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Primer Note

Development and characterization of 23 microsatellite loci for *Rhododendron ovatum* (Ericaceae)[1]

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- *Premise of the study:* To estimate the genetic variation of *Rhododendron ovatum* (Ericaceae), a monoecious evergreen shrub, 23 microsatellite markers were identified from its nuclear genome.
- *Methods and Results:* We developed 16 polymorphic and seven monomorphic microsatellite primers using the biotin-streptavidin capture method. The 16 polymorphic loci were investigated further using 89 individuals sampled from three populations in China. The number of alleles per locus ranged from four to 30, indicating a high level of polymorphism. The observed heterozygosity varied from 0.1034 to 0.9333, while the expected heterozygosity ranged from 0.1016 to 0.9542. Of these polymorphic primers, 12 were found to be functional in *R. simsii*, a congeneric species of *R. ovatum*.
- *Conclusions:* Moderate to high levels of genetic variation were found in these microsatellite loci, indicating that they can be applied in future studies of *Rhododendron* genetic structure, contributing to forest management and conservation.

Key words: Ericaceae; genetic variation; microsatellites; polymorphism; *Rhododendron ovatum*.

Evergreen broadleaf forests (EBLFs) contribute to global biodiversity and ecosystem maintenance, but are rapidly degenerating and fragmenting due to anthropogenic activities (Song and Chen, 2007). EBLF flora comprise large trees (e.g., species from the Fagaceae family) and a large variety of shrubs including many *Rhododendron* L. (Ericaceae) species (Song and Chen, 2007). Generally, EBLF fragmentation is expected to induce genetic differentiation among populations as a result of decreased gene flow, increased inbreeding, and genetic drift. This is especially true for shrub and herb species with relatively short generation times and low population densities (Zhao et al., 2006). However, very few studies have addressed this hypothesis by investigating the genetic structure of evergreen shrubs.

Rhododendron ovatum (Lindl.) Planch. ex Maxim. var. *ovatum* (subgenus *Azaleastrum* Planch. ex K. Koch) is a monoecious, self-incompatible evergreen shrub endemic to China and is one of the most prevalent shrubs in EBLFs. For the conservation and management of EBLFs, it is important to characterize the possibly dwindling genetic variation of *R. ovatum.* However, most microsatellite markers previously developed for *Rhododendron* species (e.g., Tan et al., 2009) are not functional for *R. ovatum*.

In the current study, we isolated and characterized 16 polymorphic and seven monomorphic microsatellite loci to reveal genetic variation in *R. ovatum* and to shed light on the underlying mechanisms, such as limited gene flow and historical demographics. To further study the dynamics of interspecific hybridization within the genus, we also carried out cross-amplification in *R. simsii* Planch. (subgenus *Tsutsusi* (Sweet) Pojark.), another important shrub species in EBLFs (Zhuang, 2012), thus further contributing to forest management and conservation.

METHODS AND RESULTS

Plant materials of *R. ovatum* were collected from three populations located in Tiantong and Tianmu in Zhejiang Province, China, and Jinggang in Jiangxi Province, China (Appendix 1). Microsatellite loci were developed according to the method recorded by Tong et al. (2012). Total genomic DNA was extracted from leaves dried with silica gel using the Plant Genomic DNA Kit (Tiangen, Beijing, China). After digestion with the *Mse*I restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA), approximately 250 ng of DNA was ligated to an *Mse*I-adapter pair (F: 5′-TACTCAGGACTCAT-3′, R: 5′-GACGATGAGTCCTGAG-3′). The diluted (1 : 5) ligation-digestion mixture was amplified with *Mse*I-N primers (5′-GATGAGTCCTGAGTAA-3′) in a 20-μL PCR reaction at: 95°C for 3 min, followed by 17 cycles of 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min. To enrich DNA fragments containing microsatellites, the amplification products were hybridized with the 5′-biotinylated probe $(AG)_{15}$ and the hybridization products were captured by magnetic beads coated with streptavidin (Promega Corporation, Madison, Wisconsin, USA). The enriched fragments were PCR amplified using *Mse*I-N primers for 30 cycles. After purification using the multifunctional DNA Extraction Kit (Bioteke, Beijing, China), the PCR products were transformed into *Escherichia coli* strain JM109 with the pMD 19-T vector (TaKaRa Biotechnology Co., Dalian, China) followed by transient thermal stimulation.

Of the 241 selected clones, 174 were positive after PCR with $(AG)_{10}$ and M13F/M13R as primers. The positive clones were sequenced on an ABI 3730 DNA Sequence Analyzer (Applied Biosystems, Foster City, California, USA). A total of

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51 sequences containing microsatellites were chosen for simple sequence repeat primer design using Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, California, USA). Twenty-three *R. ovatum* individuals were randomly selected from the samples mentioned above (Appendix 1) and were used to test the performance and polymorphism for all loci. PCR was performed in a 10-μL reaction system containing 50 ng of genomic DNA, 1× PCR buffer (without Mg^{2+}), 2.5 mM Mg^{2+}, 0.2 mM of each dNTP, 0.1 μM of each primer, and 1 unit of *Taq* DNA polymerase (Sangon, Shanghai, China) at 95°C for 5 min; 35 cycles of 40 s at 94°C, 45 s at 45–65°C (depending on specific locus, Table 1), and 45 s at 72°C; and a final extension at 72°C for 8 min. PCR products were separated using 8% polyacrylamide denaturing gels and visualized with silver staining using pUC19 DNA/*Msp*I (*Hpa*II) (Thermo Fisher Scientific, Waltham, Massachusetts, USA) as the ladder. A total of 16 polymorphic and seven monomorphic loci were obtained (Table 1), none of which have been previously reported in the genus *Rhododendron* based on the results of BLAST searches in GenBank.

The polymorphisms among all polymorphic loci were further surveyed with 89 individuals from the three *R. ovatum* populations (Appendix 1). We labeled the forward primers using a fluorescent dye (5′HEX, 5′ROX, or 5′6-FAM) (Sangon). PCR reactions were then performed in a 10-μL reaction system using the same thermocycling program described above. The products were scanned on an ABI 3730 automated sequencer using GeneScan 500 LIZ (Applied Biosystems) as the internal lane standard and were genotyped using GeneMapper 4.0 (Applied Biosystems).

When analyzed using the software TFPGA version 1.3 (Miller, 1997) and FSTAT 2.9.3 (Goudet, 1995), the 16 polymorphic loci displayed moderate to high levels of genetic variation in the three populations. The number of alleles ranged from four to 30 among the loci, with a mean value of 14.2, indicative of a high level of polymorphism (Table 1). The observed and expected (based on Hardy–Weinberg equilibrium) heterozygosities within the population varied from 0.1034 to 0.9333 and from 0.1016 to 0.9542, respectively (Table 2). We

Table 1. Characterization of 16 polymorphic and seven monomorphic microsatellite loci developed in *Rhododendron ovatum.*[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	*A*	T_a (°C)	Fluorescent dye[b]	GenBank accession no.
MYH2	F: ACCCAACACAACCCAACC R: AGAGAGCACCCCTTCACC	$(CT)_{22}$	200–248	20	65	ROX	KX138625
MYH3	F: TCAAAACCCTAACCAGTC R: CCACATTGCTTGCTATTC	$(TC)_{17}$	156–236	30	58	ROX	KX138626
MYH4	F: GACAGTGCCAATTGTATGC R: CAGTTTGCAACAGAGGATG	$(TC)_{13}$	113–155	20	61	6-FAM	KX138627
MYH5	F: AATCCATGGCTGCCCGTT R: TCCTCACCCCACCACTAC	$(GA)_{16}$	158–202	19	64	6-FAM	KX138628
MYH6	F: GGAAAGGAACTCTGCCAATGTCT R: ACTGATGCAAGTTGCGAGTCTGT	$(AG)_9$	203–243	8	60	HEX	KX138629
MYH7	F: GAGACCAGATAGAGAATAGCC R: TAGGAACACAGAACACACACG	$(CT)_{11}$	103–155	24	62	HEX	KX138630
MYH8	F: CATCCACCAGCGATTGAAG R: GAAGGACAGTAGTGGGAGC	$(CT)_6$	185–221	8	61	HEX	KX138631
MYH9	F: TAGAAAGAAGTGTCCCATC R: CTTGTTGCTAAACCAGTGT	$(TC)_{20}$	157–207	23	59	HEX	KX138632
MYH10	F: TGTATTCTAGTGTTGTTGCTTCCCCT R: GAACATAAACATCCAGCTAGTACTCC	$(TC)_{19}$	103–131	14	54	HEX	KX138633
MYH11	F: AGAATGCAGGAAAGGCGTACC R: CTCCCCCTTGTTTTCATCGAC	$(GA)_{20}$	123–159	19	63	HEX	KX138634
MYH12	F: CACATCATTCCAAGAAATCCTC R: TAATTTGGCTAGAACCACGAAC	$(GA)_6$	130–138	4	63	ROX	KX138635
MYH13	F: GTGCGGGTACTATTTTGT R: ATGTTGTGGTTTGTGAGG	$(CT)_{17}$	170–190	6	56	ROX	KX138636
MYH14	F: AGCAATGCGTGTGAAGTC R: ATCAGGAAATGGGGAAAC	$(CT)_8$	95–121	10	56	6-FAM	KX138637
MYH15	F: CAAATCAAAGTAGAACCACCAG R: TCAGTAGCAGACCTTCAAATGT	$(CT)_{14}$	180–204	13	65	HEX	KX138638
MYH16	F: ACATTCCACATCTCACAC R: TCACCACTTCCATCTCTT	$(CT)_{31}$	112–166	27	58	6-FAM	KX138639
MYH17	F: ACACACGAAGAGGAATAATACGC R: GTTAGCACAAAGTGGCAACATAG	$(GA)_6$	135–167	7	65	ROX	KX138640
MYH21*	F: GGTAAGAAGATAAGCCCT R: GCCCATCGTCAAAAAAAC	$(GA)_{12}$	158	1	48	—	KX424563
MYH22*	F: CCCTAAGTACACCAAGTGCTATGAG R: AGGGTAAGTTTTGTGTTATTGCTCC	$(AG)_{15}$	225	1	50	— —	KX424564
MYH23*	F: ATTGTTGTCTGTTGCCGT R: CCTGGGTCCATCTTTCAT	$(GA)_{17}$	176	1	56	— —	KX424565
MYH24*	F: AGTGAGTTCTCAAGAGCTTC R: TTCCATAGTCCATCCAAGGT	$(CT)_{16}$	228	1	48	— —	KX424566
MYH25*	F: GGTCTAGGGTTTTGTGGTTGT R: GCATCTCTCAGGTTTCTTTGT	$(AG)_{16}$	137	1	50	— —	KX424567
MYH26*	F: CAACCCATTTCTTCCTCC R: CACACAACCAACCTCACC	$(AG)_{15}$	128	1	61	— —	KX424568
MYH27*	F: GGTTTGTGTCATCTTGTGATTCTTGTG R: ATGTAGGTTATGGTCATGGGCCTTAGT	$(GA)_{16}$	200	1	65	—	KX424569

Note: *A* = number of alleles; T_a = annealing temperature.

[a] Allele size range is based on samples representing three populations located in Tiantong and Tianmu in Zhejiang Province, and Jinggang in Jiangxi Province, China (see Appendix 1).

[b] Fluorescent dyes (i.e., HEX, ROX, and 6-FAM) used to label the forward primers for fragment analysis.

* Monomorphic microsatellite loci.

Table 2. Characterization of the 16 polymorphic microsatellite loci in three *Rhododendron ovatum* populations.[a]

	Tiantong population ($n = 30$)			Tianmu population ($n = 29$)			Jinggang population ($n = 30$)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
MYH2	14	0.7333	0.8486	10	0.8214	0.8539	13	0.8966	0.8572
MYH3	12	0.6552	0.8252	15	0.5714	0.9091	24	0.8000	0.9542
MYH4	12	0.7333	0.7539	14	0.7241	0.9280	16	0.6333	0.9113
MYH5	6	0.9333	0.7616	12	0.7308	0.8371	14	0.7857	0.8805
MYH6	4	0.4333	0.4966	6	0.4400	0.4114	7	0.3103	0.3908
MYH7	14	0.6333	0.9254	15	0.6071	0.8948	19	0.5172	0.9226
MYH8	5	0.8333	0.6960	7	0.4828	0.6564	5	0.5667	0.6107
MYH9	16	0.7667	0.9282	17	0.6296	0.9182	14	0.7241	0.9250
MYH10	11	0.8667	0.8288	11	0.8214	0.8701	12	0.8276	0.8947
MYH11	14	0.8667	0.9158	14	0.7241	0.9147	16	0.8519	0.9294
MYH12	4	0.4667	0.5497	2	0.2800	0.4971	2	0.2414	0.2160
MYH13	5	0.4828	0.6788	2	0.1481	0.3913	4	0.2069	0.2523
MYH14	8	0.4667*	0.8158	6	0.1481	0.7778	7	0.2333*	0.6621
MYH15	8	0.7931	0.6842	10	0.7778	0.6988	9	0.7667	0.7915
MYH16	19	0.5000	0.9390	19	0.8077	0.9434	17	0.7143	0.9169
MYH17	4	0.4000	0.3994	4	0.1034	0.1016	4	0.2667	0.2446

Note: A = number of alleles; H_e = expected heterozygosity based on Hardy–Weinberg equilibrium; H_o = observed heterozygosity; n = number of individuals genotyped.

[a] Voucher and locality information for the populations are provided in Appendix 1.

* Indicates significant deviation from Hardy–Weinberg equilibrium ($P < 0.05$).

failed to detect any significant linkage disequilibrium for all pairs of loci in all populations. Significant deviation from Hardy–Weinberg equilibrium was only found at one locus (MYH14), and only in the Tiantong and Jinggang populations after sequential Bonferroni correction (Rice, 1989). Signs of null alleles in the loci MYH3, MYH7, MYH14, and MYH16 were detected using MICRO-CHECKER 2.2.3 (van Oosterhout et al., 2004).

We also tested the performance of these primer pairs in *R. simsii*, a closely related species to *R. ovatum* but not in the same subgenus. After scanning the PCR products in 16 *R. simsii* individuals sampled in Shanghai, China (Appendix 1), 12 polymorphic loci (except MYH3, MYH7, MYH10, and MYH13) could be used in this congeneric species. These loci revealed high levels of polymorphism and observed and expected heterozygosities (Table 3), similar to those evaluated by the polymorphic microsatellite loci specifically developed for *R. simsii* (Tan et al., 2009). However, only two of the eight microsatellite loci developed for *R. simsii* (Tan et al., 2009) could be amplified in *R. ovatum*.

Table 3. Characterization of the 16 polymorphic microsatellite loci developed for *Rhododendron ovatum* in *R. simsii*.[a]

	Shanghai Botanic Garden ($n = 16$)			
Locus	A	H_o	H_e	Allele size range (bp)
MYH2	11	0.6000	0.8529	188–222
MYH3	—	—	—	—
MYH4	11	0.6429	0.8836	127–171
MYH5	10	0.7333	0.8713	154–188
MYH6	3	0.3125	0.4940	199–203
MYH7	—	—	—	—
MYH8	3	0.5000	0.4894	159–195
MYH9	11	0.6154	0.9231	155–191
MYH10	—	—	—	—
MYH11	11	0.8125	0.8790	117–145
MYH12	3	0.2000	0.5356	130–140
MYH13	—	—	—	—
MYH14	9	0.4375	0.8609	99–121
MYH15	8	0.7333	0.7333	171–193
MYH16	12	0.8750	0.9173	110–162
MYH17	4	0.5625	0.6935	127–147

Note: A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals genotyped.

[a] Voucher and locality information for the populations are provided in Appendix 1.

CONCLUSIONS

The 23 microsatellite loci developed in the current study provide an appropriate resource to delineate the genetic variation and genetic structure of *R. ovatum* populations, thereby contributing to the management and conservation of EBLFs. These markers can also facilitate future population genetic studies at a multispecies level within the genus *Rhododendron*.

LITERATURE CITED

Goudet, J. 1995. FSTAT (version 1.2): A computer program to calculate *F*-statistics. *Journal of Heredity* 86: 485–486.

Miller, M. P. 1997. Tools for population genetic analyses (TFPGA) 1.3: A Windows program for the analysis of allozyme and molecular population genetic data. Website http://www.marksgeneticsoftware.net/tfpga.htm [accessed 30 November 2016].

Rice, W. R. 1989. Analyzing tables of statistical tests. *Evolution* 43: 223–225.

Song, Y. C., and X. Y. Chen. 2007. Degradation mechanism and ecological restoration of evergreen broad-leaved forest ecosystem in east China. Science Press, Beijing, China.

Tan, X. X., Y. Li, and X. J. Ge. 2009. Development and characterization of eight polymorphic microsatellites for *Rhododendron simsii* Planch. (Ericaceae). *Conservation Genetics* 10: 1553–1555.

Tong, X., N. N. Xu, L. Li, and X. Y. Chen. 2012. Development and characterization of polymorphic microsatellite markers in *Cyclobalanopsis glauca* (Fagaceae). *American Journal of Botany* 99: e120–e122.

van Oosterhout, C., W. F. Hutchinson, D. P. M. Wills, and P. Shipley. 2004. MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4: 535–538.

Zhao, A. L., X. Y. Chen, X. Zhang, and D. Zhang. 2006. Effects of fragmentation of evergreen broad-leaved forests on genetic diversity of *Ardisia crenata* var. *bicolor* (Myrsinaceae). *Biodiversity and Conservation* 15: 1339–1351.

Zhuang, P. 2012. Discuss on the *Rhododendron* geographical distribution types and their cause of formation in China. *Guihaia* 94: 150–156.

Appendix 1. Locality information for the *Rhododendron ovatum* and *R. simsii* samples used in this study. All voucher specimens were deposited in East China Normal University (HSNU), Shanghai, China.

Species	Locality ID	Collection locality	Geographic coordinates	Collector	Collection no.	*n*
Rhododendron ovatum (Lindl.) Planch. ex Maxim.	Tiantong	Zhejiang, China	29°48′22″N, 121°47′11″E	De-Chen Liu	ROTTZJ01–30	30
Rhododendron ovatum	Tianmu	Zhejiang, China	30°18′04″N, 119°24′32″E	De-Chen Liu	ROTMZJ01–29	29
Rhododendron ovatum	Jinggang	Jiangxi, China	26°32′34″N, 114°08′50″E	De-Chen Liu	ROJGJX01–30	30
Rhododendron simsii Planch.	Shanghai Botanic Garden	Shanghai, China	31°08′46″N, 121°26′50″E	De-Chen Liu	RSBGSH01–16	16

Note: *n* = number of individuals sampled.