Permanent Genetic Resources Notes

Isolation and characterization of twelve microsatellite loci from *Rhododendron rubropilosum* Hayata var. *taiwanalpinum* using dual-suppression-PCR method

Tsurng-Juhn Huang, Mei-Juan Wen, Tsai-Wen Hsu, Chang-Hung Chou (✉)

Research Center for Biodiversity and Graduate Institute of Ecology and Evolutionary Biology, China Medical University, Taichung, Taiwan 404

Author for correspondence: CH Chou, choumasa@mail.cmu.edu.tw, +886 (4)22071507

Running title: Microsatellite markers from *Rhododendron rubropilosum*

Abstract

Twelve polymorphic microsatellite loci were selected, isolated and characterized from a genome library of *Rhododendron rubropilosum* Hayata var. *taiwanalpinum* using a dual-suppression-PCR method. These simple sequence repeat (SSR) markers were screened in 66 samples of wild populations of *R. rubropilosum*, and also tested nine samples of its two

relative species. In *R. rubropilosum*, the number of alleles per locus ranged from 2 to 6, and expected (H_E) and observed (H_O) heterozygosity ranged from 0.2934 to 0.6677 and from 0.1515 to 0.8333, respectively. Most of these microsatellite loci are significantly deviated from Hardy-Weinberg equilibrium possibly due to the heterozygosity deficiency, indicating the habitat fragmentation. Eight loci of these markers were also amplified well in other two relative species, *R. pseudochrysanthum* Hay. ssp. *morii* (Hay.) Yamazaki and *R. pseudochrysanthum* Hayata .

Keywords: Rhododendron rubropilosum Hayata var. *tawanalpinum*, *Rhododendron pseudochrysanthum* Hay. ssp. *morii* (Hay.) Yamazaki, *Rhododendron pseudochrysanthum* Hayata, Heterozygosity, Microsatellite, dual-suppression-PCR method

Introduction

Rhododendron is the biggest genus of Ericaceae which belongs to dicotyledons and distributes in temperate, frigid, and the mountain area of subtropical zone. It is estimating at least 850 native *Rhododendron* species have been found in the world (Sleumer, 1966) and this genus has been subdivided into closed related eight subgenera (Kron et al., 1990). Within this plant genus, the relative azaleas are horticulturally important ornamental plants and have been

extensively hybridized. *Rhododendron rubropilosum* Hayata var. *taiwanalpinum* is an endemic subalpine to alpine entomophilous evergreen shrub which grows on mountain slopes at high elevations, ca. 2,500-3,500 m alt. above sea level in Taiwan. This species could be found in disturbed areas including burned or freshly exposed places. Due to the rapid social-economical development and warmer of global climate, natural habitats for *R. rubropilosum* have been severely disturbed, resulting in population fragmentation and lacking useful genetic markers in the assessing population structure of this species. Overexploitation is another reason for the quick population decline. For protecting this valuable natural resource and the extension of studies throughout this genus, investigating genetic diversity and population structure would provide information for conservation. In this study, we isolated twelve microsatellites from this endemic species and tested if these markers could cross amplify the other relative species of this genus.

In total, twelve microsatellite loci were isolated from *R. rubropilosum* by a dual suppression-PCR technique outlined in Lian et al. (2001) with modification. Briefly, total genomic DNA was extracted from liquid nitrogen ground leaf-tissue of *R. rubropilosum* Hayata var. *taiwanalpinum* using a cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). Adaptor-ligated, restricted DNA libraries were constructed and the DNA was separately digested with *Alu*I blunt-end restriction enzyme. The fragments were

then ligated to a specific adaptor (consisting of a 48-mer: 5'-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3' and an 8-mer with the 3'-end modified by amino residue: 5'- ACCAGCCC-$NH_2$3') by T4 DNA ligation kit (Promega). As a first step for isolation of microsatellite loci, fragments flanked by $(AG)_n(AC)_n$ microsatellite at one end were amplified from the constructed *Alu*I DNA library by the $(AG)_6(AC)_5$ or $(AC)_6(TC)_5$ primer and the adaptor primer AP1 (5'-GTAATACGACTCACTATAGGGC-3') which designed from the longer strand of the adaptor. The amplified DNA fragments were directed ligated into pGEM T-Easy Vector system (Promega), and the plasmids were transformed into DH5α *Escherichia coli* bacterial competent cell (Protech Technology Enterprise Co., Taiwan). Extraction of the positive plasmid DNA from *E. coli* was conducted using the plasmid purification Mini Kit (Geneaid) and then sequenced in an Applied Biosystems Model 377 A automated sequencer by SP6 or T7 primers. Among them, some fragments containing the $(AG)_n(AC)_n$ microsatellite sequences at one end were chosen for advanced analysis. The next step was to design the sequence of the flanked region of each microsatellite. Primer P1 designed from the sequenced region flanking the microsatellite and, for nested PCR, another primer P2 based on the sequence between P1 and microsatellite were also designed. As adaptor-primer for nested PCR, AP2 (5'- ACTATAGGGCACGCGTGGT-3') primer is also prepared. The primary PCR

reaction was conducted using AP1 and P1 primers. The secondary PCR reaction was conducted with 100-fold dilution of primary PCR products using AP2 and P2 primers. During nested PCR reaction, single-banded DNA fragments were usually observed in some libraries. Twelve of these fragments were cloned and sequenced as described above and obtained the opposite flanking region from microsatellite locus. The third primer P3 was then designed between the end of sequence and the microsatellite locus. Finally, we chose the P2 (forward) and P3 (reversed) primer pair sequences which flanking in both sides of each repetitive DNA sequence for PCR analysis as showed in Table 1. Specific primer pairs used above were designed according to the nucleotide sequences upstream and downstream of the repetitive DNA using Primer 3 (Rozen and Skaletsky, 2000).

To investigate the characteristics of microsatellite loci amplified by P2 and P3 primer pairs, seventy-five plant leaves were collected in Hehuan Mountain, Nantou County, Taiwan: sixty-six adults of *R. rubropilosum*, three adults of *R. pseudochrysanthum* Hay. ssp. *morii* (Hay.) Yamazaki, and six adults of *R. pseudochrysanthum* Hayata. PCR reaction was performed in a thermal cycler (Biometra T3000 model) with 25 μl volume containing 10 ng of genomic DNA, 0.2 mM dNTP, 2 mM $MgCl_2$, and 5 pmols of each primer. PCR programs was carried out as follows: initial denaturing step at 94 ^{O}C for 5 min; 35 cycles of 94 ^{O}C for 30 s, primer-specific annealing temperature (Table 1) for 30 s, 72 ^{O}C for 30 s, and a final

extension step at 72 ^{O}C for 10 min. The reaction products were electrophorezed by Agilent Bioanalyzer (Agilent Technology Co. Ltd, USA) to estimate allele size. Results of the allele number, size range, and number of bands per individual are listed in Table 1. Expected (H_E), and observed (H_O) heterozygosities were calculated using the Arlequin version 3.5 (Excoffier and Lischer, 2010). Testes of Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) were conducted using GENEPOP version 3.4 (Raymond and Rousset, 1995; Rousset, 2008) (http://genepop.curtin.edu.au/).

In summary, 12 novel polymorphic microsatellite loci in *R. rubropilosum* are presented (Table 1). The number of allele per locus ranged from 2 to 6. The expected (H_E) and observed (H_O) heterozygosities ranged from 0.2934 to 0.6677 and from 0.1515 to 0.8333, respectively. Significant departures ($P < 0.05$) from HWE (Table 1) were detected in ten of microsatellite loci with the exception of RrHT-1 and RrHT-15 loci. These deviations were due to the deficit of heterozygotes within populations (loci RrHT-3, RrHT-8, RrHT-11, RrHT-16 and RrHT-17), suggesting the random losses of genetic polymorphisms by genetic drift in fragmented populations, or possibly due to the population structuring within specimens (Wahlund effect). Microchecker version 2.2.3 (Van Oosterhout *et al.* 2004) was further used to estimate the frequency of null alleles in the microsatellite markers (Table 1). It was shown that there probably occurred null allele in five loci ($P < 0.05$), i.e. in RrHT-3, RrHT-4,

RrHT-8, RrHT-11, and RrHT-16, all of which displayed excessive homozygotes. For GENEPOP analysis of linkage disequilibrium (LD), the results from GENEPOP (Raymond and Rousset, 1995; Rousset, 2008) analysis revealed no significant LD was observed in the comparisons of all loci (data not show). For purpose of the cross reactivity of these microsatellite markers, the primer pairs were also applied to *R. pseudochrysanthum* Hay. ssp. *morii* (Hay.) Yamazaki and *R. pseudochrysanthum* Hayata. Most of isolated primer pairs from *R. rubropilosum* could cross-amplified microsatellite fingerprints in the nine specimens of these two relative species (three specimens for *R. pseudochrysanthum* Hay. ssp. *morii* (Hay.) Yamazaki and six specimens for *R. pseudochrysanthum* Hayata) with the exceptions of RrHT-1, RrHT-2, RrHT-3, and RrHT-4 loci. The application of these microsatellite loci in *R. rubropilosum* may therefore provide a powerful tool for understanding this species demongraphy and population structure during environmental change.

Acknowledgements

This work was supported by grants from National Science Council of Taiwan (NSC 96-2625-Z-039-001, NSC 97-2625-M-039-001, NSC 97-2625-M-039-002, and NSC 98-2911-I-039-001)

Data Accessibility:

Simple sequence repeat DNA sequences: Genbank accessions HQ700914-HQ700925. Final DNA sequence assembly uploaded as online supplemental material.

References

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, **19**, 11-15.

Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. *Molecular Ecology Resources*, **10**, 564-567.

Kron K.A., Gawen L.M., Chase M.W. (1990) Phylogenetic relationships within the Rhodorae (Ericaceae) with specific comments on the placement of Ledum. *Systematic Botany*, **15**, 57-68.

Lian CL, Zhou ZH, Hogetsu T (2001) A simple method for developing microsatellite markers using amplified fragments of inter-simple sequence repeat (ISSR). *Journal of Plant Research*, **114**, 381-385.

Raymond M, Rousset F (1995) GENEPOP (Version 1.2): Population Genetics Software for Exact Tests and Ecumenicism. *Journal of Heredity*, **86**, 248-249.

Rousset F. (2008) GENEPOP ' 007: a complete re-implementation of the GENEPOP software

for Windows and Linux. Molecular *Ecology Resources*, **8**, 103-106.

Rozen S, Skaletsky HJ (2000) Primer 3 on the WWW for general user and for biologist programmers. *In: Bioinformatics Methods and Protocols: Methods in Molecular Biology* (ed. Krawetz S, Misener S), pp.365-386. Human Press, Totowa, New Jersey.

Sleumer H. (1966) Ericaceae. *In: Flora Malesiana Series I*, **6**, 470-668.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, 4, 535-538.

153 **Table 1** Characterization of twelve microsatellites isolated from *Rhododendron rubropilosum* Hayata var. *taiwanalpinum*. Repeat motif, primer
154 sequence, size range, number of alleles, annealing temperature, expected (H_E) and observed (H_O) heterozygosities, probability of Hardy-Weinberg
155 equilibrium.m (HWE), and GenBank Accession number

Locus	Repeat motif	Primer sequence (5'-3')	Size range (bp)	Total number of alleles	*Ta* (^{O}C)	H_O	H_E	HWE *P*-value	Null allele frequency	GenBank Accession no.
RrHT-1	$(AC)_8$	F: CTCAAGCAGGCGTGTCCTAA R: TGCAACCACGAAGAAGCGTA	150-182	3	50	0.3788	0.4003	0.1141	0.0177	HQ700914
RrHT-2	$(AC)_8$	F: AACCACGAGGAAGCGTATTG R: CCTAACCAGAATGGCTCACC	152-168	2	52	0.7424	0.4945	<0.01	0	HQ700915
RrHT-3	$(AC)_{16}$	F: CGCTTGTTGATAACGACGAC R: ACTCAAGCAGGCGTGTCCTA	150-182	2	52	0.1515	0.4256	<0.01	0.1838*	HQ700916
RrHT-4	$(GAA)_6$	F: TTGGACTAATTGTTCTTTTTAATGC R: AGGTTGAAGTCGATGGAAGAAA	168-186	2	50	0.3182	0.4528	0.0258	0.0892*	HQ700917
RrHT-8	$(AG)_{19}$	F: GCGCTCTGTTATTCAGAACCGTA R: CCGGGCTGGTCTATCCAAAT	164-194	5	54	0.2424	0.5992	<0.01	0.2149*	HQ700918
RrHT-11	$(TA)_7$	F: CTCCTCATATCGCCTCTGGAGT R: GGGTATGGTGGAGGCTATGAGA	168-216	3	56	0.3939	0.6519	<0.01	0.1512*	HQ700919
RrHT-12	$(TG)_5TTT(GA)_5$	F: TCTCAAGTCTTCTTCCTGGCAAA R: GCTGGTCTCAAAACCCACAAATC	238-244	2	54	0.8333	0.4945	<0.01	0	HQ700920
RrHT-15	$(AC)_6$	F: GGCGACGGTAATGTTCAGTCTA R: AAGCAACAATCCTTCCCTCAAG	198-222	5	53	0.3333	0.3878	0.1116	0.0407	HQ700921
RrHT-16	$(CA)_6$	F: CACAACTGCGATATTCTGCTCT R: TTGGTCCTACCCTTTGAGTTGT	170-206	3	53	0.197	0.2934	<0.01	0.0734*	HQ700922

RrHT-17	$(CA)_5(GA)_6$	F: AGAAACGTTGAGGGGAGGTT R: GCTGCCACACCTATTCCTTC	172-216	5	52	0.5909	0.6677	<0.01	0.0355	HQ700923
RrHT-18	$(TG)_{13}(AG)_{15}$	F: GTGTTCGGTTCCAAGAGTGCT R: CTGTTCCCAACGGTTAGTGATG	118-138	3	54	0.4849	0.3737	0.0177	0	HQ700924
RrHT-19	$(AG)_{14}$	F: AAGGTGGCGGCGAGATAGAT R:TCTTAAACCTGTACCGGAGGGATT	150-206	6	54	0.5	0.6029	0.0334	0.0531	HQ700925

156 *Ta*, annealing temperature; F:, forward primer; R:, reverse primer; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE *P*-value,
157 probability of Hardy–Weinberg equilibrium.
158 *indicates significance for the existence of null alleles.

Decision Letter (MER-11-0313)

From: managing.editor@molecol.com

To: tjhuang@mail.cmu.edu.tw

CC:

Subject: Molecular Ecology Resources - Decision on Manuscript ID MER-11-0313

Body: @@date to be populated upon sending@@

Dear Dr. Huang:

It is a pleasure to accept your manuscript entitled 'Isolation and characterization of twelve microsatellite loci from Rhododendron rubropilosum Hayata var. tawanalpinum using dual-suppression-PCR method' in its current form for publication in Molecular Ecology Resources. The comments of the reviewers are included below.

To enable us to include your paper in the summary article, the primers from your manuscript must be uploaded to the Molecular Ecology Resources database as soon as possible. The database is available online at http://tomato.biol.trinity.edu/. The access password is ATGCGAAT. If you need help with this process, please contact our database manager Kevin Livingstone (klivings@trinity.edu).

Please note that all PGR Note submissions are not published in Molecular Ecology Resources as individual papers. Instead, each issue will carry a summary article announcing the primers developed for each organism. The first author will be listed as “Molecular Ecology Resources Primer Development Consortium”, and contributing authors will be listed in alphabetical order thereafter. An example paper can be found http://tomato.biol.trinity.edu/micro.pdf . The text of the PGR Note itself will be made available on the Molecular Ecology Resources Primer database at http://tomato.biol.trinity.edu/, where it will be directly linked to the database entry for loci themselves.

We are always looking for suitable images for the cover of Molecular Ecology Resources. Cover images can be photographs of organisms or habitats; other possibilities are montages of photographs and/or diagrams, maps or data. If you have any candidate photographs that you would like to be considered for the cover please send the image and a brief, informative caption and photo credit (max 100 words) to editorial.office@molecol.com.

Thank you for your fine submission. On behalf of the editors of Molecular Ecology Resources, we look forward to your continued contributions to the Journal.

All the best,

Dr. Tim Vines
Managing Editor, Molecular Ecology Resources
6270 University Blvd
Vancouver, BC
V6T 1Z4
Canada
E-mail: managing.editor@molecol.com

Editor Comments to Author

The authors' explanation for my biggest concern - that the majority of the markers display a deficit of heterozygotes - is convincing. Indeed, the fact that these markers have similar behavior, may be due to population demographic status rather than technical problems regarding the allele scoring.

Therefore, I'm happy to suggest the acceptance of this paper for publication.

Prof. Albano Beja-Pereira
Subject Editor, Molecular Ecology Resources

Date Sent: 25-Oct-2011