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PERMANENT GENETIC RESOURCES

Development of microsatellite markers in a large perennial herb, *Veratrum album* ssp. *oxysepalum*

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Abstract

We developed 11 polymorphic microsatellite [simple sequence repeat (SSR)] loci from genomic DNA of *Veratrum album* ssp. *oxysepalum* using a dual-suppression polymerase chain reaction technique and an improved method. These markers, with four to 17 alleles per locus, identified 47 genotypes in 48 samples collected from a population in Hokkaido, Japan. The observed and expected heterozygosities ranged from 0.042 to 0.938 and from 0.448 to 0.909, respectively. These SSR markers will be available to identify genets and evaluate genetic diversity of *V. album* ssp. *oxysepalum*.

Keywords: andromonoecious, dual-suppression PCR, genetic structure, mast seeding, microsatellite, vegetative reproduction

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Veratrum album L. ssp. *oxysepalum* (Turcz.) Hultén (Melanthiaceae) is a large perennial herb distributed in northeastern Asia. Flowering of this andromonoecious species is synchronized in a population, which causes mast seeding (Kelly 1994). Vegetative ramets are also produced from a rhizome of a flowering shoot that will die after seed reproduction (Tani 2005). Thus, in order to analyse the effects of such reproductive characteristics on the species' population genetic structure and reproductive success, we isolated microsatellite markers from *V. album* ssp. *oxysepalum*.

Total DNA was extracted from fresh leaves of *V. album* ssp. *oxysepalum* with a modified cetyltrimethyl ammonium bromide method (Lian *et al*. 2006). We isolated microsatellites using a dual-suppression polymerase chain reaction (PCR) method (Lian & Hogetsu 2002) and an improved technique (Lian *et al*. 2006). Briefly, in order to construct adaptor-ligated DNA libraries, DNA was digested separately using the restriction enzymes *Afa*I, *Alu*I, *Eco*RV, *Hae*III, and *Ssp*I. The fragments produced by each restriction enzyme were then ligated to an unequal-length adaptor, using TaKaRa DNA Ligation Kit version 2.1. Fragments flanked by a microsatellite at one end were amplified from the *Afa*I and *Hae*III DNA libraries using simple sequence repeat (SSR) primer $(AC)_6(AG)_5$, $(AG)_6(AC)_5$, $(AC)_6(TC)_5$ or $(TC)_6(AC)_5$ and an adaptor primer AP2 (5′-CTATAGGGC-ACGCGTGGT-3′) according to Araki *et al*. (2006). The fragments amplified from *Hae*III DNA library were cloned using PCR Cloning Kit (QIAGEN), and the plasmids were transformed into *Escherichia coli* JM109 Competent Cells (QIAGEN). The cloned fragments were amplified using the M4 forward and M13 reverse primers from the plasmid DNA of positive clones. These products were purified by QIAquick PCR Purification Kit (QIAGEN), and then, sequenced directly using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) with the ABI PRISM 3100 automated sequencer (Applied Biosystems).

For each fragment amplified by the $(AC)_6(AG)_5$, $(AG)_6(AC)_5$, $(AC)_6(TC)_5$ or $(TC)_6(AC)_5$ primer, a locus-specific primer (IP1) was designed from the sequenced region flanking the SSR. The primer pairs of IP1 primer and SSR primer were used as a SSR marker (Lian *et al*. 2006).

To analyse polymorphisms of isolated SSR markers, we collected leaves of 48 individuals from a *V. album* ssp. *oxysepalum* population in Hokkaido, Japan. PCR amplification was carried out with the GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) in a reaction mixture (10 μL) containing 5 ng 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 two primers: IP1 (0.5 μM) and the SSR primer (fluorescent dye-labelled, 0.5 μM) (Araki *et al*. 2006). The PCR condition was 9 min at 94 °C, 35 cycles of 30 s at

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Table 1 Characteristics of 11 microsatellite loci from *Veratrum album* ssp. *oxysepalum*

Locus	Repeat	Primer sequence (5′–3′) and fluorescent label	T_a (°C)	Size range (bp)	No. of alleles	H_O	H_E	GenBank Accession no.
Ve04	$(TC)_6(AC)_5$	CATGCAGAGGAATGCCACCATAGA (NED)TCTCTCTCTCTCACACACACAC	60	158–175	5	0.708	0.663	AB331671
Ve16	$(AC)_6(AG)_7$	CTTTGATATTGATGAGATGAGAC (FAM)ACACACACACACAGAGAGAGAG	53	117–123	4	0.458	0.448	AB331673
Ve20	$(AC)_6(TC)_5$	CCCGGATGGGTAGTTATTTGGTA (VIC)ACACACACACACTCTCTCTCTC	60	64–119	17	0.646	0.883*	AB331674
Ve35	$(AG)_6(AC)_5$	CCTGCTCAAGGTATCCAAAAACCG (PET)AGAGAGAGAGAGACACACACAC	60	125–148	7	0.042	0.666*	AB331675
Ve43	$(AC)_6(TC)_8$	CCTCACATACTCACAAACCTCCA (VIC)ACACACACACACTCTCTCTCTC	60	88–128	15	0.604	0.909*	AB331676
Ve48	$(AC)_6(AG)_{15}$	CACTGCATTAATGTATGTTATCAC (FAM)ACACACACACACAGAGAGAGAG	54	93–154	16	0.708	0.880	AB331667
Ve49	$(AC)_6(AG)_8$	CCCTCTGGTTCTCCCGTTTCTTGAA (FAM)ACACACACACACAGAGAGAGAG	61	75–108	14	0.938	0.835	AB331678
Ve52	$(AG)_6(AC)_6$	GGAGTCGGTGTAATCGTTATCAAGG (PET)AGAGAGAGAGAGACACACACAC	61	114–116	2	0.500	0.496	AB331679
Ve53	$(AC)_6(TC)_7$	CAAAGTGGTCCATAAACAATGAGGG (VIC)ACACACACACACTCTCTCTCTC	59	88–102	7	0.271	0.757*	AB331680
Ve54	$(AC)_6(TC)_5$	TGGTGGTTCCCGCGATGACTGTT (VIC)ACACACACACACTCTCTCTCTC	62	98–102	3	0.208	0.587*	AB331681
Ve58	$(AC)_6(AG)_6$	TCGACATGTTGTCCAGGCGTGGAT (VIC)ACACACACACACAGAGAGAGAG	63	74–109	7	0.438	0.766*	AB331682

T_a, annealing temperature of the primer pair; H_O, observed heterozygosity; H_E, expected heterozygosity. *Indicates significant deviation from Hardy–Weinberg equilibrium ($P < 0.01$).

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 an ABI PRISM 3100 automated sequencer (Applied Biosystems).

We successfully isolated 11 SSR loci with polymorphism and codominance from *V. album* ssp. *oxysepalum*. Forty-seven genotypes were detected in 48 samples analysed by these markers. The characteristics of the 11 SSR markers are shown in Table 1. These markers had a range of alleles from two to 17. The observed and expected heterozygosities calculated using FSTAT version 2.9.3 (Goudet 2001) ranged from 0.042 to 0.938 and from 0.448 to 0.909, respectively. Statistical tests for Hardy–Weinberg equilibrium (HWE) and gametic disequilibrium were conducted with GENEPOP (Raymond & Rousset 2004) using default values for the Markov chain parameters. We also estimated the frequency of null alleles using MICRO-CHECKER version 2.2.3 (Van Oosterhout *et al*. 2004). Six of the 11 loci showed significant deviation from HWE ($P < 0.01$). All these deviations were due to deficiency of heterozygosities, and the probability of null alleles at these loci were estimated to be from 0.0914 to 0.8823 ($P < 0.01$). There were no significant gametic disequilibria between the 11 loci ($P < 0.01$). These markers are currently being used to analyse the genetic structure and reproductive success of *V. album* ssp. *oxysepalum* populations in Hokkaido, Japan.

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