Development of microsatellite markers for the semi-natural grassland herb Veronicastrum japonicum (Plantaginaceae)\textsuperscript{1}

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\begin{itemize}
\item \textbf{Premise of the study:} Veronicastrum japonicum (Plantaginaceae) grows in grasslands on Honshu Island, Japan, and is threatened by habitat loss because of rapid land development over recent decades. For the genetic characterization of the remaining populations, microsatellite markers were developed.
\item \textbf{Methods and Results:} Twelve polymorphic microsatellite loci were developed using next-generation sequencing. The number of alleles per locus ranged from two to 24 (mean 7.7), and the expected heterozygosity per locus ranged from 0.35 to 0.94 (mean 0.68).
\item \textbf{Conclusions:} These markers can be used for genetic studies in conservation, such as the evaluation of genetic diversity and genetic structure.
\end{itemize}

\textbf{Key words:} conservation; grassland herb; next-generation sequencing; Plantaginaceae; Veronicastrum japonicum.

Veronicastrum japonicum (Nakai) T. Yamaz. (Plantaginaceae) is a perennial plant species growing in the grasslands of mountainous regions on Honshu Island, Japan (Ohwi, 1978). In recent decades, the number of V. japonicum populations has rapidly decreased, and this species is now classified as near threatened or higher in five of the 47 Prefectural Red Lists of Japan (Association of Wildlife Research and EnVision, 2007). The decline of V. japonicum populations can be attributed to shrinking grasslands resulting from land development or the abandonment of traditional management. Furthermore, serious grazing damage by deer, which has explosively increased in the past few decades, has threatened V. japonicum populations (Takatsuksi, 2009; Nagaie et al., 2014). Veronicastrum japonicum is also the main larval food plant species of Melitaea ambigua (Butler) (Nymphalidae), a rare butterfly species classified as endangered in the Japanese Red List (Ministry of the Environment of Japan, 2012). Accordingly, knowledge of the genetic diversity and structure of V. japonicum populations is important for the conservation management of V. japonicum and M. ambigua. Thus, 12 nuclear microsatellite markers for V. japonicum were developed using next-generation sequencing.

\section*{METHODS AND RESULTS}

A fresh leaf sample of V. japonicum was collected from an individual plant growing in Gunma Prefecture, Japan, and genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Germantown, Maryland, USA). A DNA fragment library was constructed with an Ion Xpress Plus Fragment Library Kit (Life Technologies, Foster City, California, USA), amplified with an Ion PGM Template OT2 400 Kit, and subsequently sequenced with an Ion PGM Sequencing 400 Kit and an Ion 318 Chip v2 (both from Life Technologies), yielding 161,880 reads (size range 8–618 bp; mean 220 bp). The reads were screened using Primer3 software (Rozen and Skaletsky, 1999) embedded in MSATCOMMANDER version 0.8.2 (Faircloth, 2008) to identify dinucleotide and trinucleotide loci of at least eight and seven repeats, respectively. After screening, 284 putative microsatellite loci were identified. Using these loci, a total of 57 primer pairs with melting temperatures (T\textsubscript{m}) of 57–61°C, GC content of 35–75%, and fragment sizes of 150–350 bp were designed.

Amplification and specificity of 34 out of the 57 primer pairs were tested using four V. japonicum individuals. For all confirmed microsatellite loci, the forward primers were synthesized using one of the three different M13 sequences, 5\textsuperscript{'}-CACGACCGTTGAATACGAC-3\textsuperscript{′}, 5\textsuperscript{'}-CTATAGGGACCGTGTTG-3\textsuperscript{′}, or 5\textsuperscript{'}-TGTGGAATTGTGAGCGG-3\textsuperscript{′}. One specimen from each population was collected and dehydrated for assistance with the sampling. We also thank Tomohiro Furuta for help with making the voucher specimens.

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Table 1. Characteristics of microsatellite loci for Veronicastrum japonicum. All values are based on 48 samples from two populations in Gunma and Nagano prefectures in Japan.a

| Locus    | Primer sequences (5′–3′) | Repeat motif | Fluorescent labelb | Allele size range (bp) | GenBank accession no. |
|----------|--------------------------|--------------|--------------------|------------------------|-----------------------|
| Veja004  | F: AGGAGCCGCTGTTGCTAC    | (CT)9        | FAM                | 211–221                | LC030210              |
|          | R: CAAATGCTTAATGCTGACC   |              |                    |                        |                       |
| Veja005  | F: CCCTCTTCTGGAATTTGAGC  | (TGT)6       | VIC                | 232–237                | LC030211              |
|          | R: ACAGCTCTCTCTCTCTCTCC  |              |                    |                        |                       |
| Veja007  | F: GGGACATTGGAAGGAGGC    | (TCT)6       | VIC                | 200–221                | LC030214              |
|          | R: AATCTGCTGTTGCTGCCG    |              |                    |                        |                       |
| Veja009  | F: CATTAAATATCTCTCAGTTCC | (CT)6        | NED                | 233–253                | LC030213              |
|          | R: GCTCTCTTCAAGAGTTCCC   |              |                    |                        |                       |
| Veja011  | F: TGAAGCTGCTGACGTTCC    | (GCT)6       | VIC                | 200–221                | LC030214              |
|          | R: AACCTGAGCTGCTGCCG     |              |                    |                        |                       |
| Veja012  | F: TTCCCTTCACCAACAGGGG   | (GT)6        | NED                | 308–332                | LC069371              |
|          | R: AACAGGACCTGCTGCTCGAG  |              |                    |                        |                       |
| Veja015  | F: CTGCCCGACCTTTAGATTCCC| (TC)6        | NED                | 187–193                | LC030215              |
|          | R: GTCCCTTCACAACTGCTCCC  |              |                    |                        |                       |
| Veja018  | F: ACAACATCTTGGCCAAACCC  | (GA)12       | NED                | 251–321                | LC030216              |
|          | R: ACAACGCCTTTATGACTTAC  |              |                    |                        |                       |
| Veja021  | F: TGACACATCAACAGATTCCC  | (TAT)7       | NED                | 194–209                | LC030217              |
|          | R: TCCTGATTCAACAGGAGACC  |              |                    |                        |                       |
| Veja025  | F: AGCCATCAACATATTACGAC  | (CA)9        | FAM                | 173–192                | LC030218              |
|          | R: ACCGGTAGAGGCTGTTGAGCC |              |                    |                        |                       |
| Veja029  | F: GCACAGCGTTAAGAGAGCC   | (TGT)6       | VIC                | 178–196                | LC030219              |
|          | R: CGTGACACGCGCATACCC    |              |                    |                        |                       |
| Veja032  | F: CACGCGACGCGCATACAC    | (GT)10       | VIC                | 238–265                | LC030220              |
|          | R: TTTGAGAGGAAAGGACCCA   |              |                    |                        |                       |

aAnnealing temperature was 57°C for all loci.
bSequence of the fluorescent labels: FAM = 5′-CACGACGTTGTAAAACGAC-3′, NED = 5′-CTATAGGGCACGCGTGGT-3′, VIC = 5′-TGTGGAATTGTGAGCGG-3′.

(mean 0.66) and from 0.35 to 0.94 (mean 0.68), respectively (Table 2). Only one locus (Veja009) in the Gunma population showed significant deviation from HWE (P < 0.05). Significant LD was found in the Nagano population for only one pair of loci (Veja004 and Veja021; P < 0.05).

CONCLUSIONS

The microsatellite markers described in this study will be useful for conservation genetic studies of Veronicastrum japonicum, such as those evaluating genetic diversity and structure within and between populations. Assessment of their genetic information will also contribute to elucidating how Veronicastrum japonicum genetic diversity and structure has been affected by declining populations and habitat management practices. Understanding the impacts of habitat management will help the conservation of both Veronicastrum japonicum and the endangered butterfly M. ambigua, because maintenance of Veronicastrum japonicum population size is crucial for survival of this butterfly species.

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Table 2. Results for primer screening of all samples for 12 microsatellite loci in two populations of Veronicastrum japonicum.a,b

| Locus    | A  | H0  | H1  | FIS  | A  | H0  | H1  | FIS  |
|----------|----|-----|-----|------|----|-----|-----|------|
| Veja004  | 7  | 0.81| 0.75| −0.09| 5  | 0.48| 0.59| 0.19 |
| Veja005  | 2  | 0.43| 0.39| −0.11| 3  | 0.37| 0.60| 0.39 |
| Veja007  | 6  | 0.62| 0.71| 0.13 | 6  | 0.70| 0.65| −0.08| 12  |
| Veja009  | 8  | 0.48| 0.70| 0.32 | 8  | 0.70| 0.80| 0.12 |
| Veja011  | 6  | 0.62| 0.53| −0.17| 5  | 0.59| 0.64| 0.08 |
| Veja012  | 13 | 0.86| 0.82| −0.05| 12 | 0.78| 0.77| −0.01| 12  |
| Veja015  | 4  | 0.52| 0.67| 0.22 | 4  | 0.30| 0.35| 0.15 |
| Veja018  | 18 | 0.95| 0.91| −0.04| 24 | 1.00| 0.94| −0.06| 24  |
| Veja021  | 7  | 0.62| 0.73| 0.15 | 6  | 0.67| 0.71| 0.06 |
| Veja025  | 9  | 0.57| 0.67| 0.14 | 10 | 0.93| 0.85| −0.09| 9   |
| Veja029  | 5  | 0.52| 0.52| −0.01| 3  | 0.70| 0.60| −0.17| 5   |
| Veja032  | 5  | 0.86| 0.73| −0.17| 9  | 0.63| 0.67| 0.06 |

Note: A = number of alleles; FIS = fixation index; H1 = expected heterozygosity; H0 = observed heterozygosity; N = sample size.

aPopulations: Gunma Prefecture: 36°26′12″N, 138°25′27″E; Nagano Prefecture: 35°57′25″N, 137°37′49″E. Vouchers deposited in the Kyoto University Museum herbarium (accession numbers: KYO_00019997 and KYO_00019998, respectively).

bNumbers in boldface show deviations from Hardy–Weinberg equilibrium (P < 0.05).

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