Molecular Ecology Notes (2006) **6**, 1135–1137 doi: 10.1111/j.1471-8286.2006.01462.x

PRIMER NOTE

Characterization of six microsatellite markers in *Trillium camschatcense* using a dual-suppression-polymerase chain reaction technique

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Abstract

***Trillium camschatcense* is a herbaceous perennial plant distributed in Hokkaido and northern Honshu, Japan. Geographical variations in the breeding system (partial selfing or obligate outcrossing) are reported in the populations of Hokkaido. We isolated six polymorphic microsatellite loci from this species. The number of allele per locus ranged from four to 12, whereas the expected heterozygosities ranged from 0.69 to 0.83. These markers may allow further investigations to reveal the evolutionary and ecological processes of mating system in *T. camschatcense*.**

Keywords: dual-suppression PCR, mating system evolution, microsatellite, self-compatibility, *Trillium camschatcense*

Received 17 March 2006; revision received 10 April 2006; accepted 09 May 2006

Trillium camschatcense Ker Gawler (Trilliaceae) is a hermaphroditic perennial herb that mainly occurs in the understorey of the broad-leaved deciduous forests of Hokkaido and northern Honshu, Japan. Breeding experiments in the field (bagging, emasculation and so on) conducted at various populations in Hokkaido have detected geographical variations in the breeding system among populations; populations located in northern and southern Hokkaido are self-compatible, exploiting a mixed system of outbreeding and inbreeding, whereas eastern populations are self-incompatible, and seed production obligatory results from outbreeding (Ohara *et al*. 1996). Although the self-compatible plants are capable of both selfing and outcrossing, the mating system in natural conditions will differ among populations due to the pollinator behaviours, levels of pollen limitation and extent of inbreeding depression. The microsatellite markers developed here will allow further investigations to reveal the evolutionary and ecological processes of mating system in *T. camschatcense*.

Microsatellite regions were isolated using a dual-suppression-polymerase chain reaction (PCR) technique according to Lian & Hogetsu (2002) with some modifications.

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Total genomic DNA was extracted from the frozen leaves of *T. camschatcense* using a cetyl trimethyl ammonium bromide (CTAB) miniprep procedure (Stewart & Via 1993). To construct adaptor-ligated, restricted DNA libraries, DNA was separately digested with six restriction enzymes *Eco*RV, *SSP*I, *Alu*I, *Afa*I, *Acc*II and *Hae*III. The fragments produced by each restriction enzyme were then ligated to an unequal-length adaptor, consisting of a 48-mer and an 8-mer (see, Lian & Hogetsu 2002), using a DNA Ligation Kit (TaKaRa). The ligated fragments were further treated with ddGTP by Ampli*Taq* Gold DNA polymerase (Applied Biosystems).

As a first step, the DNA fragments with a microsatellite at one end were amplified from the *Eco*RV library using primers $(AC)_{10}$, $(AC)_6(AG)_5$, $(AG)_6(AC)_5$, $(AC)_6(TC)_5$ or $(TC)_6(AC)_5$ and an adaptor primer AP2 (shown below) which was designed from the longer strand of the adaptor. The amplified fragments ranging from 300 to 600 bp were separated using SeaPlaque GTG Agarose gel (Cambrex) and purified by GENECLEAN SPIN Kit (Qbiogene). The purified DNA fragments were cloned using QIAGEN PCR Cloning Kit, and the plasmids were transformed into *Escherichia coli* JM109 Competent Cells (TaKaRa). The cloned fragments were amplified using the M13 forward and reverse primers from the plasmid DNA of positive

Table 1 Characteristics of six microsatellite loci from *Trillium camschatcense*

Locus	Repeat motif	Primer sequences (5′–3′)	T_a (°C)	Size range (bp)	Fluorescent label	N	A	H_O	H_E	P value (HWE)	DDBJ Accession no.
TC2	$(GA)_{10}T(AG)_5$ $(AC)_{10}(AG)_{16}$	F: CCGATCAACCACATAAATAGG R: TTACTGGCTTTGATGGACAA	53.0	194–203	6-FAM	31	5	0.65	0.69	0.99	AB250298
TC15	$(GA)_{22}$	F: CATCACCCATCACCATACAC R: CGACGGGCTCTGACATC	56.0	235–279	VIC	31	12	0.81	0.83	0.26	AB250299
TC36	$(AG)_{11}(AC)_8$ $(AG)_9AC(AG)_8$	F: GTCCGAATAGTCGTCTGTCA R: GCTTTGCATGGCAGGAACT	56.0	191–205	NED	31	4	0.61	0.75	0.00*	AB250300
TC44	$(GA)_8(CA)_{11}$ $(GA)_{13}$	F: GATCGGTGATCTTCTTGAGC R: TTGAGAGGTGAACCCAGGA	53.0	230–287	VIC	31	10	0.71	0.76	0.28	AB250301
TC48	$(TC)_{15}TT(TC)_4$	F: CAACCCGCAAGTATTTCAA R: GAAATTAACTAAAGAAAGATTAGAGAGA	56.0	148–182	VIC	31	8	0.71	0.78	0.78	AB250302
TC69	$(TC)_{10}\ldots$ $(AC)_6(TC)_9$	F: TTCATTACCCCTCGTCTCTC R: CTCGTAGTGGAGTTGGAGAA	56.0	167–187	6-FAM	31	8	0.77	0.72	0.56	AB250303

T_a, annealing temperature of the primer pair; N, sample size; A, allele number; H_O, observed heterozygosity; H_E, expected heterozygosity; *indicates significant deviation from Hardy–Weinberg equilibrium (HWE) ($P < 0.001$).

clones. The PCR products were sequenced directly using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) with the ABI PRISM 3100 automated sequencer (Applied Biosystems). Six, 11, 12 and seven fragments (36 in total) containing $(AC)_6(AG)_5$, $(AG)_6(AC)_5$, $(AC)_6(TC)_5$ or $(TC)_6(AC)_5$ microsatellite sequence at one end, respectively, were chosen for the next step analysis.

As a second step, primers IP1 and IP2 were designed for the nested PCR. Primer IP1 was designed from the region flanking the microsatellite, and another primer IP2 was based on the sequence between IP1 and the microsatellite. Primers AP1 (5′-CCATCGTAATACGACTCACTATAGGGC-3′) and AP2 (5′-CTATAGGGCACGCGTGGT-3′) were also prepared as adaptor-primers. The primary nested PCR was conducted with each of six DNA libraries using primers IP1 and AP1, at the annealing temperature of 61 °C. The secondary reaction was conducted with a 100-fold dilution of the primary PCR products using primers IP2 and AP2, at the annealing temperature of 57 °C. Single-banded fragments were usually observed in some of the libraries. These products were purified by QIAquick PCR Purification Kit (QIAGEN), and then, cloned and sequenced, as described above. Primer IP3 was designed for each 26 loci from the newly identified sequence between the AP2 binding site and the microsatellite. Primers IP2 and IP3 were used as microsatellite markers.

To investigate microsatellite polymorphism, 32 leaves were collected from Niikappu (self-incompatible) population of *T. camschatcense* in Hokkaido, Japan. In the polymorphism analysis, 5′ end of each IP3 (forward) primer was tailed with U19 sequence (5′-GGTTTTCCCAGTCACGACG-3′) (Schuelke 2000; Islam *et al*. 2005). PCR amplification was performed with the GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) in a reaction mixture (10 μL) containing 0.5 μL of template DNA, 0.2 mM of each dNTP, 1× PCR buffer with 1.5 mM $MgCl_2$ (Applied Biosystems), 0.25 U of Ampli*Taq* Gold DNA polymerase (Applied Biosystems), and three primers; a forward primer with the tail of U19 (0.1 μM), a reverse nontailed primer (0.5 μM) and a fluorescent dye-labelled U19 primer (0.5 μM; Applied Biosystems). The PCR conditions were 9 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at the annealing temperature, and 1 min at 72 °C, followed by 72 °C for 7 min. The PCR products were detected with the ABI PRISM 3100 automated sequencer and GENESCAN analysis software (Applied Biosystems).

Six of 26 loci designed for *T. camschatcense* were codominant and polymorphic (Table 1). The forward primers of these loci were later labelled with fluorescent dyes (Applied Biosystems). The number of alleles per locus ranged from four to 12 per locus, with an average of 7.83 (Table 1). CERVUS version 2.0 (Marshall *et al*. 1998) was used to calculate the observed and expected heterozygosities. Statistical tests for Hardy–Weinberg equilibrium and linkage disequilibrium were conducted with GENEPOP (Raymond & Rousset 2004) using default values for the Markov chain parameters. The observed and expected heterozygosities ranged from 0.61 to 0.81 and from 0.69 to 0.83, respectively (Table 1). One locus, TC36 significantly deviated from the Hardy–Weinberg equilibrium due to an excess of homozygotes (Table 1). There was no significant linkage disequilibrium between the six loci ($\alpha = 0.05$). Six microsatellites presented here are suitable tool for genetic studies of *T. camschatcense*.

Acknowledgements

We acknowledge the considerable advice of Dr Chunlan Lian, The University of Tokyo. This study was supported in part by grants

from Suhara Memorial Foundation, the Foundation for Earth Environment, Grants-in-Aid from the Japanese Society for the Promotion of Science (JSPS) for Scientific Research (nos. 15370006 and 16370007), and the Ministry of Education, Culture, Sports, Science and Technology for the 21st Century Center of Excellence Program (E-01).

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