties of 12 newly developed microsatellites of *Quillaja saponaria*.[a]

	Angostura (n = 11)				Astillero (n = 14)				Cuesta Batuco (n = 14)			
Locus	A	H_o	H_e	HWE	A	H_o	H_e	HWE	A	H_o	H_e	HWE
Qsa7	3	0.778	0.582	0.712	5	0.385	0.729	0.003	3	0.545	0.537	1.000
Qsa11	3	0.545	0.437	1.000	3	0.500	0.489	0.141	3	0.917	0.620	0.035
Qsa13	3	0.909	0.589	0.043	4	0.929	0.664	0.092	3	0.667	0.627	1.000
Qsa16	3	0.750	0.633	0.703	7	0.857	0.746	0.328	6	0.917	0.728	0.559
Qsa17	3	0.300	0.689	0.028	4	0.286	0.704	0.002	4	0.214	0.680	0.004
Qsa18	3	0.818	0.541	0.115	3	0.429	0.421	0.632	3	0.714	0.540	0.420
Qsa19	8	0.818	0.861	0.200	9	0.692	0.846	0.018	8	0.786	0.786	0.470
Qsa24	2	0.545	0.416	0.506	2	0.429	0.349	1.000	2	0.583	0.489	0.593
Qsa26	3	0.182	0.450	0.009	2	0.000	0.492	0.0003*	3	0.286	0.579	0.005
Qsa28	4	0.636	0.567	0.322	4	0.923	0.591	0.0009*	5	0.786	0.659	0.0013*
Qsa66	3	0.100	0.279	0.052	3	0.000	0.283	0.002*	4	0.214	0.431	0.000*
Qsa70	3	0.364	0.450	0.085	2	0.357	0.304	1.000	2	0.571	0.423	0.506

Note: A = number of alleles sampled; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = P values of the exact test of Hardy–Weinberg equilibrium; n = number of individuals sampled.

[a] See Appendix 1 for geographic coordinates and voucher information. All three populations are located in Chile.

* Locus showed significant deviations from Hardy–Weinberg equilibrium, after Bonferroni correction ($P < 0.001$).

LITERATURE CITED

DOYLE, J. J., AND J. L. DOYLE. 1990. Isolation of plant DNA from fresh tissue. *Focus* 12: 13–15.

EXCOFFIER, L., G. LAVAL, AND S. SCHNEIDER. 2005. Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* 1: 47–50.

GARCÍA, N., AND C. ORMAZABAL. 2008. Árboles nativos de Chile. Enersis S.A., Santiago, Chile.

KENSIL, C. R., U. PATEL, M. LENNICK, AND D. MARCIANI. 1991. Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* Molina cortex. *Journal of Immunology* 146: 431–437.

KUBITZKI, K. 2007. Quillajaceae. *In* K. Kubitzki [ed.], The families and genera of vascular plants: Flowering plants, Eudicots, vol. 9, 407–408. Springer, Berlin, Germany.

MITTERMEIER, R. A., N. MYERS, J. B. THOMSEN, G. A. B. DA FONSECA, AND S. OLIVIERI. 1998. Biodiversity hotspots and major tropical wilderness areas: Approaches to setting conservation priorities. *Conservation Biology* 12: 516–520.

MONTENEGRO, G., F. SALAS, R. C. PEÑA, AND R. PIZARRO. 2009. Actividad antibacteriana y antifúngica de mieles monoflorales de *Quillaja saponaria*, especie endémica de Chile. *Phyton* 78: 141–146.

MYERS, N., R. A. MITTERMEIER, C. G. MITTERMEIER, G. A. B. DA FONSECA, AND J. KENT. 2000. Biodiversity hotspots for conservation priorities. *Nature* 403: 853–858.

ROZEN, S., AND H. J. SKALETSKY. 1999. Primer3 on the WWW for general users and for biologist programmers. *In* S. Misener and S. A. Krawetz [eds.], Methods in molecular biology, vol. 132: Bioinformatics methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.

SAN MARTÍN, R., AND R. BRIONES. 1999. Industrial uses and sustainable supply of *Quillaja saponaria* (Rosaceae) saponins. *Economic Botany* 53: 302–311.

APPENDIX 1. Voucher information for *Quillaja saponaria* samples used in this study. Vouchers are deposited at Herbarium CONC, Departamento de Botánica, Facultad de Ciencias Naturales y Oceanográficas, Universidad de Concepción, Chile.

ID	Locality[a]	Geographic coordinates[b]	Altitude (m a.s.l.)	Collector	Collection date	Accession no.	*n*	Use
1	Coquimbo Region, Angostura	31°27′00″S, 71°31′32″W	341	Letelier L. & A. Valderrama	10/29/2011	201105	11	Determining genetic properties
2	Coquimbo Region, Cuesta El Espino	31°21′28″S, 71°05′26″W	1413	Letelier L. & A. Valderrama	11/01/2011	201106	1	Initial testing
3	Coquimbo Region, Cuesta Los Cristales	31°40′43″S, 71°08′41″W	669	Letelier L. & A. Valderrama	11/01/2011	201107	1	Initial testing
4	Valparaiso Region, Santa Marta	32°19′07″S, 71°11′35″W	200	Letelier L. & A. Valderrama	11/02/2011	201108	1	Initial testing
5	O'Higgins Region, Coya	34°12′19″S, 70°36′33″W	904	Letelier L. & A. Valderrama	03/25/2012	201203	1	Development of SSR library
6	O'Higgins Region, Fundo La Rosa	34°19′10″S, 71°14′28″W	390	Letelier L. & A. Valderrama	10/10/2011	201103	1	Initial testing
7	O'Higgins Region, La Gatera	34°49′12″S, 70°56′03″W	449	Letelier L. & A. Valderrama	10/17/2011	201104	1	Initial testing
8	Maule Region, Astillero	35°21′48″S, 71°15′48″W	303	Letelier L. & A. Valderrama	08/25/2011	201102	14	Determining genetic properties
9	Maule Region, Cuesta Batuco	35°17′46″S, 71°58′14″W	267	Letelier L. & A. Valderrama	08/11/2011	201101	14	Determining genetic properties
10	Bío-Bío Region, San Fabián de Alico	36°30′52″S, 71°37′37″W	450	Letelier L. & A. Valderrama	03/20/2012	201202	1	Initial testing
11	Bío-Bío Region, Santa Bárbara	37°36′37″S, 72°07′42″W	200	Letelier L. & A. Valderrama	03/18/2012	201201	1	Initial testing

Note: *n* = number of individuals.

[a] State (region) and locality in Chile, the order of localities follows the north–south orientation of the states and within each state the sites are listed in alphabetical order.

[b] Datums: World Geodetic System 1984 (WGS84).