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Primer Note

Characterization of microsatellites for the endangered *Ruta oreojasme* (Rutaceae) and cross-amplification in related species[1]

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- *Premise of the study: Ruta oreojasme* is an endangered species endemic to Gran Canaria (Canary Islands, Spain), where it occurs in small populations with disjunct distribution. Nothing is known about the genetic structure of these populations.
- *Methods and Results:* Using a microsatellite-enriched library method, 10 microsatellite markers have been developed from *R. oreojasme*, all of which showed polymorphism. The transferability of the 10 markers was tested in two other Canarian endemic species, *R. microcarpa* and *R. pinnata*, as well as in the widespread species *R. montana*.
- *Conclusions:* Our results demonstrate the value of these newly developed microsatellite markers to investigate the genetic structure in *R. oreojasme* and show their potential applicability for population genetic studies in other *Ruta* species.

Key words: genetic diversity; microsatellites; *Ruta oreojasme*; Rutaceae.

The genus *Ruta* L. (Rutaceae) includes nine species of perennial shrubs with three species endemic to the Canary Islands: *R. oreojasme* Webb. & Berthel. in Gran Canaria, *R. pinnata* L. in La Palma and Tenerife, and *R. microcarpa* Svent. in La Gomera. The three Canarian endemics have a monophyletic origin, *R. montana* Mill. being the sister species of the Canarian group (Salvo et al., 2010). All Canarian species are characterized by small population size and a highly fragmented distribution. Both *R. oreojasme* and *R. microcarpa* are listed in the Red List of Spanish vascular flora (Moreno, 2008) as endangered and critically endangered, respectively.

Gaining a better understanding of the genetic structure of the three Canarian endemics is fundamental for preserving these species. Within a conservation genetics project, microsatellite markers were newly developed for *R. oreojasme* to assess the genetic structure of populations of this species. The applicability of the markers has also been tested for the other two Canarian endemics and for the closely related *R. montana*.

METHODS AND RESULTS

Voucher specimens were deposited at the herbarium of the University of Zurich (Z) and at the herbarium of the Botanical Garden of Gran Canaria (LPA). Total genomic DNA was isolated from dried leaves using the DNeasy Plant Mini Kit (QIAGEN, Hombrechtikon, Switzerland) according to the manufacturer's protocol. Because the plants generated very viscous cell lysate, minor modifications were applied to the protocol to optimize results. Modifications included an increase in volume of buffer AP1 (from 400 to 600 μL), buffer AP2 (from 130 to 200 μL), and RNase A (from 4 to 6 μL), as well as a longer incubation time with buffer AP1 (at 15 min) for cell lysis.

DNA isolated from one specimen of *R. oreojasme* (voucher no. Z-85501) was used by Genetic Marker Services (Brighton, United Kingdom; http://www.geneticmarkerservices.com) to develop an enriched library and to design and test microsatellite primer pairs. Enrichment involved incubating adapter-ligated restricted DNA with filter-bonded synthetic repeat motifs, $(AG)_{17}$, $(AC)_{17}$, $(AAC)_{10}$, $(CCG)_{10}$, $(CTG)_{10}$, and $(AAT)_{10}$. Eighteen positive library colonies were selected for sequencing, from which 11 microsatellites were designed and tested. The primer sets were designed using the primer design software Primer3 (Rozen and Skaletsky, 2000), and were developed to amplify products ranging from 100 to 300 bp to help minimize later overlap ambiguities during multiloading genotyping projects. Each primer pair was tested for specificity, polymorphism, and cross-species amplification on high-resolution agarose gels before continuing to fluorescent-labeled genotyping. Screening of microsatellites was carried out using eight individuals of *R. oreojasme* from three different populations. PCRs were performed in 25 μL and contained approximately 50 ng of DNA, 5 pmol of each unlabeled primer, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 1× PCR buffer, and 0.5 U of SupraTherm DNA Polymerase (GeneCraft, Cologne, Germany). All PCRs were carried out under the following conditions: 60 s denaturation at 95°C, followed by 25 cycles of 95°C for 60 s, annealing at 55°C for 60 s and 72°C for 60 s, and a final extension at 72°C for 5 min. Ten microsatellite markers were easily amplified. The markers were then thoroughly screened by amplification with fluorescent-labeled primers and separation of PCR products by capillary electrophoresis. The screening was carried out using 30 individuals of *R. oreojasme* from three different populations: Arteara (27°50′36.7″N, 15°34′02″W; voucher no. Z-85501; 10 individuals), Montaña La Gorra (27°49′18.2″N, 15°36′07.6″W; voucher no. LPA-28995; 10 individuals), and Barranco de Las Palmas (27°50′47.3″N, 15°30′39.1″W; voucher no. LPA-29007; 10 individuals). Amplifications were performed following the two-step method described by Schuelke (2000). PCR was carried out in a final volume of 25 μL using the following components: approximately 20 ng of genomic DNA, 2.5 μL of 10× reaction buffer, 0.5 μL of each dNTP (10 mM), 1 μL of $MgCl_2$ (50 mM), 0.2 μL of the forward primer with M13(–21) tail at the 5′ end (10 μM), 0.5 μL of the reverse primer (10 μM), 0.5 μL of the fluorescently labeled M13(–21) primer (FAM, NED, VIC, PET; 10 μM), and 0.1 μL of *Taq* DNA polymerase (5 U/μL; Bioline GmbH, Luckenwalde, Germany). PCRs were performed in a T1 Thermocycler (Biometra GmbH, Goettingen, Germany) under the following conditions: initial denaturation at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, at the annealing temperature for 45 s (see Table 1), and 72°C for 1 min. The incorporation

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Table 1. Characteristics of 10 microsatellite markers developed in *Ruta oreojasme*.

Locus	GenBank accession no.	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Size range (bp)	Label[a]
RO57	JX187409	F: CACGTCCAATGTTAATGAAGC R: TCGAGGAAATGGAGTGAAGA	$(GA)_{12}$	55	195–210	FAM
RO59	JX187411	F: GTACAACTACAAAGCCAAAGC R: GACAAGATGAGACTATGAAAGAGC	$(GA)_{10}$	55	171–187	FAM
RO62	JX187414	F: TGAACAGAGGGCTCGTTTCT R: CGACATGTATATATTAATTACCTTACC	$(CT)_{12}$	55	134–188	FAM
RO66	JX187418	F: TTCTGCTTCTGATGTTACCCCTA R: CAAGAAACATGGGCGAACTC	$(CT)_{15}$	54	174–204	NED
RO70	JX187422	F: GGTTGAAGGAGGAACAGGTG R: TGCCTACACATGCAAGGATT	$(CT)_{12}$	55	140–188	VIC
RO71	JX187423	F: CCTCTAATCATTTACTGATCATCACC R: AGTCTGGTGGCATCTCCAAC	$(GA)_{16}$	55	113–149	NED
RO72	JX187424	F: GTAGCCGCCGTTTTGAGACT R: TTTCTCTCCCCTGTGCAAGT	$(TGT)_{9}$	55	227–246	PET
RO77	JX187429	F: GGGAGGCCAGAAGATGTAGA R: TCACGATCACCACCCAATAA	$(CA)_{9}$	55	159–165	PET
RO78	JX187430	F: TGAGCATAGGAGCCAGGTGT R: GACAAAAACAGAGACGCGGTA	$(GT)_{29}$	55	257–261	NED
RO79	JX187431	F: GTGCGGATTCTTTTCCCTAA R: GAGCAAACGGCTACAATACC	$(GA)_{14}$	54	217–241	PET

Note: T_a = annealing temperature when run individually.
[a] Fluorescent label attached to reverse end of primer.

of the fluorescently labeled M13(–21) primer occurred in the following eight cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 1 min, followed by a final extension step of 72°C for 5 min. Up to four PCR products of different primer sets were pooled for each individual and separated by capillary electrophoresis on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, California, USA). Alleles were sized against the internal size standard GeneScan LIZ500 (Applied Biosystems) and scored using GeneMapper software version 4.0 (Applied Biosystems). The observed allele size of all individuals genotyped was reduced by 18 bp to account for the M13(–21) universal sequence tag (Table 1). All 10 microsatellites showed a clear amplification pattern after capillary electrophoresis. Even though *R. oreojasme* is a tetraploid species (Stace et al., 1993), only up to two alleles were detected for individuals in all populations and for all loci, suggesting that a diploidization had occurred in their genome. For this reason, the species was treated as diploid for statistical analyses. The program GENEPOP 4.0 (Rousset, 2008) was used to calculate the observed and expected heterozygosity at each locus in the three populations (Table 2). The total number of observed alleles per locus per population ranged from 1 (locus RO78 in populations Montaña La Gorra and Barranco de Las Palmas) to 11 (locus RO62 in populations Montaña La Gorra and Barranco de Las Palmas, locus RO66 in population Montaña La Gorra; Table 2). Observed heterozygosity per locus ranged from 0.00 (locus RO78 in populations Montaña La Gorra and Barranco de Las Palmas) to 0.91 (locus RO78 in population Montaña La Gorra; Table 2). Gene diversity, inferred from Nei's heterozygosity (H_e), ranged from 0.00 (locus RO78 in populations Montaña La Gorra and Barranco de Las Palmas) to 0.92 (locus RO78 in population Montaña La Gorra; Table 2). Preliminary analyses suggest that these loci reveal high levels of genetic diversity in the three studied populations of *R. oreojasme*.

Cross-species amplification was tested in three related taxa using 15 individuals per species (*R. microcarpa*, voucher no. Z-85504; *R. pinnata*, voucher no. Z-85503; *R. montana*, voucher no. Renaux349-Z). All markers except for RO78 amplified *R. microcarpa* and *R. pinnata* samples; eight markers showed polymorphism in *R. microcarpa*, and seven markers showed polymorphism in *R. pinnata* (no data on polymorphism are available for locus RO66 in *R. pinnata*). Seven markers amplified *R. montana* samples, no data on polymorphism are available for this species. Results on cross-amplification tests are shown in Table 3.

Table 2. Results of primer screening in three populations of *Ruta oreojasme*.[a]

	Arteara pop. (N = 10)			Montaña La Gorra pop. (N = 10)			Barranco de Las Palmas pop. (N = 10)		
Locus	A	H_o	H_e	A	H_o	H_e[b]	A	H_o	H_e[b]
RO57	4	0.44	0.40	4	0.27	0.26	2	0.22	0.21
RO59	3	0.67	0.51	5	0.82	0.65	4	0.40	0.49
RO62	7	0.78	0.85	11	0.82	0.86	11	0.60	0.92**
RO66	8	0.56	0.81	11	0.91	0.92	7	0.60	0.85*
RO70	5	0.56	0.76	5	0.55	0.64	6	0.70	0.68
RO71	7	0.78	0.83	7	0.82	0.85	9	0.70	0.90
RO72	6	0.78	0.86	3	0.36	0.60**	5	0.70	0.79
RO77	4	0.67	0.77	3	0.55	0.57	2	0.40	0.33
RO78	3	0.22	0.22	1	0.00	0.00	1	0.00	0.00
RO79	6	0.78	0.69	9	0.73	0.89	8	0.60	0.89**

Note: A = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity.
[a] Locus RO65 showed an unclear amplification pattern, and thus was not included in these analyses.
[b] Statistically significant departure from Hardy–Weinberg equilibrium is indicated with $^*P < 0.05$ and $^{**}P < 0.01$, respectively.

CONCLUSIONS

The present investigation developed and tested 10 new unique polymorphic dinucleotide and trinucleotide nuclear DNA microsatellite loci. These markers represent useful tools for studying genetic diversity and population structure of *R. oreojasme*, *R. microcarpa*, *R. pinnata*, and *R. montana*. Information on the distribution of genetic variation in these species will contribute to their management and conservation as well as to the study of their history and evolution.

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Table 3. The cross-amplification of 10 microsatellite markers developed for *Ruta oreojasme* in three closely related species of the same genus.

Species	RO57	RO59	RO62	RO66	RO70	RO71	RO72	RO77	RO78	RO79
R. microcarpa	P	P	P	P	P	P	M	P	–	P
R. pinnata	P	P	P	+	P	P	M	P	–	P
R. montana	–	–	+	+	+	+	+	+	–	+

Note: + = successful amplification but no information on whether the locus is polymorphic or not; – = failed amplification; M = monomorphic locus; P = polymorphic locus.

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