Cross-amplification and Characterization of Microsatellite Loci for the Genus *Rhododendron*

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Abstract. To assess cross-species amplification, we tested 38 microsatellite loci previously developed for *Rhododendron delavayi* Franch. and *R. decorum* Franch. in eight species representative of the genus *Rhododendron*. Sixteen pairs can be amplified successfully in all species, whereas two failed amplification in all species. Nine loci were polymorphic across six examined species with one to 11 alleles per locus. The observed and expected heterozygosity per locus varied from 0.07 to 0.65 and 0.44 to 0.81, respectively. Cross-application of these microsatellite loci will provide a potentially useful tool to investigate the genetic structure, gene flow, and evolutionary relationships in genus *Rhododendron*.

The genus *Rhododendron* includes widely distributed flowering plants found throughout the world with the exception of Africa and South America and contains over 1000 species (Chamberlain et al., 1996). Centers with highest diversity and endemism of the genus are the Himalayas and Malaysia (Fang and Min, 1995; Sleumer, 1966). There are 571 species distributed in China, of which 409 species are endemic (Fang et al., 2005). Many *Rhododendron* species are particularly valued for horticulture because of their large and impressive flowers (Sleumer, 1949). *Rhododendron*'s high diversity and wide geographical range have made it an important genus for fundamental and applied research and biodiversity conservation. However, there was considerable morphological over-

lap between the species making discrimination problematic, and more than 1000 horticultural hybrids in existence (Bean, 1976) showed the weakness of genetic barriers toward hybridization in this genus.

Microsatellites are repeating sequences of one to six nucleotides that typically exhibit high levels of polymorphism and are randomly dispersed in the genomes of all prokaryotes and eucaryotes (Litt and Luty, 1989; Tautz, 1989). It is important to determine the extent to which a set of simple sequence repeat (SSR) primers can be used across species within a given genus as a result of the expensive and time-consuming de novo microsatellites isolation. Several studies have been conducted on the use of newly developed primers for cross-specific amplification on related species (Lemes et al., 2007; Nevill et al., 2008; Pinheiro et al., 2009).

A dozen pairs of microsatellite primers (Dendauw et al., 2001; Naito et al., 1998; Tan et al., 2009; Wang et al., 2009) were developed for *Rhododendron* but have not been screened against other members in the genus. There were many previous studies that suggested microsatellite loci could be a useful tool to study hybridization (Duputie et al., 2007; Schrey et al., 2007; Zhang et al., 1994). We report the ability of these markers to amplify SSR loci in closely related taxa with the goal of identifying a set of polymorphic markers that can be used to investigate the genetic structure and diversity, assess the degree of genetic

introgression, and determine the parentage of suspected hybrid individuals for species of this genus.

Total genomic DNA was extracted from leaf tissues using the cetyltrimethyl ammonium bromide method (Milligan, 1992). We tested two sets of microsatellite markers previously developed from *Rhododendron delavayi* Franch. (14 loci) and *R. decorum* Franch. (24 loci). A total of 38 primer pairs were initially screened in eight species representative of the genus *Rhododendron* using two individuals from each species (Table 1). Polymerase chain reaction (PCR) was done in a 20 µL volume using a PTC0200 thermal cycler (MJ Research, Ashland, OR). Each reaction was performed using 20 ng DNA, 1 µM of each dNTP, 1 µM each primer, 1× *Taq* buffer, and 1 U of *Taq* polymerase (TaKaRa). Amplifications were carried out according to the following protocol: initial denaturing step at 95 °C for 5 min, 30 cycles of 94 °C for 30 s, annealing temperature (°C) for each locus/species for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 8 min. The products were electrophoresed in 1.5% agarose gel and sized with a 1-kb DNA ladder (Fermentas, Ontario, Canada). After PCR optimization, the loci that showed clear and robust band amplification were selected for further analysis of polymorphisms.

We focused our effort on six species belonging to different sections of the genus *Rhododendron* (Fang et al., 2005): *R. irroratum* Franch. (Sect. *Ponticum* G. Don), *R. molle* (Blume) G. Don (Sect. *Pentanthera* G. Don), *R. simsii* Planchon (Sect. *Tsutsusi* Sweet), *R. pachypodum* I. B. Balfour & W. W. Smith (Sect. *Rhododendron*), *R. spiciferum* Franch. (Sect. *Trachyrhodon* Sleumer), and *R. fuyuanensis* Z. H. Yang (Sect. *Rhodobotrys* Sleumer). *R. irroratum* and *R. pachypodum* were natural populations located in Yangbi County and Fuming County (Yunnan, China), respectively. Other species were cultivated populations from Kunming Botanical Garden (Yunnan, China). Leaves of 10 random plants were sampled from each population. The PCR products were stored at 4 °C until analysis using the automated capillary electrophoresis QIAxcel system (Qiagen, Hilden, Germany), which uses a preassembled cartridge (cartridge type QIAxcel DNA high-resolution cartridge) to simultaneously run samples and collect data. The PCR samples were automatically loaded into an individual capillary and voltage was applied. A detector in the instrument detected the nucleic acid molecules as they migrated toward the positively charged terminus of the capillary. These data were passed through a photomultiplier before being converted to an electropherogram and gel image. To enable accurate size measurements, a QX Alignment Marker (15/500 bp) and a QX DNA Size Marker (25 to 450 bp) were added to the analysis (Fig. 1). Data were analyzed on a PC running BioCalculator software according to the manufacturer's instructions (Qiagen, Hilden, Germany).

Among the 38 primer pairs tested, 16 pairs were amplified successfully in all species,

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Table 1. Cross-species amplification of 38 microsatellite loci tested for eight related *Rhododendron* species.

Locus (species)	<i>R. irroratum</i>	<i>R. agastum</i>	<i>R. araiophyllum</i>	<i>R. molle</i>	<i>R. simsii</i>	<i>R. pachypodum</i>	<i>R. spiciferum</i>	<i>R. fuyuanensis</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	+	+	+	+	—	—	+	+
R 111	—	+	+	—	—	—	+	—
R 140	—	+	+	—	—	—	—	—
R 147	—	—	—	+	+	—	—	—
R 163	+	—	+	+	+	+	+	+
R 166	+	+	+	+	+	—	—	+
R 172	+	+	+	—	+	—	—	+
R 210	—	—	—	—	—	—	—	+
R 299	—	—	+	+	—	+	+	+
R 318	—	+	+	—	—	—	+	+
R 320	+	+	+	+	+	+	+	+
R 335	—	+	+	+	+	—	+	+
R 432	+	+	+	+	+	+	+	+
R 544	+	+	+	+	+	+	+	+
R 557	+	+	+	+	+	+	+	+

+ = successful; — = unsuccessful amplification.

Fig. 1. Example of sizing of alleles in this study. All samples were amplified with primer RDW16: lanes 1 to 5 were samples of *R. spiciferum* and lanes 6 to 11 were samples of *R. fuyuanensis*. A DNA size marker (25 to 450 bp) and an alignment marker (15/500 bp) were used to size the different alleles.

whereas two failed amplification in all species (Table 1). Of the 38 loci, 28 (73%) gave consistent cross-amplification in *R. irroratum*, 29 (76%) in *R. agastum* I. B. Balfour & W. W. Smith, 30 (79%) in *R. araiophyllum* I. B. Balfour & W. W. Smith, 27 (71%) in *R. molle*, 26 (68%) in *R. simsii*, 21 (55%) in *R. pachypodum*, 27 (71%) in *R. spiciferum*, and 34 (89%) in *R. fuyuanensis* (Table 1). Our results did not reveal significant differences in the transferability of all the microsatellites among the eight species. This is likely the result of the high conservation of the primer

binding sites in the coding regions assayed in the present study.

A total of nine microsatellite exhibited ideal features for using as codominant molecular markers for further studies in the six examined species (Table 2). 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://wbionmed.curtin.edu.au/genepop/>) (Raymond and Rousset, 1995). The number of alleles per locus ranged from one to 11 (overall mean 4.53 alleles).

Natural populations had more average alleles than cultivated populations. The average alleles of *R. irroratum* and *R. pachypodum* were 6.00 and 5.11, respectively, followed by *R. fuyuanensis* (4.89), *R. spiciferum* (4.78), *R. simsii* (3.44), and *R. molle* (2.89). Two loci (RDW31 and RDW38) in *R. molle* and two loci (RDW46 and R557) in *R. simsii* were monomorphic (Table 3). The percentage of polymorphic loci in the six *Rhododendron* species ranged from 78% to 100% with an average of 93%. The observed and expected heterozygosities (H_O and H_E) ranged from 0.07 to 0.65 and 0.44 to 0.81

Table 2. Characteristics of nine polymorphic microsatellite loci for *Rhododendron* species.

Locus	Repeat motif	Primer sequence (5'-3')	Size range (bp)	GeneBank accession no.
RDW1	(TC) ₉	R GAAGGTGATCGTGTGCGGAAT F GCCTCTAACTACTTGCTCCA	241–249	FJ903451
RDW16	(GA) ₉	R CACCAAGCATCATGCTCTA F GGTGATCGTGTGCGGAATACA	249–255	FJ903460
RDW31	(GA) ₈	R TGCCTCTAACTACTTGCTCC F AAGGTGATCGTGTGCGGAATA	240–245	FJ903465
RDW35	(TC) ₅ (CT) ₆	R GTGACTTCGGATTCTCGTGAG F TAAGGTTGGTGTAGCGTGTGA	210–219	FJ903468
RDW38	(TAGAG) ₄ (AG) ₇	R AACAGCGACGAGAAAAGC F GTGTTTGAATTTGTCGGC	140–148	FJ903469
RDW46	(AGAGAT) ₃ (CTT) ₃ (GA) ₁₁	R AGCAAGATAGAAAACCTGTAAAC F TCTCCAGAAGTACGCAAAAT	303–312	FJ903473
R-432	(CT) ₁₄	R CTGGTCCATTCTCCAAGTA F CCGTTTGAGTATCTTCCC	128–147	GU338307
R-544	(CT) ₆	R AATGGAGTAAATGGGGTG F TCTGGACTTCAAGCAACA	144–170	GU338308
R-557	(CT) ₉ (TG) ₆	R TTCCGAACCTCCTTACCAG F CGAAACTCAGAACCTCCG	204–245	GU338309

Table 3. Genetic parameters for the microsatellite loci transferred to six *Rhododendron* species.

Locus	<i>R. irroratum</i>					<i>R. molle</i>					<i>R. simsii</i>				
	Size range (bp)	A	<i>T_m</i> (°C)	H _E	H _O	Size range (bp)	A	<i>T_m</i> (°C)	H _E	H _O	Size range (bp)	A	<i>T_m</i> (°C)	H _E	H _O
RDW1	237–243	3	60	0.35	0.40	241–243	2	60	0.34	0.00*	233–252	3	60	0.63	0.90
RDW16	248–254	3	56	0.59	0.40	249–251	2	56	0.19	0.00	240–261	5	56	0.70	0.90
RDW31	234–242	4	56	0.63	0.50	239	1	56	—	—	230–248	4	56	0.77	0.90
RDW35	200–221	8	56	0.85	0.80	207–226	7	56	0.76	0.50	216–229	4	56	0.70	0.10*
RDW38	126–140	5	60	0.62	0.60	138	1	60	—	—	126–136	3	60	0.53	0.50
RDW46	269–343	11	60	0.82	0.50*	274–286	3	60	0.63	0.90	268	1	60	—	—
R432	113–133	9	50	0.83	0.70	112–126	4	50	0.61	0.40	111–153	7	50	0.81	0.90
R544	149–155	4	60	0.63	0.20*	154–160	4	60	0.66	1.00*	148–152	3	60	0.49	0.00*
R557	210–226	7	50	0.70	0.10*	204–206	2	50	0.44	0.00*	197	1	50	—	—

Locus	<i>R. pachypodum</i>					<i>R. spiciferum</i>					<i>R. fuyuanensis</i>				
	Size range (bp)	A	<i>T_m</i> (°C)	H _E	H _O	Size range (bp)	A	<i>T_m</i> (°C)	H _E	H _O	Size range (bp)	A	<i>T_m</i> (°C)	H _E	H _O
RDW1	235–237	2	60	0.27	0.10	237–245	4	60	0.74	0.40*	237–244	3	60	0.36	0.20*
RDW16	243–249	4	56	0.66	0.20*	244–256	5	57	0.81	0.60*	245–252	3	57	0.28	0.20
RDW31	233–235	2	56	0.51	0.00*	236–242	2	56	0.52	0.50	235–243	4	56	0.62	0.20
RDW35	198–222	7	56	0.78	0.50	202–228	9	56	0.91	1.00	203–230	8	56	0.85	1.00
RDW38	118–139	3	60	0.59	0.80	126–134	4	60	0.78	0.30*	123–134	6	60	0.82	0.60
RDW46	274–322	9	60	0.90	0.40*	262–272	3	60	0.53	0.30	223–275	4	60	0.65	0.30*
R432	105–132	11	50	0.84	0.70	110–145	8	52	0.80	0.50*	117–141	9	50	0.73	0.60
R544	149–153	3	60	0.68	0.80	148–154	4	62	0.47	0.50	151–172	4	62	0.70	0.70
R557	190–200	5	50	0.58	0.30*	199–222	4	51	0.40	0.00*	197–201	3	51	0.51	0.00*

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

(averages of 0.59 and 0.42, respectively) (Table 3). Among the nine microsatellite screened on six species, 20 (37%) showed significant deviation from HWE (*P* < 0.01; Table 3), probably as a result of deficiency of heterozygote or the limitation of sample size. Tests for linkage disequilibrium 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. The size range of amplification products were similar to those found in species for which the primers were originally developed except for one primer pairs RDW46 (Tables 2 and 3).

In this study, we have demonstrated successful cross-species amplification of nine microsatellite loci among species of the genus *Rhododendron*. The markers tested here should be of potential use in future studies of genetic diversity, population structure, hybridization, and molecular breeding of species in this genus.

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