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# Microsatellite markers in Rhodiola (Crassulaceae), A MEDICINAL HERB GENUS WIDELY USED IN TRADITIONAL Chinese medicine ${ }^{1}$ 

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- 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 QinghaiTibet 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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## 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 RsaI and XmnI (New England Biolabs, Beijing, China), and then ligated with Super SNX24 adapters ( $5^{\prime}$-GTTAAGGCC-TAGCTAGCTAGCAGAATC-3' and $5^{\prime}$-pGATTCTGCTAGCTAGGCCTTAAC-AAAA-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 $5^{\prime}$-biotinylated oligonucleotides $(\mathrm{CCG})_{6},(\mathrm{AAG})_{8},(\mathrm{AGG})_{6},(\mathrm{CT})_{13}$, $(\mathrm{AGC})_{6},(\mathrm{AC})_{10}$, 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 DH5 $\alpha$ 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 $94^{\circ} \mathrm{C}$; followed by 30 cycles of 1 min at $94^{\circ} \mathrm{C}, 45 \mathrm{~s}$ at $55^{\circ} \mathrm{C}$, and 40 s at $72^{\circ} \mathrm{C}$; and a final extension of 10 min at $72^{\circ} \mathrm{C}$. Reactions were carried out in a volume of $20 \mu \mathrm{~L}$ containing $9.6 \mu \mathrm{~L}$ double-distilled water, $2 \mu \mathrm{~L} 10 \times$ Taq reaction buffer, $2.4 \mu \mathrm{~L} \mathrm{dNTP}, 2.4 \mu \mathrm{~L} \mathrm{Mg}{ }^{2+}, 1.2 \mu \mathrm{~L}$ M13 forward primers, 1.2 $\mu \mathrm{L}$ M13 reverse primers, $0.2 \mu \mathrm{~L} 0.5 \mathrm{U} / \mu \mathrm{L}$ Taq DNA polymerase (Aidlab Biotechnologies Co. Ltd., Beijing, China), and $1 \mu \mathrm{~L}$ 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. ${ }^{\text {a }}$

| Locus | R. crenulata |  |  |  |  |  |  |  |  | R. sacra |  |  |  |  |  |  |  | R. fastigiata |  |  |  |  |  |  |  | R. bupleuroides |  |  |  |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | $\mathrm{RC1}(N=24)$ |  |  |  | RC2 ( $N=24$ ) |  |  |  |  | RS1 $(N=24)$ |  |  |  | RS2 ( $N=24$ ) |  |  |  | RF1 ( $N=24$ ) |  |  |  | RF2 ( $N=24$ ) |  |  |  | RB1 $(N=24)$ |  |  |  | RB2 ( $N=24$ ) |  |  |  |
|  | A | $H_{0}$ | ${ }_{\text {o }} H_{\text {e }}$ | $F$ | A | A | $H_{\text {o }}$ | $\mathrm{H}_{\text {e }}$ | $F$ | A | $H_{\text {o }}$ | $\mathrm{H}_{\text {e }}$ | $F$ | A | $H_{\text {o }}$ | $H_{\text {e }}$ | $F$ | A | $H_{\text {o }}$ | $H_{\text {e }}$ | $F$ | A | $H_{\text {o }}$ | $H_{\text {e }}$ | $F$ | A | $H_{\text {o }}$ | $H_{\text {e }}$ | $F$ | A | $H_{0}$ | $H_{\text {e }}$ | $F$ |
| Rs1 | 3 | 0.875 | 75 0.518* | -0.688 | 4 |  | 0.429 | 0.480* | 0.106 | 3 | 0.042 | 20.119* | 0.65 | 2 | 0.043 | 30.043 | -0.022 | 1 | - |  | - 4 | 4 | 0 | 0.238* | * | 5 | 0 | 0.361* | 1 | 2 | 0 | 0.080* | , |
| Rs2 | 7 | 0.75 | 750.747 | -0.003 | 5 |  | 0.708 | 0.699* | -0.014 | 5 | 0.208 | 8.262 | 0.205 |  | 0.217 | 70.493 | 0.559 | 4 | 1 | 0.576* | -0.735 |  | 0.609 | 0.472 | -0.291 | 5 | 0.208 | 80.194 | -0.071 | 6 | 0.5 | 0.587* | 0.148 |
| Rs3 | 8 | 0.304 | 0.737* | 0.587 | 9 |  | 0.111 | $10^{0.867 *}$ | 0.872 | 3 | 0 | 0.156* | 1 | 3 | 0.083 | 3 0.594* | 0.86 | 3 | 0.208 | 0.536* | 0.611 |  | 0.208 | 0.609* | * 0.658 | 11 | 0.042 | 2 0.829* | 0.95 | 10 | 0.167 | 0.847* | * 0.803 |
| Rs4 | 10 | 0.833 | 33 0.757* | -0.101 |  |  | 0.667 | 0.822 | 0.189 | 5 | 0.333 | 3 0.359* | 0.07 | 5 | 0.875 | 5 0.683* | -0.281 | 5 | 0.75 | 0.635* | -0.182 |  | 0.917 | 0.752* | -0.219 | 12 | 0.292 | $20.661 *$ | 0.559 | 5 | 0.208 | 0.497* | * 0.581 |
| Rs5 | 2 | 0 | 0.091* | 1 | 1 |  | - | - | - | 2 | 0 | 0.5 | 1 | 6 | 0.111 | 1 0.753* | 0.852 | 7 | 0.571 | 0.709* | 0.194 |  | 0.3 | 0.665* | 0.549 | 3 | 0.333 | 3 0.542* | 0.385 | 3 | 0 | 0.500* |  |
| Rs6 | 4 | 0.1 | 0.685* | 0.854 | 8 |  | 0.1 | 0.804* | 0.876 | 3 | 0.043 | 3 0.124* | 0.649 |  | 0.059 | 9 0.839* | 0.93 | 4 | 0.05 | 0.414* | 0.879 |  | 0.87 | 0.681* | *-0.276 | 11 | 0.125 | 5 0.891* | 0.86 | 6 | 0 | 0.813* |  |
| Rs7 | 3 | 0.5 | 0.546 | 0.084 | 4 |  | 0.167 | 0.594* | 0.719 | 3 | 0.042 | 2 0.119* | 0.65 | 4 | 0.478 | 8 0.585* | 0.183 | 3 | 0.083 | 0.081 | -0.032 |  | 0.417 | 0.677* | 0.385 | 2 | 0.125 | 50.249 | 0.498 | 3 | 0.333 | 0.601* | 0.445 |
| Rs8 | 6 | 0.091 | 1 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 | 7 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* | , | 4 | 0 | 0.475* | 1 | 3 | 0 | 0.480* |  |
| Rs 10 | 7 | 0.333 | 3330.479 | 0.304 | 3 |  | 0.087 | 0.084 | -0.034 | 8 | 0.458 | 80.484 | 0.052 | 8 | 0.75 | 0.753* | 0.003 | 5 | 0.833 | 3 0.633* | -0.317 | 6 | 0.792 | 0.678 | -0.168 | 5 | 0.696 | 60.513 | -0.355 1 |  | 0.583 | 0.803* | 0.274 |
| Rs11 | 9 | 0.75 | 50.828 | 0.094 | 9 |  | 0.583 | 0.849* | 0.313 | 8 | 0.542 | 2 0.804* | 0.326 |  | 0.917 | 7 0.661* | -0.386 | 9 | 0.458 | 0.857* | 0.465 |  | 0.167 | 0.578* | 0.712 | 7 | 0.208 | 8 0.658* | 0.683 | 5 | 0.042 | 0.296* | 0.859 |
| Rs 12 | 3 | 0.167 | 67 0.352* | 0.526 | 4 |  | 0.208 | 0.381* | 0.453 | 3 | 0.125 | 5 0.442* | 0.717 |  | 0.542 | 20.61 | 0.112 | 8 | 0.25 | 0.505* | 0.505 |  | 0.083 | 0.566* | * 0.853 | 5 | 0.292 | 20.464 | 0.371 | 8 | 0.208 | 0.679* | * 0.693 |
| Rs 13 | 4 | 0.125 | 250.289 | 0.568 | 9 |  | 0.429 | 0.705* | 0.392 | 9 | 0.75 | 0.746 | -0.006 |  | 0.792 | 2 0.553* | -0.432 | 9 | 0.667 | 0.780* | 0.146 |  | 0.455 | 0.689* | 0.34 | 4 | 0.087 | 7 0.306* | 0.7161 |  | 0.391 | 0.758* | * 0.484 |
| Rs 14 | 2 | 0.083 | 083 0.08 | -0.043 | 7 |  | 0.417 | 7 0.772* | 0.46 | 5 | 0.333 | 0.580* | 0.425 |  | 0.708 | 80.644 | -0.1 | 4 | 0.667 | 0.568 | -0.174 |  | 0.5 | 0.71 | 0.296 | 4 | 0.458 | 80.607 | 0.245 | 8 | 0.458 | 0.713* | * 0.357 |
| Rs 15 | 2 | 0.042 | 20 0.041 | -0.021 | 3 |  | 0.125 | 5 0.414* | 0.698 | 2 | 0.042 | 20.041 | -0.021 |  | 0.458 | 80.353 | -0.297 | 3 | 0.792 | 0.588* | -0.347 |  | 0.083 | 0.258* | * 0.677 | 3 | 0.167 | 70.258 | 0.354 | 4 | 0.13 | 0.271* | * 0.519 |
| Rs 16 | 3 | 0.087 | 870.162 | 0.462 | 5 |  | 0.045 | 0.650* | 0.93 | 3 | 0 | 0.405* |  | 3 | 0.435 | 50.553 | 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 | 48 0.804* | 0.941 | 7 |  | 0.05 | 0.786* | 0.936 | 7 | 0.095 | 5 0.680* | 0.86 | 4 | 0 | 0.525* | - 1 | 8 | 0.25 | 0.723* | 0.654 |  | 0.364 | 0.851* | * 0.573 | 7 | 0.111 | $10.821^{*}$ | 0.865 |  | 0.545 | 0.893 | 0.389 |
| Mean | 4.824 | 0.299 | 990.473 | 0.378 |  | . 88 | 20.262 | 2.567 | 0.438 | 4.353 | 0.177 | 70.363 | 0.505 | 4.824 | 40.395 | 50.552 | 0.281 | 5.176 | 0.412 | 0.553 | 0.23 | 5.882 | 0.363 | 0.578 | 0.437 |  | 240.201 | 10.517 | 0.555 | 6.23 | 50.22 | 0.547 | 0.631 |

[^1]Table 2. Characteristics of 17 microsatellite primers developed in Rhodiola.

| Locus | Primer sequences ( $5^{\prime}-3^{\prime}$ ) | Repeat motif | $T_{\mathrm{a}}\left({ }^{\circ} \mathrm{C}\right)$ | Allele size (bp) | GenBank accession no. |
| :---: | :---: | :---: | :---: | :---: | :---: |
| Rs1 | F: TTGGGCGGATTGTTCCTGTT | $(\mathrm{TGA})_{3}$ | 55 | 181-223 | JQ857096 |
|  | R: TCCACCCTTCATCTCCTTCA |  |  |  |  |
| Rs2 | F : GCACGATGACAATTTATACGA | $(\mathrm{TCC})_{4}$ | 55 | 134-224 | JQ857092 |
|  | R: GGTTAGGGTTTGGAGGTGAC |  |  |  |  |
| Rs3 | F: TTCCAAATAAGCCAATCCTC | $(\mathrm{CAA})_{4}$ | 55 | 183-288 | JQ857095 |
|  | R: TGTGCAACTGTAACCATCGA |  |  |  |  |
| Rs4 | F: ACCCTTCATCTTGTCCCTCA | $(\mathrm{CT})_{8}$ | 55 | 119-150 | JQ857089 |
|  | R: CACCCTTTTCTGTCCCACTC |  |  |  |  |
| Rs5 | F: GGAGGAAGAACTTCCCATTT | $(\mathrm{CCT})_{4}$ | 55 | 163-239 | JQ857082 |
|  | R: GTGGTGGTGATTGCTTTGAT |  |  |  |  |
| Rs6 | F: GAGTCAGGTGGTGGAGAATG | $(\mathrm{AGG})_{4}$ | 55 | 157-262 | JQ857094 |
|  | R: CAAAAGATTAGAAACTCAAAACG |  |  |  |  |
| Rs7 | F: TTGTGGACTTTGTGGGACTC | $(\mathrm{GTT})_{5}$ | 57.8 | 262-277 | JQ857087 |
|  | R: TGGATAAATTGCTGCCTGAC |  |  |  |  |
| Rs8 | F: CTGACGCTGAAGCAGTTGAT | (TGC) ${ }_{5}$ | 57.8 | 131-206 | JQ857085 |
|  | R: CCCAATGGAGGACCGATGAT |  |  |  |  |
| Rs9 | F: CTTCATCATTTACATCTTGCTC | $(\mathrm{CCT})_{6}$ | 55 | 177-204 | JQ857084 |
|  | R: TTTTGTTACTTGACTGTGGC |  |  |  |  |
| Rs10 | F: TGCGTCAAACGGATCAAACC | $(\mathrm{CAG})_{4}$ | 55 | 117-186 | JQ857088 |
|  | R: TCGCTCAGCCCCTTCTCAAT |  |  |  |  |
| Rs11 | F: GTTGTTGCTTAGGCTGCTGT | $(\mathrm{GCT})_{4}$ | 55 | 265-313 | JQ857097 |
|  | R: AACTTCTATGGAAATGTGGC |  |  |  |  |
| Rs12 | F: AAAAGACAGTATAGCCTCACC | (TCA) ${ }_{4}$ | 55 | 112-148 | JQ857091 |
|  | R: TGTAGACTGATGCTGCTGAT |  |  |  |  |
| Rs13 | F: GAATAAGGTGGCTGGAGGTT | $(\mathrm{GGA})_{5}$ | 55 | 156-234 | JQ857089 |
|  | R: GATGAGGGACAAGATGAAGG |  |  |  |  |
| Rs14 | F: CAGAAGCGGATTCCTCATCA | $(\mathrm{AGG})_{4}$ | 55 | 140-200 | JQ857083 |
|  | R: CGAATCACCCGTAACCCTAA |  |  |  |  |
| Rs15 | F: CCACAGAAGCGAGTCAGGTT | $(\mathrm{GCATCA})_{5}$ | 55 | 146-164 | JQ857090 |
|  | R: GTCCCGGCAAATACAAAAGT |  |  |  |  |
| Rs16 | F: AACAAGGCAGAGTCGAGAAA | $(\mathrm{GCA})_{4}$ | 55 | 116-173 | JQ857086 |
|  | R: CATCTTTGAACCCTAATCCA |  |  |  |  |
| Rs17 | F: ATTCTTCATCTCAGCCGTCC | $(\mathrm{GA})_{8}$ | 55 | 126-310 | JQ857093 |
|  | R: CACAGCCATTAGAGCCAACT |  |  |  |  |

Note: $T_{\mathrm{a}}=$ 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 $10 \mu \mathrm{~L}$, including $\sim 20 \mathrm{ng}$ genomic $\mathrm{DNA}, 6.5 \mu \mathrm{~L}$ double-distilled water, $1 \mu \mathrm{~L} 10 \times$ Taq reaction buffer, $0.8 \mu \mathrm{~L}$ dNTP, $0.8 \mu \mathrm{~L} \mathrm{Mg}^{2+}$, $0.25 \mu \mathrm{~L}$ forward primers, $0.25 \mu \mathrm{~L}$ reverse primers, and $0.05 \mu \mathrm{~L} 0.5 \mathrm{U} / \mu \mathrm{L}$ Taq DNA polymerase. A Biometra thermocycler was used with the following cycling conditions: $94^{\circ} \mathrm{C}$ for $5 \mathrm{~min} ; 35$ cycles at $94^{\circ} \mathrm{C}$ for $40 \mathrm{~s}, 55-57.8^{\circ} \mathrm{C}$ (marker dependent, see Table 1) for 30 s , and $72^{\circ} \mathrm{C}$ for 40 s ; and a final elongation step of $72^{\circ} \mathrm{C}$ 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 $\left(H_{\mathrm{o}}\right)$ and expected heterozygosity $\left(H_{\mathrm{e}}\right)$ 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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[^1]:     et al. RS2-6, Z. P. Song et al. RC1-2, Z. P. Song et al. RC2-1).

    * Indicates that $H_{\mathrm{o}}$ departed significantly from $H_{\mathrm{e}}$ under Hardy-Weinberg equilibrium (HWE) according to sequential Bonferroni testing.

