DEVELOPMENT AND EVALUATION OF MICROSATELLITE MARKERS FOR Acer miyabei (Sapindaceae), a Threatened Maple Species in East Asia

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• Premise of the study: Twelve microsatellite markers were developed and characterized in a threatened maple species, Acer miyabei (Sapindaceae), for use in population genetic analyses.

• Methods and Results: Using Ion Personal Genome Machine (PGM) sequencing, we developed microsatellite markers with perfect di- and trinucleotide repeats. These markers were tested on a total of 44 individuals from two natural populations of Acer miyabei subsp. miyabei f. miyabei in Hokkaido Island, Japan. The number of alleles per locus ranged from two to eight. The observed and expected heterozygosities per locus ranged from 0.05 to 0.75 and from 0.05 to 0.79, respectively. Some of the markers were successfully transferred to the closely related species A. campestre, A. platanoides, and A. pictum.

• Conclusions: The developed markers will be useful in characterizing the genetic structure and diversity of Acer miyabei and will help to understand its spatial genetic variation, levels of inbreeding, and patterns of gene flow, thereby providing a basis for conservation.

Key words: Acer miyabei; Ion PGM sequencing; maple; microsatellite; Sapindaceae; threatened species.

Acer miyabei Maxim. (Sapindaceae) is a deciduous tree species that grows in temperate forests in East Asia. The species comprises three infraspecific taxa: A. miyabei Maxim. subsp. miyabei f. miyabei, A. miyabei subsp. miyabei f. shibatae (Nakai) K. Ogata, and A. miyabei subsp. miaotaiense (Tsongo) A. E. Murray. Each subspecies has a characteristic distribution (Ogata, 1965; van Gelderen et al., 1994). Acer miyabei subsp. miyabei f. miyabei grows in Hokkaido and northern and central Honshu, Japan. Its occurrence is strongly associated with river floodplain ecosystems, and some of the isolated southern populations are considered a relic of glacial times. Acer miyabei subsp. miyabei f. shibatae is also endemic to Japan, although its range is restricted to parts of Honshu. Acer miyabei subsp. miaotaiense was found in 1954 in Shaanxi Province in northwestern China (Tsongo, 1954). The discovery of this taxon is important because its distribution is likely a biogeographic stepping stone to A. campestre L., a morphologically similar European species (Ogata, 1967). Yet, the phylogenetic relationships among the subspecies, forms, and their related species have not been examined at the molecular level. Because of their limited range and habitat decline, all three infraspecific taxa of A. miyabei are listed in national or IUCN Red Lists (Ministry of the Environment, Government of Japan, 2012; IUCN, 2014). Natural populations of A. miyabei in Japan are typically fragmented by urban and rural development, which affects seed production and gene flow (Hotta, 2004; Nagamitsu et al., 2014).

Here, we present 12 microsatellite markers for A. miyabei to facilitate evolutionary and conservation studies. These markers were developed from two forms of A. miyabei subsp. miyabei, and tested on two natural populations of A. miyabei subsp. miyabei f. miyabei and an individual of A. miyabei subsp. miaotaiense. We also examined the transferability of the markers to three species that belong to the same section (sect. Platanoidae) as A. miyabei (Renner et al., 2007; Grimm and Denk, 2014): A. campestre, A. platanoides L., and A. pictum Thunb.

METHODS AND RESULTS

Microsatellite markers were developed for A. miyabei with an Ion Personal Genome Machine (PGM; Life Technologies, Carlsbad, California, USA). Library preparation, PGM sequencing, and genotyping were conducted at the Sugadaira Montane Research Center, University of Tsukuba, Japan. Total genomic DNA was extracted from dried leaves of a single A. miyabei subsp. miyabei f. miyabei individual from Sugadaira with a DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). The voucher specimen was stored at the Herbarium of Sugadaira Montane Research Center (no. 05507). The concentration of genomic DNA was determined with a Qubit 2.0 Fluorometer (Life Technologies). Microsatellite markers were kindly provided by the University of British Columbia Botanical Garden. This research was funded by the Japan Society for the Promotion of Science (grant no. 25890002) and the Fujiwara Natural History Foundation.

doi:10.3732/apps.1500020

1 Manuscript submitted 27 February 2015; revision accepted 24 April 2015.

The authors thank S. Yamaguchi, Y. Yamaguchi, O. Harada, R. Oyama, Dr. S. Kondoh, Dr. T. Hiura, Dr. T. Nagamitsu, Dr. H. Matsumura, and Dr. B. V. Barnes for their valuable support. The leaf specimen of Acer miyabei subsp. miaotaiense was kindly provided by the University of British Columbia Botanical Garden. This research was funded by the Japan Society for the Promotion of Science (grant no. 25890002) and the Fujiwara Natural History Foundation.

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and purification of the ligated DNA were conducted with an Ion Plus Fragment Library Kit (Life Technologies). Fragments of 300–350 bp were selected with an E-Gel Agarose Gel Electrophoresis System (Life Technologies), followed by library amplification with an Ion Plus Fragment Library Kit. The library was assessed and quantified with a BioAnalyzer (Agilent Technologies, Palo Alto, California, USA), and then diluted to 26 pM for template preparation. The library was enriched with an Ion PGM Template OT2 400 kit (Life Technologies) and sequenced with an Ion PGM Sequencing 400 Kit (Life Technologies) according to the manufacturer’s protocol. Single processing and base calling were performed using Torrent Suite 3.6 (Life Technologies), and a library-specific FASTQ file was generated. A total of 557,106 reads were obtained and registered in the DNA Data Bank of Japan (DDBJ) Sequence Read Archive (DRA001873).

The data sets were collated and applied to the QDD bioinformatics pipeline (Meglécz et al., 2010) to filter sequences containing microsatellites with appropriate flanking sequences to define PCR primers. QDD detected 4909 loci, each containing a microsatellite consisting of at least five repeats. Based on this information, we chose 58 primer pairs for loci consisting of either di- or trinucleotide repeats. For initial primer screening by PCR, we used four DNA samples from three A. miyabei subsp. miyabei individuals from the Bibi, Kushiro, and Sugadaira populations and one A. miyabei subsp. miyabei f. shibatae individual from the Sugadaira population (Appendix 1).

Each forward primer was labeled with either FAM, HEX, or TAMRA fluorescent dye. We also prepared unlabeled forward primers and mixed them with fluorescent ones. The ratio was initially set at 1 (fluorescent) to 24 (unlabeled) but was changed later as described below, following Suyama (2012). All reverse primers used in PCR contained a fluorescent dye and were labeled with either FAM, HEX, or TAMRA.

### Table 1. Characteristics of 12 polymorphic microsatellite markers developed for *Acer miyabei*.

| Locus | Primer sequences (5’-3’) | Repeat motif | Allele size (bp) | $T_\text{a}$ (°C) | Fluorescent dye<sup>a</sup> (Multiplex set no.) | Primer ratio<sup>b</sup> | GenBank accession no. |
|-------|-------------------------|--------------|------------------|-----------------|--------------------------------|--------------------------|----------------------|
| Acni2 | F: TCACTCCACCTCTCTCT+CTCA | (CT)<sub>15</sub> | 108 | 60 | HEX (1) | 1:39 | KP825168 |
| Acni3 | F: GTTCTCTGAGAAATGTTTGTG | (AG)<sub>16</sub> | 147 | 60 | HEX (2) | 1:39 | KP825169 |
| Acni4 | F: GCATATGATGGTAGTGGCAAA | (AG)<sub>14</sub> | 151 | 60 | PET (2) | 1:39 | KP825170 |
| Acni5 | F: TACAGGCTGTTGAATGTC | (AT)<sub>12</sub> | 226 | 60 | HEX (2) | 1:39 | KP825172 |
| Acni6 | F: TGGAGAAAGAGAGAGGAG | (AG)<sub>10</sub> | 137 | 60 | FAM (2) | 1:1.5 | KP825176 |
| Acni7 | F: CATTGCTGATTCATCATTCA | (TCT)<sub>11</sub> | 111 | 60 | FAM (1) | 1:79 | KP825175 |
| Acni8 | F: TTTCCCTGCAACATGTTTGTG | (CT)<sub>12</sub> | 217 | 60 | HEX (1) | 1:4 | KP825177 |
| Acni9 | F: TTTCTGTAATGCTGATGCG | (AT)<sub>10</sub> | 218 | 60 | PET (1) | 1:0.25 | KP825178 |
| Acni10 | F: AACAGTACACCACATCTTATCAG | (AT)<sub>10</sub> | 218 | 60 | PET (1) | 1:0.25 | KP825178 |
| Acni11 | F: TCTACTAGCATAAACCAAC | (AT)<sub>10</sub> | 268 | 60 | PET (2) | 1:9 | KP825179 |

<sup>a</sup>Fluorescent label used for two sets of multiplex PCR.

<sup>b</sup>Ratio of fluorescent and unlabeled forward primers for multiplex PCR. See text for details.

### Table 2. Genetic diversity of 12 microsatellite loci in two natural populations of *Acer miyabei* (Bibi and Kyouwa) in Hokkaido, Japan.

| Locus | Bibi (n = 22) | Kyouwa (n = 22) | Overall (n = 44) |
|-------|--------------|----------------|-----------------|
| Acni2 | 3 | 0.500 | 0.637 | 0.119 | 4 | 0.364 | 0.388 | −0.000 | 5 | 0.432 | 0.560 | 0.134 | 110−122 |
| Acni3 | 4 | 0.909 | 0.754 | −0.105 | 3 | 0.591 | 0.63 | 0.038 | 4 | 0.750 | 0.738 | −0.013 | 134−149 |
| Acni10 | 4 | 0.818 | 0.698 | −0.090 | 3 | 0.364 | 0.369 | −0.012 | 5 | 0.591 | 0.581 | −0.001 | 153−181 |
| Acni11 | 6 | 0.818 | 0.789 | −0.034 | 5 | 0.455 | 0.508 | 0.061 | 8 | 0.636*** | 0.774 | 0.103 | 159−179 |
| Acni23 | 2 | 0.409 | 0.333 | −0.113 | 2 | 0.409 | 0.511 | 0.099 | 2 | 0.409 | 0.468 | 0.062 | 225−228 |
| Acni28 | 2 | 0.364 | 0.406 | 0.044 | 3 | 0.409 | 0.443 | 0.085 | 3 | 0.386 | 0.557 | 0.184 | 274−284 |
| Acni29 | 3 | 0.682 | 0.524 | −0.151 | 4 | 0.136 | 0.133 | −0.026 | 4 | 0.409 | 0.364 | −0.066 | 266−280 |
| Acni33 | 2 | 0.091 | 0.089 | −0.014 | 1 | 0 | 0 | — | 2 | 0.045 | 0.045 | −0.004 | 100−103 |
| Acni38 | 3 | 0.591 | 0.545 | −0.052 | 4 | 0.864* | 0.701 | −0.131 | 4 | 0.727 | 0.673 | −0.040 | 130−136 |
| Acni45 | 5 | 0.500* | 0.682 | 0.131 | 6 | 0.810 | 0.769 | −0.041 | 8 | 0.651* | 0.790 | 0.080 | 211−229 |
| Acni46 | 4 | 0.864 | 0.687 | −0.125 | 6 | 0.636 | 0.643 | −0.004 | 7 | 0.750 | 0.681 | −0.055 | 218−230 |
| Acni53 | 3 | 0.409 | 0.464 | 0.081 | 3 | 0.682 | 0.63 | −0.063 | 3 | 0.545 | 0.565 | 0.009 | 269−273 |

**Note:** $A$ = number of alleles; $H_e$ = expected heterozygosity; $H_o$ = observed heterozygosity; Null = null allele frequency estimate (Marshall et al., 1998; Kalinowski et al., 2007).

* Asterisks indicate significant deviation from Hardy–Weinberg equilibrium after Bonferroni correction (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Note that there were no deviations at the $P < 0.01$ level.
primers were PIG-tailed by adding GTTTCTT to obtain consistent addition of adenine by Taq DNA polymerase (Brownstein et al., 1996). DNA (ca. 10 ng) was placed into wells of 96-well plates and dried at room temperature over several hours. Singleplex PCR was performed with a single pair of primers in 2 μl of 1× Type-It Multiplex PCR Kit Master Mix (QIAGEN) and 0.2 μM of each primer, overlaid with 6 μl of mineral oil as described in Kenta et al. (2008). The thermal cycler program was 95°C for 5 min; followed by 35 cycles of 95°C for 30 s, 60°C for 90 s, and 72°C for 30 s, and 72°C for 30 min. PCR products were mixed with 0.25 μl of GeneScan 500 LIZ Size Standard (Applied Biosystems) and 9.25 μl of Hi-Di formamide (Applied Biosystems). Samples were run on an ABI 3130 Genetic Analyzer (Applied Biosystems), and PCR products were examined in GeneMapper ver. 4.0 (Applied Biosystems). If fluorescent signal intensity was too high or too low, the ratio of the fluorescent forward primer to the unlabeled one was optimized (Table 1). However, even at high ratios of fluorescent forward primers, products labeled with TAMRA were relatively poorly detectable, and thus we excluded the corresponding loci. Screening resulted in 18 primer pairs that consistently amplified clear bands. High ratios of fluorescent forward primers, products labeled with TAMRA were nearly zero or negative (Appendix 1). Polymorphic variation was consistently detected in 10 microsatellite loci in A. miyabei. These markers will help to characterize the genetic structure and diversity of the species. They will also help to understand its spatial genetic variation, levels of inbreeding, and patterns of gene flow, thereby providing a basis for conservation. Some of the markers were successfully transferred to closely related species. High transferability to A. campestre agrees with its morphological similarity to A. miyabei.

### Table 3. Cross-amplification of 12 microsatellite loci in species closely related to *Acer miyabei.*

| Locus | A. campestre (n = 4) | A. platanoides (n = 4) | A. pictum (n = 4) |
|-------|---------------------|------------------------|-----------------|
|       | A | H<sub>e</sub> | H<sub>o</sub> | A | H<sub>e</sub> | H<sub>o</sub> | A | H<sub>e</sub> | H<sub>o</sub> |
| Acmi2 | 5 | 0.500 | 0.857 | 5 | 0.750 | 0.857 | 5 | 0.750 | 0.786 |
| Acmi8 | — | — | — | — | — | — | — | — | — |
| Acmi10 | 5 | 0.500 | 0.857 | 2 | 0 | 0.571 | — | — | — |
| Acmi11 | 5 | 0.750 | 0.893 | 3 | 0.750 | 0.607 | — | — | — |
| Acmi23 | — | — | — | — | — | — | — | — | — |
| Acmi28 | 5 | 1.000 | 0.857 | — | — | — | — | — | — |
| Acmi33 | 4 | 0.500 | 0.821 | 4 | 0.750 | 0.750 | 4 | 0.500 | 0.786 |
| Acmi38 | 2 | 0 | 0.533 | — | — | — | — | — | — |
| Acmi45 | 5 | 0.750 | 0.786 | — | — | — | 3 | 0.250 | 0.750 |
| Acmi46 | 3 | 0.500 | 0.679 | 3 | 1.000 | 0.750 | — | — | — |
| Acmi53 | 2 | 0.250 | 0.250 | 3 | 1.000 | 0.750 | 6 | 1.000 | 0.929 |
| Average | 3.800 | 0.525 | 0.696 | 3.400 | 0.650 | 0.707 | 4.500 | 0.625 | 0.813 |

Note: — = amplification failed or nonspecific (three or more polymorphic bands detected); A = number of alleles; H<sub>e</sub> = expected heterozygosity; H<sub>o</sub> = observed heterozygosity.

*Testing for Hardy–Weinberg equilibrium and estimation of null allele frequency were not performed because of small sample sizes.

### CONCLUSIONS

Using next-generation sequencing with the Ion PGM system, we developed 12 microsatellite markers for the threatened maple *A. miyabei*. These markers will help to characterize the genetic structure and diversity of the species. They will also help to understand its spatial genetic variation, levels of inbreeding, and patterns of gene flow, thereby providing a basis for conservation. Some of the markers were successfully transferred to closely related species. High transferability to *A. campestre* agrees with its morphological similarity to *A. miyabei*.

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APPENDIX 1. Voucher information for species used in the development and evaluation of microsatellite markers for Acer miyabei.

| Taxon | Population | Location | Geographic coordinates | N | Voucher no.* |
|-------|------------|----------|------------------------|---|--------------|
| A. miyabei Maxim. subsp. miyabei f. miyabei | Bibi | Bibi, Chitose, Hokkaido, Japan | 42.80°N, 141.72°E | 22 | IOS10138–IOS10159 |
| | Kyouwa | Kyouwa, Chitose, Hokkaido, Japan | 42.88°N, 141.76°E | 22 | IOS10160–IOS10181 |
| | Kushiro | Onbetsu, Kushiro, Hokkaido, Japan | 43.00°N, 143.89°E | 1 | IOS10182 |
| | Sugadaira | Sugadaira, Ueda, Nagano, Japan | 36.52°N, 138.34°E | 1 | IOS10183 |
| | | | 36.53°N, 138.31°E | 1 | IOS10184 |
| A. miyabei subsp. miyabei f. shibatae (Nakai) K. Ogata | Cultivar | University of British Columbia Botanical Garden, Vancouver, Canada. (Living specimen grown from seeds collected in Tianshui, Gansu, China.) | — | 1 | NACPEC11-064 |
| A. campestre L. | Tiefenbronn | Tiefenbronn, Germany | 48.82°N, 8.80°E | 1 | IOS10185 |
| | Mühlhausen | Mühlhausen, Germany | 48.80°N, 8.82°E | 1 | IOS10186 |
| | Lichtenstein Strasse | Traifelberg, Germany | 48.41°N, 9.27°E | 1 | IOS10187 |
| | Kandern | Johannes-August-Sutter Strasse, Kandern, Germany | 47.71°N, 7.67°E | 1 | IOS10188 |
| A. platanoides L. | Pforzheim | Pforzheim, Germany | 48.87°N, 8.72°E | 1 | IOS10189 |
| | Stuttgart-Weilimdorf | Stuttgart-Weilimdorf, Germany | 48.82°N, 9.12°E | 1 | IOS10190 |
| | Château du Haut | Château du Haut Koenigsbourg, France | 48.25°N, 7.34°E | 1 | IOS10191 |
| | Koenigsbourg | Stoffelberg, Germany | — | 1 | IOS10192 |
| A. pictum Thunb. | Ikawa | Ikawa University Forest (University of Tsukuba), Shizuoka, Japan | 35.34°N, 138.23°E | 2 | IOS10193–IOS10194 |
| | Yatsugatake | Yatsugatake University Forest (University of Tsukuba), Nagano, Japan | 35.93°N, 138.50°E | 1 | IOS10195 |
| | Shizunai | Hokkaido University Shizunai Livestock Farm, Hokkaido, Japan | 42.43°N, 142.480°E | 1 | IOS10196 |

Note: — = unknown; N = number of samples.

*All vouchers except for Acer miyabei subsp. miaotaiense were deposited at Makino Herbarium (MAK), Tokyo Metropolitan University, Japan. Acer miyabei subsp. miaotaiense is a living specimen.