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Applications in Plant Sciences

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Primer Note

Characterization of nuclear microsatellite markers for *Rumex bucephalophorus* (Polygonaceae) using 454 sequencing[1]

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- *Premise of the study:* Nuclear microsatellite markers were developed in *Rumex bucephalophorus* subsp. *canariensis* (Polygonaceae) to investigate its genetic diversity and structure.
- *Methods and Results:* Sixteen polymorphic microsatellite markers were obtained using 454 next-generation sequencing with di-, tri-, and tetranucleotide repeats. The average number of alleles was 5.688 and 3.813 for *R. bucephalophorus* subsp. *canariensis* var. *canariensis* and var. *fruticescens*, respectively. Slightly higher levels of mean genetic diversity were found in var. *canariensis* (expected heterozygosity = 0.600) than in var. *fruticescens* (expected heterozygosity = 0.514). Cross-amplifications in related taxa within *R. bucephalophorus* showed good amplification and polymorphic patterns.
- *Conclusions:* These 16 novel nuclear microsatellite markers are the first in the genus *Rumex* and may serve as valuable tools to carry out studies on genetic diversity and structure as well as progeny studies.

Key words: heterocarpy; Macaronesian; Mediterranean; Polygonaceae; *Rumex bucephalophorus*; simple sequence repeat (SSR).

The genus *Rumex* L. (Polygonaceae) includes nearly 200 species mostly distributed in both Europe and North America with an intricate taxonomy (Talavera et al., 2011). *Rumex bucephalophorus* L. is a Mediterranean-Macaronesian species with mostly annual, hermaphrodite or gynomonoecious, self-incompatible, and anemophilous populations. It shows an enormous heterocarpic diversity, in which up to four different diaspore types have been described (Talavera et al., 2011). Indeed, based on diaspore morphology, the most recent systematic treatment encompassed its variability into four subspecies (Press, 1988): *R. bucephalophorus* subsp. *bucephalophorus* (heterocarpic with larger diaspores than in other taxa and with two to three pairs of wide teeth per valve); subsp. *canariensis* (Steinh.) Rech. f. (homocarpic with entire valves or having four to eight pairs of straight teeth); subsp. *gallicus* (Steinh.) Rech. f. (heterocarpic with both entire and toothed valves); and subsp. *hispanicus* (Steinh.) Rech. f. (homocarpic with four to six pairs of uncinated teeth per valve). Additionally, lower taxonomic entities were proposed in two subspecies to describe perennial and suffrutescent populations (subsp. *canariensis* var. *fruticescens* (Bornm.) Press) or plants with basal fruits (subsp. *gallicus* var. *subaegaeus* Maire; Talavera et al., 2011). However, previous molecular attempts based on amplified fragment length polymorphism (AFLP) and internal transcribed spacer (ITS) markers failed at delimiting these taxa (Talavera et al., 2011). Concretely, two subspecies (subsp. *gallicus* and subsp. *hispanicus*) are not clearly distinguished. We have characterized 16 nuclear microsatellite loci from *R. bucephalophorus* subsp. *canariensis* and analyzed their transferability into closely related taxa, with the aim of assessing population genetic diversity levels and genetic structure, and delimiting systematic taxa.

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METHODS AND RESULTS

Total DNA was extracted from silica gel–dried leaves using the Invisorb Spin Plant Mini Kit (Invitek, Berlin, Germany) from one individual of *R. bucephalophorus* subsp. *canariensis* (Appendix 1). Size-selected microsatellite enrichment was performed following a Dynabeads-based protocol (Glenn and Schable, 2005) that has been successfully applied in other plants (Sánchez-Robles et al., 2012; Jiménez-López et al., 2015). Genomic DNA was digested with *Rsa*I and *Bst*UI (New England Biolabs, Ipswich, Massachusetts, USA) and enriched for $(AC)_{12}$, $(AG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$, $(AAAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(AATC)_6$, $(ACAG)_6$, $(ACTG)_6$, $(AAAC)_6$, $(AATG)_6$, $(AGAT)_8$, $(AACT)_8$, $(AAGT)_8$, $(AAAT)_8$, and $(ACAT)_8$. Fragments were sequenced on a 454 Genome Sequencer FLX System (454 Life Sciences, a Roche Company, Branford, Connecticut, USA) at the Savannah River Ecology Laboratory (Aiken, South Carolina, USA; see Abdelkrim et al., 2009). The 454 sequencing reads were assembled into contigs using CAP3 at 98% sequence identity and a minimum overlap of 75 bp (Huang and Madan, 1999). Microsatellite repeat arrays were found in 3173 contigs (2206 di-, 416 tri-, and 551 tetranucleotides), from which 451 contained enough flanking sequences to design primers (191 di-, 117 tri-, and 143 tetranucleotides). Primers were designed with 5′-tails (CAG or M13R; Boutin-Ganache et al., 2001; Glenn and Schable, 2005) and 5′-PIG-tail (GTTT) to the second primer to promote adenylation (Brownstein et al., 1996). We discarded pairs of primers with high self- and pair product complementary parameters, inadequate product size (upper limit 400 bp), or melting temperature difference higher than 1°C, and obtained 157 optimal primers (72 di-, 24 tri-, and 61 tetranucleotides). We tested up to 34 pairs of primers until we obtained a set of 16 polymorphic loci.

PCRs were optimized for each locus under the following conditions (Table 1): an initial denaturation step of 4 min at 95°C; followed by 35 cycles of 95°C for 30 s, 49–55°C for 30 s, and 72°C for 60 s, or by a touchdown procedure of

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TABLE 1. Characteristics of 16 microsatellite loci developed in *Rumex bucephalophorus* subsp. *canariensis*.

Locus	Primer sequences (5′–3′)[a]	Repeat motif	T_a (°C)	Allele size range (bp)	GenBank accession no.
441	Fm: GGAAACAGCTATGACCATCACTCGACATCACTCCAATC R: **GTTT**AGCGAATGATGAAGAATGAC	$(AG)_9$	TD 49.5	181–193	KT351066
510	Fm: GGAAACAGCTATGACCATAATGAAGGAAATGACGACTG R: **GTTT**AGGTCAGTTCGATTGTGTTG	$(AC)_9$	52	127–181	KT351068
679	Fm: GGAAACAGCTATGACCATACTCACACTCACGCATGTC R: **GTTT**GTTGATCCTGTCGAATGTTG	$(ACT)_7$	49	148–202	KT351064
1087	F: **GTTT**ACCGTCCGATAACAATCTAG Rm: GGAAACAGCTATGACCATCTATAAGTATCGTGCGTGTTG	$(ACTC)_6$	52	196–204	KT351056
1322	F: **GTTT**ATTTACATGGTGCGAGAAAG Rm: GGAAACAGCTATGACCATCACAATGCATGACGTTTAAC	$(AG)_{10}$	55	301–403	KT351065
1327	F: **G**TTTCCACGAGTTGTAACACAC Rm: GGAAACAGCTATGACCATCTATAAGTATCGTGCGTGTTG	$(AC)_{12}$	52	88–153	KT351067
1648	Fc: CAGTCGGGCGTCATCAAATTCGAGCAACAGTAAG R: **GTTT**GGCACCTTAGACACATTCAC	$(AAAC)_7$	52	176–204	KT351061
1806	Fm: GGAAACAGCTATGACCATCCCACACTCTTCTGTCATG R: **GTT**TAGAGGCTGCATACAACAAG	$(AAC)_{15}$	55	264–294	KT351053
1959	F: **GTTT**GAGTCACAGCCCAAGATTAG Rc: CAGTCGGGCGTCATCAGGTGGTCGAGAAAGTAGTG	$(AAC)_7$	55	304–340	KT351055
1965	F: **GTTT**GGCGAGTTATTCCTCTTATG Rm: GGAAACAGCTATGACCATAATCCAAGACTTCTGCAATC	$(AAAC)_7$	52	160–180	KT351062
2031	Fm: GGAAACAGCTATGACCATCTATAAGTATCGTGCGTGTTG R: **GTTT**AACTCTATATGTTTGCCTCCTC	$(ACTC)_{15}$	52	124–128	KT351057
BHZNV	Fm: GGAAACAGCTATGACCATTCCCTCCACCTAACAAAG R: **GTTT**ATCACACGCCGTTAAATATC	$(ACAT)_8$	TD 49.5	124–128	KT351060
CUN6O	Fm: GGAAACAGCTATGACCATCCACCCTACAAATCATGAG R: **GTTT**ACCTTCATAATCTGCCAAAG	$(AAC)_9$	55	165–201	KT351054
D8UYR	Fm: GGAAACAGCTATGACCATTTAATCCCTTGCATTCTTC R: **GTT**TACCCAAGAGAGCTGGTTAG	$(AAAG)_7$	52	160–196	KT351058
D90ZA	F: **GTT**TCTGATTACACCTTCGGAAC Rc: CAGTCGGGCGTCATCATTTGTCTAAGCCGTTCAAG	$(AAAG)_8$	49	119–263	KT351063
EJ5P7	F: **GTTT**ATGAGGTTCCTACGTTGTTG Rm: GGAAACAGCTATGACCATACGTACCGATGAAGAAGTTG	$(ACAT)_8$	TD 49.5	188–216	KT351059

Note: T_a = annealing temperature; TD = touchdown PCR procedure.
[a] m = modified primer with underlined M13R motif; c = modified primer with underlined CAG motif. PIG-tail primers are set boldface.

21 cycles of 95°C for 20 s, 60°C for 20 s (decreased 0.5°C per cycle), and 72°C for 30 s; followed by 21 cycles of 95°C for 20 s, 49.5°C for 30 s, and 72°C for 30 s; and in both cases a final step of 10 min at 72°C. PCR reactions, for a total volume of 25 µL, contained 2.5 µL of PCR Buffer 10×, 1 µL of $MgCl_2$ (25 mM), 1 µL of dNTP (10 mM), 0.2 µL of KAPA Taq DNA polymerase (5 U/µL) (Kapa Biosystems, Wilmington, Massachusetts, USA), 1 µL of primer

TABLE 2. Results of initial primer screening in two populations of *Rumex bucephalophorus* subsp. *canariensis*.[a]

	var. *canariensis* (N = 22)						var. *fruticescens* (N = 11)					
Locus	A	H_o	H_e	PIC	r	F_{IS}[b]	A	H_o	H_e	PIC	r	F_{IS}[b]
441	4	0.682	0.739	0.671	0.016	0.079**	6	0.455	0.823	0.673	0.247	0.459^{ns}
510	5	0.636	0.547	0.484	–0.122	–0.169^{ns}	3	0.909	0.610	0.508	–0.2510	–0.527^{ns}
679	5	0.476	0.592	0.529	0.112	0.200^{ns}	3	0.200	0.279	0.247	0.246	0.294^{ns}
1087	2	1.000	0.519	0.375	–0.333	–1.000**	2	1.000	0.556	0.375	—	–1.000^{ns}
1322	12	0.409	0.818	0.782	0.309	0.506**	7	0.364	0.866	0.803	0.393	0.592**
1327	4	0.773	0.697	0.580	0.133	–0.112**	7	0.818	0.831	0.701	—	0.016**
1648	7	0.546	0.650	0.607	0.083	0.164^{ns}	1	0.000	0.000	0.000	—	—
1806	7	0.500	0.554	0.520	0.043	0.099^{ns}	8	0.727	0.866	0.805	0.070	0.167*
1959	8	0.500	0.801	0.752	0.231	0.382*	5	0.455	0.801	0.726	0.245	0.444*
1965	6	0.550	0.603	0.532	0.058	0.089^{ns}	2	0.250	0.250	0.195	—	0.000^{ns}
2031	2	0.136	0.130	0.119	–0.027	–0.050^{ns}	1	0.000	0.000	0.000	—	—
BHZNV	2	0.227	0.206	0.181	–0.055	–0.105^{ns}	1	0.000	0.000	0.000	—	—
CUN60	6	0.381	0.767	0.711	0.320	0.509*	2	0.000	0.173	0.152	0.888	1.000^{ns}
D8UYR	5	0.364	0.328	0.306	–0.091	–0.113^{ns}	4	0.818	0.680	0.582	–0.123	–0.216*
D90ZA	9	0.500	0.852	0.813	0.251	0.419**	6	0.636	0.801	0.693	0.045	0.213^{ns}
EJ5P7	7	0.818	0.795	0.745	–0.024	–0.030^{ns}	3	0.636	0.688	0.583	0.016	0.079^{ns}
Mean	5.688	0.531	0.600	0.544	0.057	0.054	3.813	0.454	0.514	0.440	0.178	0.117
SD	2.701	0.218	0.218	0.210	0.172	0.360	2.373	0.351	0.338	0.299	0.315	0.506

Note: A = number of alleles; F_{IS} = inbreeding within populations; H_e = unbiased expected heterozygosity; H_o = observed heterozygosity; PIC = polymorphic information content; r = null allele frequency.
[a] See Appendix 1 for voucher specimens and locality information.
[b] Significant departure from Hardy–Weinberg equilibrium: *P < 0.05, **P < 0.01; ns = not significant.

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TABLE 3. Cross-amplification and transferability results of 16 microsatellite loci in closely related taxa of *Rumex bucephalophorus*.[a]

	subsp. *bucephalophorus* ($N = 15$)		subsp. *hispanicus* ($N = 14$)		subsp. *gallicus* var. *gallicus* ($N = 13$)		subsp. *gallicus* var. *subaegaeus* ($N = 12$)	
Locus	*A*	Allele size range (bp)	*A*	Allele size range (bp)	*A*	Allele size range (bp)	*A*	Allele size range (bp)
441	10	153–207	3	183–195	5	181–189	4	185–193
510	2	127–129	2	127–129	2	127–129	2	127–129
679	5	184–196	0	—	3	184–196	2	184–196
1087	7	184–196	0	—	0	—	0	—
1322	7	251–449	12	303–423	8	299–403	11	333–519
1327	3	85–112	2	99–112	2	85–99	0	—
1648	3	176–192	1	180	3	176–196	3	180–192
1806	6	276–288	4	279–291	7	264–291	2	279–282
1959	7	310–331	6	313–343	5	313–343	11	306–355
1965	5	168–208	0	—	6	160–180	2	172–176
2031	2	128–130	1	128	1	128	1	128
BHZNV	3	124–132	1	124	1	124	1	124
CUN6O	11	153–201	5	177–192	2	192–198	3	186–195
D8UYR	2	164–166	3	156–166	4	160–168	3	156–166
D90ZA	8	119–185	2	127–129	5	121–129	5	125–263
EJ5P7	5	184–200	4	184–200	4	184–196	3	184–196

[a] See Appendix 1 for voucher specimens and locality information.

with 5′-GTTT tail (10 μM), 0.3 μL of primer with 5′-CAG or M13R tail (10 μM), and 0.5 μL of CAG or M13R with FAM, NED, PET, or VIC fluorescent label (10 μM). Sixteen primer pairs correctly amplified with the expected size and were run on an ABI 3730 automated sequencer (Applied Biosystems, Foster City, California, USA) using LIZ 500 as the internal lane size standard. Fragment lengths were assigned to allelic classes with GeneMarker 1.71 software (SoftGenetics, State College, Pennsylvania, USA).

Genotypic data were obtained for two populations of *R. bucephalophorus* subsp. *canariensis*, including the population chosen for the microsatellite enrichment (var. *canariensis*) and a second population of var. *fruticescens* (Appendix 1). Genetic diversity indexes (number of alleles [*A*], observed and unbiased expected heterozygosities [H_o and H_e], and polymorphic information content [PIC]) and null allele frequencies (*r*) were calculated in GENETIX 4.05 (Belkhir et al., 2004) and CERVUS 3.0.7 (Marshall et al., 1998; Kalinowski et al., 2007). Deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were calculated with GENEPOP version 4.0 (Rousset, 2008) using 1000 permutations.

All of the 16 simple sequence repeat (SSR) loci amplified in both varieties of *R. bucephalophorus* subsp. *canariensis*, although the var. *fruticescens* population showed three monomorphic loci. None of the 120 pairwise comparisons showed significant LD ($P < 0.5$) for both populations after Bonferroni correction. Considering the polymorphic loci, the number of alleles per locus ranged from two to 12 in var. *canariensis* and from two to eight in var. *fruticescens*, with means of 5.688 and 3.813, respectively. Without considering locus 1087 with alleles fixed in all individuals (see Table 2), H_o, H_e, and PIC per locus ranged from 0.227 to 0.818, from 0.130 to 0.852, and from 0.119 to 0.813 in var. *canariensis* and from 0.000 to 0.909, from 0.173 to 0.866, and from 0.152 to 0.805 in var. *fruticescens*, respectively. Mean genetic diversity values were slightly higher in var. *canariensis* than in var. *fruticescens* (Table 2). Both populations showed significant deviations from HWE (Table 2) that could be explained through subpopulation structuring or by the presence of null alleles. The null allele frequencies varied from 0.016 to 0.393 in those loci that significantly deviated from HWE (Table 2).

Cross-amplifications of these 16 loci into other *R. bucephalophorus* taxa (Appendix 1) were completely successful in subsp. *bucephalophorus*, whereas amplifications failed in two loci in subsp. *gallicus* and in three loci in subsp. *hispanicus* (Table 3). The 16 SSR loci were polymorphic in subsp. *bucephalophorus*, with two to 11 alleles per locus. However, in subsp. *gallicus* and subsp. *hispanicus*, two and three loci were monomorphic, and considering the remaining polymorphic markers, alleles per locus ranged from two to 11 and two to 12, respectively (Table 3).

CONCLUSIONS

Sixteen SSR loci have been characterized in *R. bucephalophorus* subsp. *canariensis*. Transferability of these loci into coinfraspecific taxa was successful in both amplification and polymorphism patterns. These are the first SSR markers described for the genus *Rumex*, and may constitute a remarkable tool for population genetic studies of related taxa. Concretely, these markers may also be valuable in studies of the mating system in heterocarpic taxa and in unravelling the systematics of the *R. bucephalophorus* complex.

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Appendix 1. Voucher and location information for *Rumex bucephalophorus* populations used in this study. All vouchers were deposited in the herbarium of the Universidad de Sevilla (SEV), Seville, Spain.

Voucher no.	Taxon	Locality	Geographic coordinates	Altitude (m a.s.l.)
SEV279249–279254	*R. bucephalophorus* subsp. *bucephalophorus*	Greece: Crete, Genari	38°21′66″N, 24°20′32″E	1
SEV249375–249376	*R. bucephalophorus* subsp. *canariensis* var. *canariensis*	Spain: Tenerife, Icod de los Vinos	28°21′31″N, 16°42′47″W	447
SEV285642	*R. bucephalophorus* subsp. *canariensis* var. *fruticescens*	Portugal: Madeira, between Poiso and Pico de Arieiro	32°43′58.31″N, 16°55′47.46″W	1500
SEV275565–275567	*R. bucephalophorus* subsp. *hispanicus*	Portugal: Azores, Terceira, Porto Judeu	38°38′53″N, 27°08′36″W	50
SEV285914	*R. bucephalophorus* subsp. *gallicus* var. *gallicus*	France: Les Mayons, Réserve Naturelle Plaine des Maures	43°20′32.34″N, 6°23′46.58″E	95
SEV285913	*R. bucephalophorus* subsp. *gallicus* var. *subaegaeus*	Spain: Huelva, Almonte, Doñana	37°7′48.44″N, 6°31′30.34″W	12

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