Isolation and Characterization of Twenty-four Microsatellite Loci for *Rhododendron decorum* Franch. (Ericaceae)

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Abstract. *Rhododendron decorum* is a common species in southwest China and northeast Myanmar, in which the flowers have been eaten as a favorite vegetable. We isolated and characterized 24 microsatellite primer pairs from this species. The number of alleles ranged from two to seven. The observed and expected heterozygosities (H_O and H_E) were 0.3830 to 0.7855 and 0 to 0.7917, respectively. Eleven loci were significantly deviated from Hardy-Weinberg equilibrium as a result of the heterozygote deficiency. Cross-species amplification in another eight *Rhododendron* species showed their potential use for evolutionary and conservation studied in this genus. These markers will be useful to reveal the genetic population structure and genetic diversity of *R. decorum*.

Rhododendron L. is a large and diverse genus with a nearly worldwide distribution, and the center of diversity of the genus is in the Himalayas (Kron and Judd, 1990). This genus contains seven subgenus in all, including *Hymenanthus*, which has 23 subsections with ≈ 270 species (Fang et al., 2005), and all the species are diploids ($2n = 26$) (Min and Fang, 1990). Most species in subgenus *Hymenanthus* are world-famous ornamental plants as a result of the evergreen leaves and large and colorful flowers (Min, 1984). *Rhododendron decorum* belongs to subgenus *Hymenanthus*. It is a beautiful evergreen shrub or small tree, commonly distributed in southwest China and northeast Myanmar (Fang et al., 2005). Its flower is a favorite edible vegetable for local ethnic people in

southwest China (Yong and Chong, 1980). In common with other species of subgenus *Hymenanthus*, *R. decorum* is highly interfertile, which was supported by molecular evidence (Zha et al., 2008; Zhang et al., 2007). However, until now, little research has been conducted related to population genetics of *R. decorum*. To study more deeply the genetic diversity within this species and the effect of hybridization on the speciation process of this species, we developed 24 microsatellite markers for this species to investigate the genetic diversity and genetic structures among populations and provide a potential tool for studying molecular breeding in *R. decorum*.

Genomic DNA was extracted from leaf tissues using the cetyltrimethyl ammonium bromide method (Milligan, 1992). Approximately 300 ng genomic DNA was completely digested with *MseI* restriction enzyme (Fermentas). The digested DNA was ligated to the *MseI* adaptor pair (Vos et al., 1995), then 5 μ L of the adapter-ligated fragments acted as templates to perform polymerase chain reaction (PCR) in a volume of 20 μ L using *MseI*-N (5'-GAT GAG TCC TGA GTA AN-3') as the primer and following the program: 95 °C for 3 min, 30 cycles of 94 °C for 30 s, 53 °C for 60 s, 72 °C for 60 s, followed by 72 °C for 5 min.

For enrichment, the PCR products were denatured at 95 °C for 5 min, then hybridized with a 5'-biotinylated probe (AG) 15 in 250 μ L hybridization solution ($20 \times$ SSC, 10% SDS, 100 pmol/ μ L probe) at 48 °C for 2 h. The DNA hybridized to the probe was separated and captured by streptavidin-coated magnetic beads at room temperature for 20 min, followed by two washing steps, including three times in TEN_{100} for 15 min and three times in TEN_{1000} for 24 min. The separated single-stranded DNA was subjected to a second round of PCR according to the same procedure as the first round of PCR. The PCR products, after being purified with the E.Z.N.A Gel Extraction Kit (Omega Bio-Tek, Atlanta, GA), were ligated into PMD18-T vector (TaKaRa) according to the manufacturer's instructions and then transformed into *Escherichia coli* strain JM109 (Sangon, Shanghai). The positive clone was picked out by blue-white screening and tested by PCR using (AG) 10 and M13+/M13- as primers, respectively. One hundred ten of 292 screened clones contained potential microsatellite motifs.

A total of 50 clones were found to contain simple sequence repeats and then subjected to primer designing using the Primer 5.0 (Clarke and Gorley, 2001). Twenty-four individuals from two wild populations, two semicultivated and cultivated populations, were used to screen polymorphism. PCR reaction was done in 20 μ L volume using a PTC200 thermal cycler (MJ Research, Ashland). Each reaction was performed using 20 ng of genomic DNA, 1 μ M of each dNTP, 1 μ M each primer, $1 \times$ Taq buffer [100 mM Tris-HCl, pH 8.8, 2.0 mM $MgCl_2$, 200 mM $(NH_4)_2SO_4$, 0.1% Tween 20] and 1 U of Taq polymerase (TaKaRa). The PCR programs took place as follows: initial denaturing step at 95 °C for 5 min, 30 cycles of 94 °C for 30 s, primer-specific annealing temperature 55 to 62 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 8 min. The PCR products were electrophoresed in denaturing 6% polyacrylamide gels using a 25-bp DNA ladder molecular size standard (Fermentas) to estimate allele size by silver staining.

Of the 50 new primers designed for *R. decorum*, 32 successfully amplified the target regions and 24 of them displayed polymorphism. The number of alleles per locus, observed (H_O) and expected heterozygosity (H_E), and deviation from Hardy-Weinberg equilibrium (HWE) were assessed using GENEPOP Version 3.4 (<http://wbio.med.curtin.edu.au/genepop/>) (Raymond and Rousset, 1995). The number of alleles per locus ranged from two to seven with an average of 4.1 (Table 1). The observed and expected heterozygosities (H_O and H_E) ranged from 0.3830 to 0.7855 and from 0 to 0.7917 with averages of 0.6121 and 0.4531, respectively. Among the 24 microsatellite markers, 11 loci show significant deviation from HWE ($P < 0.01$, Table 1), probably as a result of deficiency of heterozygote or the limitation of sample size. Tests for linkage disequilibrium

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Table 1. Characteristics of 24 polymorphic microsatellite loci for *Rhododendron decorum*.

Locus	Repeat motif	Primer sequence (5'–3')	Size range (bp)	A	T _m (°C)	H _O	H _E	Hardy-Weinberg equilibrium (P value)	GeneBank accession no.
RDW1	(TC) ₉	R GAAGGTGATCGTGTGCGGAAT F GCCTCTAACTACTTGCTCCA	241–249	3	60	0.5044	0.3750	0.1925	FJ903451
RDW3	(TG) ₈ (TG) ₃	R CAGTTGTTATCATGCAGTTC F ACCCTTATGATTGGTATGTA	356–366	4	56	0.6800	0.3333	0.0005*	FJ903452
RDW4	(GA) ₉	R CGAACAGAACCAACCATAT F ACGAGAATAAGTTGGGAAAT	194–200	3	61	0.5027	0.5833	0.6547	FJ903453
RDW6	(GA) ₁₀	R AATGAAGCCCCCTAAAAGTAA F CTAGCAGTATGGTGGGTTGT	160–170	5	55	0.7349	0.5833	0.0004*	FJ903454
RDW8	(TC) ₁₀	R GAAAGAATCTCGCATGGACT F AATGCCTTTCACTTCTCACC	285–295	4	61	0.7243	0.3750	0.0000*	FJ903455
RDW11	(GA) ₉	R TGCCTCTAACTACTTGCTCC F AAGGTGATCGTGTGCGGAATA	240–250	4	56	0.5434	0.5417	0.1650	FJ903456
RDW13	(GA) ₇ (GA) ₅	R TTTTGTCTATCCCCATCACA F ACTTAAACACGACCATCAGC	185–200	2	61	0.4672	0.6250	0.1706	FJ903457
RDW14	(AG) ₁₀	R AACCAACAAAGCAGACCC F GTTGTAGATGCCGAGGAG	207–213	4	62	0.7340	0.5000	0.1130	FJ903458
RDW15	(CT) ₁₅	R GCATAACAACAGCAACACAG F GAATCGAAAATAAGCCTTGG	206–223	7	56	0.7855	0.6667	0.0066*	FJ903459
RDW16	(GA) ₉	R CACCAAGCATCATGCCTCTA F GGTGATCGTGTGCGGAATACA	249–255	4	61	0.5771	0.7500	0.4694	FJ903460
RDW17	(CT) ₁₄ (CA) ₆	R TGCCTCTGGAAGTAAACCTA F TTTTCTACTCCTCCCTTAT	236–243	4	58	0.7367	0.7083	0.5719	FJ903461
RDW18	(AG) ₈	R CATCTGCTGAATAATGTGGG F AGGGACTGAGGGAGTAACAA	270–276	4	60	0.4087	0.0417	0.0000*	FJ903462
RDW22	(AG) ₁₁ (GA) ₅	R CCTATTTTGAATTAGCCTTT F GACATTATTGTTGCTTTGGT	173–183	4	58	0.3927	0.2917	0.0460	FJ903463
RDW27	(GT) ₆ (GA) ₉	R TCTACAATTCATGGATGGC F TGTGAATGTGAGTGAGCGTG	184–190	4	60	0.6800	0.2917	0.0000*	FJ903464
RDW31	(GA) ₈	R TGCCTCTAACTACTTGCTCC F AAGGTGATCGTGTGCGGAATA	240–245	4	56	0.5842	0.7917	0.1598	FJ903465
RDW33	(GA) ₁₉	R GATTGGAAAGTTTGCTGTT F TGTGTTGCCATTCCTCTTCT	227–243	6	58	0.7677	0.5417	0.0453	FJ903466
RDW34	(AC) ₁₄ (GA) ₉ (GA) ₅	R GCAAGTGTACAAACAAAGC F CCACCATCATTACTCTTCCT	193–199	4	61	0.4238	0.2917	0.2135	FJ903467
RDW35	(TC) ₅ (CT) ₆ (ATA) ₃	R GTGACTTCGGATTCGTGGAG F TAAGGTTGGTGTAGCGTGTA	210–219	5	56	0.7234	0.5417	0.0919	FJ903468
RDW38	(TAGAG) ₄ (AG) ₇ (AGAGAT) ₃	R AACAGCGACGAGAAAAGC F GTGTTTGAATTTGTGCGGC	140–148	4	60	0.7234	0.4167	0.0007*	FJ903469
RDW39	(TC) ₁₅	R AGGAAATATGCTAGTCCACAA F CTTTGGGAAGATTTGATGG	194–200	4	58	0.6436	0.3333	0.0004*	FJ90370
RDW43	(CT) ₇	R AATGTGAGATATGCGTGGGT F GTTTGGTCTGTACAGAAGT	188–198	2	58	0.3830	0.0000	0.0000*	FJ903471
RDW44	(AGG) ₃ (AG) ₉	R CAAAACCCACTTGTAGAT F AGATCCGTATTTCTTGAGG	205–215	4	56	0.5833	0.6667	0.0662	FJ903472
RDW46	(CTT) ₃ (GA) ₁₁	R AGCAAGATAGAACTCTGTAAC F TCTCCAGAAGTACGCAAT	303–312	4	60	0.7074	0.4167	0.0008*	FJ903473
RDW51	(AG) ₁₁	R GGTAAGTTTGGGCGAGAAGT F TGAAAACCACTAGTAGTTATCC	168–180	5	60	0.6800	0.2083	0.0000*	FJ903474

T_m = annealing temperature of primer pair; A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity. Statistically significant deviation from Hardy-Weinberg expectation is indicated by the asterisk ($P < 0.01$).

were run in FSTAT Version 2.9.3.2 (Goudet, 1995). Significance levels were adjusted using sequential Bonferroni corrections (Rice, 1989). No loci showed significant linkage disequilibrium after Bonferroni correction. For cross-species application, these 24 new primer pairs were tested in the other eight important horticultural species and close taxa such as *R. irroratum*, *R. agastum*, *R. delavayi*, *R. araiophyllum*, *R. molle*, *R. simsii*, *R. pachypodu*, and *R. spinuliferum*. Twelve pairs of them can be amplified successfully in all species, whereas the RDW18 failed amplification in all species (Table 2). These polymorphic microsatellite loci presented here would provide a useful tool for studying the population genetic structure and genetic diversity of *R. decorum*, and it will also be valuable for studying other species in this group.

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Table 2. Cross-species amplification of 24 new microsatellite loci from *Rhododendron decorum* in other related *Rhododendron* species.

Locus (species)	<i>R. irroratum</i>	<i>R. agastum</i>	<i>R. delavayi</i>	<i>R. araiophyllum</i>	<i>R. molle</i>	<i>R. simsii</i>	<i>R. pachypodum</i>	<i>R. spinuliferum</i>
RDW1	+	+	+	+	+	+	+	+
RDW3	+	+	+	–	–	+	–	–
RDW4	+	–	+	+	–	+	–	+
RDW6	+	+	+	+	+	–	+	+
RDW8	+	–	–	–	+	–	+	–
RDW11	+	+	+	+	+	+	+	+
RDW13	+	–	+	–	–	–	–	+
RDW14	+	+	+	+	+	+	+	+
RDW15	+	+	+	+	+	+	–	+
RDW16	+	+	+	+	+	+	+	+
RDW17	+	+	+	+	+	+	+	+
RDW18	–	–	–	–	–	–	–	–
RDW22	+	+	–	+	–	–	–	–
RDW27	–	+	+	+	+	+	+	+
RDW31	+	+	+	+	+	+	+	+
RDW33	+	+	+	+	+	+	+	+
RDW34	–	–	+	–	–	–	–	–
RDW35	+	+	+	+	+	+	+	+
RDW38	+	+	+	+	+	+	+	+
RDW39	+	+	+	+	+	+	+	+
RDW43	+	+	+	–	+	+	–	–
RDW44	+	+	+	+	+	+	+	+
RDW46	+	+	+	+	+	+	+	+
RDW51	+	+	+	+	+	–	–	+

^aThe plus symbols (+) indicate successful polymerase chain reaction (PCR) amplification and the minus symbols (–) suggested failed PCR amplification.

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