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

Received 29 October 2007; revision accepted 13 January 2008

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 AfaI, AluI, EcoRV, HaeIII, and SspI. 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 AfaI and HaeIII 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-ACGCCGTGGT-3') according to Araki et al. (2006). The fragments amplified from HaeIII 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 MgCl2 (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*

<table>
<thead>
<tr>
<th>Locus</th>
<th>Repeat</th>
<th>Primer sequence (5’–3’) and fluorescent label</th>
<th>(T_a) (°C)</th>
<th>Size range (bp)</th>
<th>No. of alleles</th>
<th>(H_O)</th>
<th>(H_E)</th>
<th>GenBank Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ve04</td>
<td>(TC)&lt;sub&gt;3&lt;/sub&gt;(AC)&lt;sub&gt;5&lt;/sub&gt;</td>
<td>CATCACAGAGATTTCCATCAG</td>
<td>60</td>
<td>158–175</td>
<td>5</td>
<td>0.708</td>
<td>0.663</td>
<td>AB331671</td>
</tr>
<tr>
<td>Ve16</td>
<td>(AC)&lt;sub&gt;2&lt;/sub&gt;(AG)&lt;sub&gt;7&lt;/sub&gt;</td>
<td>CTTTTGATTTAGATAATTGAC</td>
<td>53</td>
<td>117–123</td>
<td>4</td>
<td>0.458</td>
<td>0.448</td>
<td>AB331673</td>
</tr>
<tr>
<td>Ve20</td>
<td>(AC)&lt;sub&gt;2&lt;/sub&gt;(TC)&lt;sub&gt;5&lt;/sub&gt;</td>
<td>CCGAGTGGATGTTATGTTTA</td>
<td>60</td>
<td>64–119</td>
<td>17</td>
<td>0.646</td>
<td>0.883*</td>
<td>AB331674</td>
</tr>
<tr>
<td>Ve35</td>
<td>(AG)&lt;sub&gt;4&lt;/sub&gt;(AC)&lt;sub&gt;5&lt;/sub&gt;</td>
<td>CCTCTGCAAGTATCCAAACCCG</td>
<td>60</td>
<td>125–148</td>
<td>7</td>
<td>0.042</td>
<td>0.666*</td>
<td>AB331675</td>
</tr>
<tr>
<td>Ve43</td>
<td>(AC)&lt;sub&gt;4&lt;/sub&gt;(TC)&lt;sub&gt;5&lt;/sub&gt;</td>
<td>CTTTCAGATCCTCACATTC</td>
<td>60</td>
<td>88–128</td>
<td>15</td>
<td>0.604</td>
<td>0.909*</td>
<td>AB331676</td>
</tr>
<tr>
<td>Ve48</td>
<td>(AC)&lt;sub&gt;3&lt;/sub&gt;(AO)&lt;sub&gt;15&lt;/sub&gt;</td>
<td>CATTGCAATATGATATGATCAC</td>
<td>54</td>
<td>93–154</td>
<td>16</td>
<td>0.708</td>
<td>0.880</td>
<td>AB331667</td>
</tr>
<tr>
<td>Ve49</td>
<td>(AG)&lt;sub&gt;4&lt;/sub&gt;(AO)&lt;sub&gt;8&lt;/sub&gt;</td>
<td>CTTTCTCTCCCTCCCCCTGTGA</td>
<td>61</td>
<td>75–108</td>
<td>14</td>
<td>0.938</td>
<td>0.835</td>
<td>AB331678</td>
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<tr>
<td>Ve52</td>
<td>(AC)&lt;sub&gt;4&lt;/sub&gt;(AC)&lt;sub&gt;6&lt;/sub&gt;</td>
<td>GAGCTGCTGTAATTATAGGAAG</td>
<td>61</td>
<td>114–116</td>
<td>2</td>
<td>0.500</td>
<td>0.496</td>
<td>AB331679</td>
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<tr>
<td>Ve53</td>
<td>(AC)&lt;sub&gt;4&lt;/sub&gt;(TC)&lt;sub&gt;7&lt;/sub&gt;</td>
<td>CAAGATCTGATTAACATGAGGAA</td>
<td>59</td>
<td>88–102</td>
<td>7</td>
<td>0.271</td>
<td>0.757*</td>
<td>AB331680</td>
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<tr>
<td>Ve54</td>
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<td>GCTGTTGTGATGTTAGGATTC</td>
<td>62</td>
<td>98–102</td>
<td>3</td>
<td>0.208</td>
<td>0.587*</td>
<td>AB331681</td>
</tr>
<tr>
<td>Ve58</td>
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<td>TCTGATCTTTCGACTCGTCTAT</td>
<td>63</td>
<td>74–109</td>
<td>7</td>
<td>0.438</td>
<td>0.766*</td>
<td>AB331682</td>
</tr>
</tbody>
</table>

\(T_a\): 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 4 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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**Acknowledgements**

This study was supported by the JSPS for Scientific Research (nos 18405010 and 1957001107) and research fellowships of the JSPS for Young Scientists to K. Araki and S. Kubota.

**References**


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