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 Canary 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}, (CGG)_{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 Skaltsky, 2000), and were developed to amplify products ranging from 100 to 300 bp to help minimize later overlap ambiguities during multilocus 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′36.76″ N, 15°34′02″ W; voucher no. Z-85501; 10 individuals), Montaña La Gorra (27°49′18.22″ N, 15°36′07.67″ 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 of

---

1 Manuscript received 12 July 2012; revision accepted 3 October 2012. The authors thank the Swiss National Science Foundation (SNSF) for financial support, and Juli Caujapé Castells, Moisés Soto-Medina, and Águedo Marrero for help in field collection.

2 Institute of Systematic Botany, University of Zurich, Zollikerstrasse 107, 8008 Zurich, Switzerland; and 3 Department of Environmental Systems Science, ETH Zurich, Universitätstrasse 6, 8006 Zurich, Switzerland.

3 Institute of Systematic Botany, University of Zurich, Zollikerstrasse 107, 8008 Zurich, Switzerland; and 4 Department of Environmental Systems Science, ETH Zurich, Universitätstrasse 6, 8006 Zurich, Switzerland.

4 Author for correspondence: marilena.meloni@systbot.uzh.ch

doi:10.3732/apps.1200347
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). The observed allele size of all individuals genotyped was reduced (Applied Biosystems) and scored using GeneMapper software version 4.0 (Applied Biosystems). 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 (Hs), 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. Renau349-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.

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.

LITERATURE CITED

MORENO, J. C. [ed.]. 2008. Lista Roja 2008 de la flora vascular española. Dirección General de Medio Natural y Política Forestal (Ministerio de Medio Ambiente, y Medio Rural y Marino, y Sociedad Española de Biología de la Conservación de Plantas), Madrid, Spain.

ROUSSET, F. 2008. GENEPOP’007: A complete re-implementation of the GENEPOP software for Windows and Linux. Molecular Ecology Resources 8: 103–106.
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.

ROZEN, S., AND H. SKALETSKY. 2000. Primer3 on the WWW for general users and for biologist programmers In S. Krawetz and S. Misener [eds.], Methods in molecular biology, vol. 132: Bioinformatics methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.

SALVO, G., S. Y. W. HO, G. ROSEBAUM, R. REE, AND E. CONTI. 2010. Tracing the temporal and spatial origins of island endemics in the Mediterranean region: A case study from the *Citrus* family (*Ruta* L., Rutaceae). *Systematic Biology* 59: 705–722.

SCHUELKE, M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* 18: 233–234.

STACE, H. M., J. A. ARMSTRONG, AND S. H. JAMES. 1993. Cytoevolutionary pattern in Rutaceae. *Plant Systematics and Evolution* 187: 1–28.