

Development and Characterization of 15 Microsatellite Loci for *Rhododendron delavayi* Franch. (Ericaceae)

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Abstract. *Rhododendron delavayi* Franch. is an important ornamental plant and often plays a role in natural hybridization with other sympatric species in *Rhododendron* subgenus *Hymenanthes*. Fifteen microsatellite loci were developed and characterized in this species. The average allele number of these microsatellites was four per locus, ranging from three to six. The ranges of expected (H_E) and observed (H_O) heterozygosities were 0.0365 to 0.7091 and 0.0263 to 0.9512, respectively. Cross-species amplification in *R. agastum* and *R. decorum* showed that a subset of these markers holds promise for congeneric species study. These sets of markers are potentially useful to investigate the genetic structure and gene flow of *R. delavayi* and other congeneric species.

Rhododendron, renowned for its ornamental value, is one of the largest and most widespread woody plant genera with over 1000 species distributed from the northern temperate zone, throughout tropical southeast Asia to northeastern Australia (Chamberlain, 2003; Cox, 1994; Ng and Corlett, 2000). There are nine subgenera in *Rhododendron* (Fang et al., 2005), and many species have been introduced and cultivated for ornamental and landscape purposes. Over 1000 rhododendron cultivars have been bred or selected in the world, especially for species from *Rhododendron* subgenus *Hymenanthes* (Chamberlain, 1982). *Rhododendron delavayi* Franch. is an important ornamental tree species with red flowers; it belongs to *Rhododendron* subgenus *Hymenanthes*. It distributes from southwest China to southeast Asia, especially in the Himalayan region, and it is often involved in natural hybridization with other sympatric *Rhododendron* species

such as *R. agastum* Balf.f. & W.W.Sm., *R. irroratum* Franch., *R. cyanocarpum* Franch. & W.W.Sm., and *R. decorum* Franch. (Zha et al., 2008; Zhang et al., 2007). Actually, the true extent of hybridization is certainly much greater in areas where species boundaries appear incomplete (Milne et al., 1999). More recently, some natural hybrid individuals have been selected from *R. cyanocarpum* and *R. delavayi* (Zhang et al., unpublished data). We believe that selecting new hybrids from nature may be a good way toward sustainable use for *Rhododendron*.

Simple sequence repeats (SSRs; microsatellites) are the favored type of molecular marker for identifying plant germplasm (Dikshit et al., 2007). From a horticultural perspective, *R. delavayi* provides abundant genetic resources for breeding new cultivars. However, until now, little research has been conducted on this species. Therefore, it is urgent to investigate the genetic structure and gene flow of *R. delavayi*.

We sampled 34 *R. delavayi* individuals among five populations (two populations were from Yangbi and the remaining three from Kunming, Zhanyi, and Shizong), four *R. agastum* and *R. decorum* individuals among three populations, respectively (the three populations of *R. agastum* and *R. decorum* were from Zhanyi, Shizong, and Yangbi), across Yunnan province, southwest China. Genomic DNA of *R. delavayi* was used for the construction of all genomic libraries. In total, 42 *Rhododendron* individuals were used to detect SSR loci polymorphism and to test the transferability of microsatellite markers. A microsatellite enriched library was conducted by using a modified biotin–streptavidin capture method (Chen et al., 2008). Briefly, the genomic DNA (≈500 to 800 ng) was completely digested with *Mse*I restriction enzyme (NEB) and then the digested fragments were ligated to a *Mse*I amplified fragment length polymorphism adaptor followed by amplification with adaptor-specific primers (5'-GAT GAG TCC TGA GTA AN-3') (Huang et al., 2009). For enrichment of the fragments (≈300 to 800 bp) containing SSR, the polymerase chain reaction (PCR) products were hybridized to a mixture of biotinylated probes [(AAG)₁₀(AC)₁₅, (AG)₁₅] (Zane et al., 2002). The purified PCR products were ligated into PGEM-T vector (Promega, Beijing, China) and transformed into *Escherichia coli* strain DH5a (Tiangen, Beijing, China). The positive clones were picked out and tested using (AAG)₇/(AC)₁₀/(AG)₁₀ and SP6/T7 vector primers, respectively. A total of 462 clones were chosen for sequencing with an ABI PRISM 3730XL SEQUENCER (Shanghai, China). In all, 240 clones (52%) were found to contain microsatellite sequences. Finally, 90 pairs of SSR primers were selected for primer designing using Primer Premier 5.0 software (Premier, Canada) (Clarke and Gorley, 2001). Primer pairs were assessed in 34 wild *R. delavayi* individual samples from southeast to southwest Yunnan, China. Microsatellite loci were amplified in a final volume of a 15-μL reaction containing 7.5 μL 29 Taq PCR MasterMix [Tiangen; 0.1 U Taq polymerase/μL, 0.5 mM dNTP each, 20 mM Tris-HCl (pH 8.3), 100 mM KCl, 3 mM MgCl₂], 0.6 μM of each primer, and ≈50 ng genomic DNA. The amplification profiles included initial denaturation at 94 °C for 3 min followed by 35 to 40 cycles of 30 s at 94 °C, 30 s at 54 to 68 °C, and 1 min at 72 °C and then final extension

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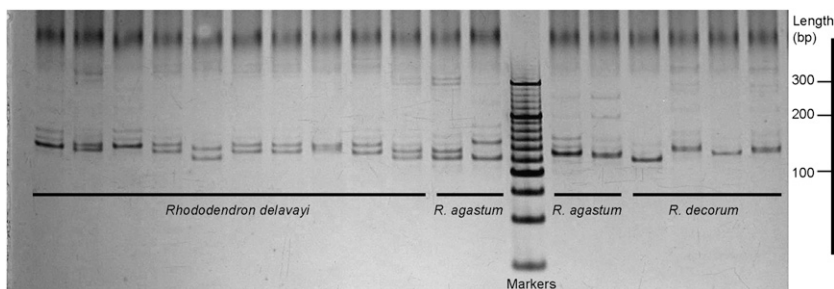


Fig. 1. The amplification profile with primer R-432.

at 72 °C for 7 min. The amplified products were then separated on 6% denaturing polyacrylamide gels and visualized by silver staining.

A 20-bp DNA ladder standard (Fermentas, Shenzhen, China) was used as the standard for scoring.

Cross-species amplification of *R. agastum* and *R. decorum* was further investigated using four wild individuals from three populations for each species (Table 1; Fig. 1). From the 22 tested markers, 15 pairs showed clear polymorphic PCR products across five populations of *R. delavayi* and six pairs in *R. decorum* and nine pairs in *R. agastum*, respectively (Table 1). The variability at each locus was measured in terms of number of alleles (A), observed (H_O), and expected heterozygosity (H_E) for the 15 microsatellite loci using GENEPOP Version 4.0 (Raymond and Rousset, 1995). The same software was used to test the deviations from Hardy-Weinberg equilibrium (HWE) and pairwise linkage disequilibrium. The number of alleles per locus (A) was three to six with an average of four; values for observed (H_O) and expected (H_E) heterozygosities ranged from 0.0263 to 0.9512 (averaged at 0.3122) and from 0.0365 to 0.7091 (averaged at 0.4032), respectively (Table 2). Seven loci (R-111, R-112, R-147, R-299, R-320, R-335, and R-544) deviated significantly from the HWE ($P < 0.01$). No significant linkage disequilibrium was detected between locus pairs except for three locus pairs: R-299 and R-544, R-166 and R-320, and R-111 and R-320. Altogether, these primers can provide a useful tool to investigate genetic relationships between these closely related *Rhododendron* species. They are also of great potential to study genetic structure, hybridization and evolution of *Rhododendron* species in this genus.

Table 1. Cross-species amplification of two congeneric species, *R. decorum* and *R. agastum*. weak amplification (W), monomorphic amplification (M), polymorphic amplification (P).

Locus	Primer sequence (5'-3')	<i>R. decorum</i>	<i>R. agastum</i>
R-111	F 5' AGCTTGAGCTTGGTGAG 3' R 5' TTGACAAGACAATGGGAG 3'	M	P
R-112	F 5' TACTGTTCTTTCTTCC 3' R 5' CTCCCACAATGTCTTCTC 3'	P	P
R-140	F 5' GAAGCCAGTGTGTGATT 3' R 5' CAGCCAACCTTGACCTAA 3'	W	M
R-147	F 5' TAATCATTACTTCTCCTAG 3' R 5' TCCTTTCTCGATCCCTCT 3'	W	P
R-163	F 5' CGTGAGAAATCGTCCAAA 3' R 5' TCCAAAAGGGGAGTCATT 3'	P	P
R-166	F 5' AACGAAACCCAGAAACT 3' R 5' TCATTTATCTCCACTCCC 3'	P	P
R-172	F 5' GCTGTCGTATTTACCTAA 3' R 5' AGATGAAGAAGTGCTGGA 3'	P	M
R-210	F 5' TTTCTGGCTCAAAGAGGG 3' R 5' ATTTGGCAGTGTCTTCTCA 3'	M	P
R-299	F 5' TACTGTGCTTAGACGCCATT 3' R 5' CCCTCTGCTGTA AAAACTG 3'	P	P
R-318	F 5' AGTCTTATCTTCGCTTCC 3' R 5' ACTATCAACTCGCAGGTC 3'	M	P
R-320	F 5' GCTGATACGGAGCTGGTG 3' R 5' GACGCAAATGGGTGAACT 3'	W	M
R-335	F 5' TCAGGCAACCGTAACAAC 3' R 5' AAACGCAACAAACAGCAA 3'	W	P
R-432	F 5' CCGTTTGAGTATCTTCCC 3' R 5' CTGGTCCATTCTCCAAGTA 3'	P	P
R-544	F 5' TCTGGACTCAAGCAACA 3' R 5' AATGGAGTAAATGGGGTG 3'	P	M
R-557	F 5' CGAAACTCAGAACCTCCG 3' R 5' TTCCGAACTCCTTACCAG 3'	P	P

Table 2. Characteristics of 15 microsatellite loci developed for *Rhododendron delavayi*.

Locus	Primer sequence (5'-3')	T_a (°C)	Expected size (bp)	Observed range (bp)	A	H_O	H_E	Repeat motif	Accession number
R-111	F5' AGCTTGAGCTTGGTGAG 3' R5' TTGACAAGACAATGGGAG 3'	59	125	104–141	5	0.1219*	0.5643	(CT) ₇	GU338310
R-112	F5' TACTGTTCTTTCTTCC 3' R5' CTCCCACAATGTCTTCTC 3'	58	164	143–186	6	0.2105*	0.7091	(AG) ₁₅	GU338311
R-140	F5' GAAGCCAGTGTGTGATT 3' R5' CAGCCAACCTTGACCTAA 3'	64	98	90–110	3	0.1717	0.1543	(AG) ₆	GU338297
R-147	F5' TAATCATTACTTCTCCTAG 3' R5' TCCTTTCTCGATCCCTCT 3'	60	180	120–160	3	0.0690*	0.3727	(AG) ₉	GU338298
R-163	F5' CGTGAGAAATCGTCCAAA 3' R5' TCCAAAAGGGGAGTCATT 3'	58	168	151–203	4	0.4000	0.0365	(TG) ₈	GU338299
R-166	F5' AACGAAACCCAGAAACT 3' R5' TCATTTATCTCCACTCCC 3'	59	248	240–273	3	0.5500	0.4038	(AG) ₇	GU338300
R-172	F5' GCTGTCGTATTTACCTAA 3' R5' AGATGAAGAAGTGCTGGA 3'	59	227	180–242	5	0.4634	0.5760	(CT) ₆ /(CT) ₇	GU338301
R-210	F5' TTTCTGGCTCAAAGAGGG 3' R5' ATTTGGCAGTGTCTTCTCA 3'	60	103	86–110	4	0.4210	0.5154	(AG) ₂₁	GU338302
R-299	F5' TACTGTGCTTAGACGCCATT 3' R5' CCCTCTGCTGTA AAAACTG 3'	62	80	65–96	5	0.4250*	0.6152	(AG) ₁₂	GU338303
R-318	F5' AGTCTTATCTTCGCTTCC 3' R5' ACTATCAACTCGCAGGTC 3'	62	86	75–100	3	0.1667	0.1549	(AC) ₈	GU338304
R-320	F5' GCTGATACGGAGCTGGTG 3' R5' GACGCAAATGGGTGAACT 3'	66	242	213–258	3	0.0263*	0.4254	(AG) ₁₂	GU338305
R-335	F5' TCAGGCAACCGTAACAAC 3' R5' AAACGCAACAAACAGCAA 3'	64	95	80–126	5	0.2195*	0.4104	(CT) ₁₁ /(CT) ₈	GU338306
R-432	F5' CCGTTTGAGTATCTTCCC 3' R5' CTGGTCCATTCTCCAAGTA 3'	64	141	128–147	4	0.4146	0.5227	(CT) ₁₄	GU338307
R-544	F5' TCTGGACTCAAGCAACA 3' R5' AATGGAGTAAATGGGGTG 3'	60	153	144–170	4	0.9512*	0.5146	(CT) ₆	GU338308
R-557	F5' CGAAACTCAGAACCTCCG 3' R5' TTCCGAACTCCTTACCAG 3'	66	211	204–245	3	0.0732	0.0714	(CT) ₉ /(TG) ₆	GU338309

Primer sequence, polymerase chain reaction (PCR) annealing temperature (T_a), expected and observed size range of PCR products, number of observed alleles (A), expected heterozygosity (H_E), and observed heterozygosity (H_O).

*The observed heterozygosity is significantly different from the expected heterozygosity under Hardy-Weinberg equilibrium ($P < 0.01$).

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