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Authors: You, Jianling, Liu, Wensheng, Zhao, Yao, Zhu, Yongqing, Zhang, Wenju, et al.

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PRIMER NOTE

MICROSATELLITE MARKERS IN *RHODIOLA* (CRASSULACEAE), A MEDICINAL HERB GENUS WIDELY USED IN TRADITIONAL CHINESE MEDICINE[1]

JIANLING YOU[2], WENSHENG LIU[2], YAO ZHAO[2], YONGQING ZHU[2], WENJU ZHANG[2], YUGUO WANG[2], FAN LU[2], AND ZHIPING SONG[2,3]

[2]Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering, Institute of Biodiversity Science, Fudan University, Shanghai 200433, People's Republic of China

- *Premise of the study:* Microsatellite loci are described for *Rhodiola*, a medicinal herb genus widely used in traditional Chinese medicine.
- *Methods and Results:* A total of 17 polymorphic microsatellite primer pairs were developed using the combined biotin capture method. The number of alleles per locus ranged from one to 12 across 192 individuals from *R. bupleuroides*, *R. crenulata*, *R. fastigiata*, and *R. sacra*, and the mean observed and expected heterozygosities ranged from 0.177 to 0.412 and from 0.363 to 0.578, respectively.
- *Conclusions:* The results demonstrate the potential use of this new set of microsatellite markers for genotyping individuals and estimating genetic diversity in *Rhodiola*.

Key words: Crassulaceae; medicinal plants; microsatellites; *Rhodiola bupleuroides*; *Rhodiola crenulata*; *Rhodiola fastigiata*; *Rhodiola sacra*.

Rhodiola L. (Crassulaceae) comprises 90 species of perennial herbaceous plants with succulent leaves distributed worldwide (Fu and Ohba, 2001). A total of 73 species of this genus are found in China, and are especially common in the Qinghai–Tibet Plateau (Fu and Ohba, 2001). Many species of *Rhodiola* have been widely used for medicinal purposes in Tibet and other regions for more than 1000 years (Zhao et al., 1998). However, the high demand of *Rhodiola* for medicinal uses has led to overexploitation of many wild populations of this genus, driving them close to local extinction in the wild. For instance, *R. crenulata* (Hook. f. & Thomson) H. Ohba, a perennial herbaceous plant mainly distributed in Tibet and widely used to reinforce immunity, improve memory, and relieve altitude sickness (Lei et al., 2006), is now considered endangered in this and neighboring regions due to overexploitation (Zhao et al., 2011). Despite the important medicinal uses of *Rhodiola* spp., little is known about their genetic background. However, such information is an indispensable prerequisite for conservation and management of such economically important plant species. Here, we isolated and characterized 17 polymorphic microsatellites for population genetic studies to infer the genetic diversity and differentiation among populations, which will provide new insights into *Rhodiola* reproductive strategies in the alpine regions and support the development of conservation plans.

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[3]Author for correspondence: songzp@fudan.edu.cn

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METHODS AND RESULTS

Four species of *Rhodiola* (*R. bupleuroides* (Wall. ex Hook. f. & Thomson) S. H. Fu, *R. crenulata*, *R. fastigiata* (Hook. f. & Thomson) S. H. Fu, and *R. sacra* (Prain ex Raym.-Hamet) S. H. Fu) were collected from the Qinghai–Tibet Plateau, China. Genomic DNA from four individuals of two populations per species were isolated and then pooled to construct microsatellite libraries. Voucher information for the sampled populations is provided in Table 1. DNA was extracted from dry leaves using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987), digested by *Rsa*I and *Xmn*I (New England Biolabs, Beijing, China), and then ligated with Super SNX24 adapters (5′-GTTAAGGCCTAGCTAGCTAGCAGAATC-3′ and 5′-pGATTCTGCTAGCTAGGCCTTAACAAAA-3′) (Sangon Biotech, Shanghai, China) by T4 DNA ligase (TaKaRa Biotechnology Co., Dalian, Liaoning, China) (Glenn and Schable, 2005). Digested, adapter-ligated DNA fragments containing potential microsatellite loci were hybridized to 5′-biotinylated oligonucleotides $(CCG)_6$, $(AAG)_8$, $(AGG)_6$, $(CT)_{13}$, $(AGC)_6$, $(AC)_{10}$, and $(ATC)_6$, and microsatellite-rich hybridized genomic fragments were recovered by capture with streptavidin-conjugated magnetic Dynabeads (Invitrogen, Carlsbad, California, USA). Captured molecules were amplified with SNX24 adapter-specific primers, ligated into pMD18-T, and transformed into the competent *Escherichia coli* DH5α host cells (TIANGEN Biotech, Beijing, China). Positive clones were identified by PCR amplification with M13 primers (Sangong Biotech, Shanghai, China). The PCR reactions were performed in a Biometra thermocycler (Biometra, Goettingen, Germany) with a denaturation period of 4 min at 94°C; followed by 30 cycles of 1 min at 94°C, 45 s at 55°C, and 40 s at 72°C; and a final extension of 10 min at 72°C. Reactions were carried out in a volume of 20 μL containing 9.6 μL double-distilled water, 2 μL 10× *Taq* reaction buffer, 2.4 μL dNTP, 2.4 μL Mg^{2+}, 1.2 μL M13 forward primers, 1.2 μL M13 reverse primers, 0.2 μL 0.5 U/μL *Taq* DNA polymerase (Aidlab Biotechnologies Co. Ltd., Beijing, China), and 1 μL template DNA. A total of 243 positive clones were sequenced on an ABI 377XL DNA sequencer (Applied Biosystems, Foster City, California, USA), and 145 (59.7%) were found to contain simple sequence repeats (SSRs). DNA sequence alignments and primer design were performed using Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA).

Primer pairs were synthesized for 66 microsatellite sequences containing a repeat region of 20–24 bases and initially screened using four samples from each *Rhodiola* species. After PCR optimization, including gradient PCR for testing

TABLE 1. Genetic diversity in eight *Rhodiola* populations based on 17 microsatellite loci.[a]

	R. crenulata								*R. sacra*								*R. fastigiata*								*R. bupleuroides*							
	RC1 (N = 24)				RC2 (N = 24)				RS1 (N = 24)				RS2 (N = 24)				RF1 (N = 24)				RF2 (N = 24)				RB1 (N = 24)				RB2 (N = 24)			
Locus	A	H_o	H_e	F	A	H_o	H_e	F	A	H_o	H_e	F	A	H_o	H_e	F	A	H_o	H_e	F	A	H_o	H_e	F	A	H_o	H_e	F	A	H_o	H_e	F
Rs1	3	0.875	0.518*	−0.688	4	0.429	0.480*	0.106	3	0.042	0.119*	0.65	2	0.043	0.043	−0.022	1	—	—	—	4	0	0.238*	1	5	0	0.361*	1	2	0	0.080*	1
Rs2	7	0.75	0.747	−0.003	5	0.708	0.699*	−0.014	5	0.208	0.262	0.205	5	0.217	0.493	0.559	4	1	0.576*	−0.735	4	0.609	0.472	−0.291	5	0.208	0.194	−0.071	6	0.5	0.587*	0.148
Rs3	8	0.304	0.737*	0.587	9	0.111	0.867*	0.872	3	0	0.156*	1	3	0.083	0.594*	0.86	3	0.208	0.536*	0.611	8	0.208	0.609*	0.658	11	0.042	0.829*	0.95	10	0.167	0.847*	0.803
Rs4	10	0.833	0.757*	−0.101	12	0.667	0.822	0.189	5	0.333	0.359*	0.07	5	0.875	0.683*	−0.281	5	0.75	0.635*	−0.182	7	0.917	0.752*	−0.219	12	0.292	0.661*	0.559	5	0.208	0.497*	0.581
Rs5	2	0	0.091*	1	1	—	—	—	2	0	0.5	1	6	0.111	0.753*	0.852	7	0.571	0.709*	0.194	5	0.3	0.665*	0.549	3	0.333	0.542*	0.385	3	0	0.500*	1
Rs6	4	0.1	0.685*	0.854	8	0.1	0.804*	0.876	3	0.043	0.124*	0.649	8	0.059	0.839*	0.93	4	0.05	0.414*	0.879	5	0.87	0.681*	−0.276	11	0.125	0.891*	0.86	6	0	0.813*	1
Rs7	3	0.5	0.546	0.084	4	0.167	0.594*	0.719	3	0.042	0.119*	0.65	4	0.478	0.585*	0.183	3	0.083	0.081	−0.032	4	0.417	0.677*	0.385	2	0.125	0.249	0.498	3	0.333	0.601*	0.445
Rs8	6	0.091	0.645*	0.859	9	0.333	0.734*	0.546	4	0	0.358*	1	9	0.25	0.593*	0.578	7	0.167	0.444*	0.624	8	0.304	0.660*	0.539	8	0.227	0.826*	0.725	5	0.083	0.298*	0.72
Rs9	2	0	0.278	1	1	—	—	—	1	—	—	—	2	0	0.153*	1	4	0	0.691*	1	2	0	0.198*	1	4	0	0.475*	1	3	0	0.480*	1
Rs10	7	0.333	0.479	0.304	3	0.087	0.084	−0.034	8	0.458	0.484	0.052	8	0.75	0.753*	0.003	5	0.833	0.633*	−0.317	6	0.792	0.678	−0.168	5	0.696	0.513	−0.355	11	0.583	0.803*	0.274
Rs11	9	0.75	0.828	0.094	9	0.583	0.849*	0.313	8	0.542	0.804*	0.326	9	0.917	0.661*	−0.386	9	0.458	0.857*	0.465	7	0.167	0.578*	0.712	7	0.208	0.658*	0.683	5	0.042	0.296*	0.859
Rs12	3	0.167	0.352*	0.526	4	0.208	0.381*	0.453	3	0.125	0.442*	0.717	4	0.542	0.61	0.112	8	0.25	0.505*	0.505	9	0.083	0.566*	0.853	5	0.292	0.464	0.371	8	0.208	0.679*	0.693
Rs13	4	0.125	0.289	0.568	9	0.429	0.705*	0.392	9	0.75	0.746	−0.006	4	0.792	0.553*	−0.432	9	0.667	0.780*	0.146	9	0.455	0.689*	0.34	4	0.087	0.306*	0.716	12	0.391	0.758*	0.484
Rs14	2	0.083	0.08	−0.043	7	0.417	0.772*	0.46	5	0.333	0.580*	0.425	4	0.708	0.644	−0.1	4	0.667	0.568	−0.174	7	0.5	0.71	0.296	4	0.458	0.607	0.245	8	0.458	0.713*	0.357
Rs15	2	0.042	0.041	−0.021	3	0.125	0.414*	0.698	2	0.042	0.041	−0.021	2	0.458	0.353	−0.297	3	0.792	0.588*	−0.347	3	0.083	0.258*	0.677	3	0.167	0.258	0.354	4	0.13	0.271*	0.519
Rs16	3	0.087	0.162	0.462	5	0.045	0.650*	0.93	3	0	0.405*	1	3	0.435	0.553	0.214	4	0.25	0.659*	0.62	4	0.111	0.539*	0.794	3	0.05	0.141*	0.646	3	0.095	0.177*	0.462
Rs17	7	0.048	0.804*	0.941	7	0.05	0.786*	0.936	7	0.095	0.680*	0.86	4	0	0.525*	1	8	0.25	0.723*	0.654	8	0.364	0.851*	0.573	7	0.111	0.821*	0.865	12	0.545	0.893	0.389
Mean	4.824	0.299	0.473	0.378	5.882	0.262	0.567	0.438	4.353	0.177	0.363	0.505	4.824	0.395	0.552	0.281	5.176	0.412	0.553	0.23	5.882	0.363	0.578	0.437	5.824	0.201	0.517	0.555	6.235	0.22	0.547	0.631

Note: A = mean number of alleles per locus; F = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; N = sample size.

[a]Vouchers of the sampled populations were deposited in Fudan University Herbarium (accessions: *Z. P. Song et al. RF1-2*, *Z. P. Song et al. RF2-1*, *Z. P. Song et al. RB1-7*, *Z. P. Song et al. RB2-3*, *Z. P. Song et al. RS1-3*, *Z. P. Song et al. RS2-6*, *Z. P. Song et al. RC1-2*, *Z. P. Song et al. RC2-1*).

*Indicates that H_o departed significantly from H_e under Hardy–Weinberg equilibrium (HWE) according to sequential Bonferroni testing.

TABLE 2. Characteristics of 17 microsatellite primers developed in *Rhodiola*.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Allele size (bp)	GenBank accession no.
Rs1	F: TTGGGCGGATTGTTCCTGTT	$(TGA)_3$	55	181–223	JQ857096
	R: TCCACCCTTCATCTCCTTCA				
Rs2	F: GCACGATGACAATTTATACGA	$(TCC)_4$	55	134–224	JQ857092
	R: GGTTAGGGTTTGGAGGTGAC				
Rs3	F: TTCCAAATAAGCCAATCCTC	$(CAA)_4$	55	183–288	JQ857095
	R: TGTGCAACTGTAACCATCGA				
Rs4	F: ACCCTTCATCTTGTCCCTCA	$(CT)_8$	55	119–150	JQ857089
	R: CACCCTTTTCTGTCCCACTC				
Rs5	F: GGAGGAAGAACTTCCCATTT	$(CCT)_4$	55	163–239	JQ857082
	R: GTGGTGGTGATTGCTTTGAT				
Rs6	F: GAGTCAGGTGGTGGAGAATG	$(AGG)_4$	55	157–262	JQ857094
	R: CAAAAGATTAGAAACTCAAAACG				
Rs7	F: TTGTGGACTTTGTGGGACTC	$(GTT)_5$	57.8	262–277	JQ857087
	R: TGGATAAATTGCTGCCTGAC				
Rs8	F: CTGACGCTGAAGCAGTTGAT	$(TGC)_5$	57.8	131–206	JQ857085
	R: CCCAATGGAGGACCGATGAT				
Rs9	F: CTTCATCATTTACATCTTGCTC	$(CCT)_6$	55	177–204	JQ857084
	R: TTTTGTTACTTGACTGTGGC				
Rs10	F: TGCGTCAAACGGATCAAACC	$(CAG)_4$	55	117–186	JQ857088
	R: TCGCTCAGCCCCTTCTCAAT				
Rs11	F: GTTGTTGCTTAGGCTGCTGT	$(GCT)_4$	55	265–313	JQ857097
	R: AACTTCTATGGAAATGTGGC				
Rs12	F: AAAAGACAGTATAGCCTCACC	$(TCA)_4$	55	112–148	JQ857091
	R: TGTAGACTGATGCTGCTGAT				
Rs13	F: GAATAAGGTGGCTGGAGGTT	$(GGA)_5$	55	156–234	JQ857089
	R: GATGAGGGACAAGATGAAGG				
Rs14	F: CAGAAGCGGATTCCTCATCA	$(AGG)_4$	55	140–200	JQ857083
	R: CGAATCACCCGTAACCCTAA				
Rs15	F: CCACAGAAGCGAGTCAGGTT	$(GCATCA)_5$	55	146–164	JQ857090
	R: GTCCCGGCAAATACAAAAGT				
Rs16	F: AACAAGGCAGAGTCGAGAAA	$(GCA)_4$	55	116–173	JQ857086
	R: CATCTTTGAACCCTAATCCA				
Rs17	F: ATTCTTCATCTCAGCCGTCC	$(GA)_8$	55	126–310	JQ857093
	R: CACAGCCATTAGAGCCAACT				

Note: T_a = optimal annealing temperature.

annealing temperature and changing the proportion of reagents, 17 (25.8%) of these loci generated stable and clear bands with independent annealing temperatures (Table 2). The 17 loci were then tested with 192 DNA samples from two populations per *Rhodiola* species (Table 1). The optimization amplifications were performed in a final volume of 10 µL, including ~20 ng genomic DNA, 6.5 µL double-distilled water, 1 µL 10× *Taq* reaction buffer, 0.8 µL dNTP, 0.8 µL Mg^{2+}, 0.25 µL forward primers, 0.25 µL reverse primers, and 0.05 µL 0.5 U/µL *Taq* DNA polymerase. A Biometra thermocycler was used with the following cycling conditions: 94°C for 5 min; 35 cycles at 94°C for 40 s, 55–57.8°C (marker dependent, see Table 1) for 30 s, and 72°C for 40 s; and a final elongation step of 72°C for 10 min. The PCR products were separated on a capillary electrophoresis genotyper (Majorbio Bio-pharm, Shanghai, China). The separated SSR fragments were examined and scored using GeneMapper version 3.7 (Applied Biosystems). Standard population genetics metrics were calculated using GenAlEx 6.4 (Peakall and Smouse, 2006).

Across all populations of the four *Rhodiola* species, the number of alleles per polymorphic locus (*A*) ranged from one to 12 (Table 1). For the polymorphic loci, average values of observed (H_o) and expected heterozygosity (H_e) varied from 0.177 to 0.412 and from 0.363 to 0.578, respectively. The fixation index (*F*) was highly variable among loci in each population (Table 1); averaged across loci for each species, it ranged from 0.230 to 0.631, which is consistent with a mixed mating system in *Rhodiola*. Among the 17 analyzed loci, eight to 16 loci exhibited significant deviation from Hardy–Weinberg equilibrium based on a sequential Bonferroni test (Table 1); this may reflect the presence of undetected null alleles or departure from equilibrium conditions of an ideal population.

CONCLUSIONS

Seventeen polymorphic microsatellite loci are characterized in eight *Rhodiola* populations. The high degree of polymorphism in these microsatellite markers gives them great potential for use in genetic studies of wild populations of this genus. These studies may increase understanding of the biology of *Rhodiola* and help to develop conservation strategies.

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