Development and Evaluation of Microsatellite Markers for Acer miyabei (Sapindaceae), a Threatened Maple Species in East Asia

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Source: Applications in Plant Sciences, 3(6)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1500020

Applications in Plant Sciences

Applications in Plant Sciences 2015 3(6): 1500020

Primer Note

Development and evaluation of microsatellite markers for *Acer miyabei* (Sapindaceae), a threatened maple species in East Asia[1]

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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 *A. 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 *A. 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* (Tsoong) 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 (Tsoong, 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. *Platanoidea*) 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).

The genomic DNA (100 ng) was sheared into fragments of 350–450 bp with Ion Shear Plus Reagents (Life Technologies), and adapter ligation, nick repair,

[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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doi:10.3732/apps.1500020

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

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size (bp)	T_a (°C)	Fluorescent dye[a] (Multiplex set no.)	Primer ratio[b]	GenBank accession no.
Acmi2	F: TCACATCCACTCTCTCTTTCACA	$(CT)_{15}$	108	60	HEX (1)	1 : 39	KP825168
	R: GTTTCTTCAGCAGCAACAACAACAACA						
Acmi8	F: CGCAGTTGACTGGTGTTCTC	$(AG)_{16}$	147	60	HEX (2)	1 : 39	KP825169
	R: GTTTCTTCGACGTTGTTTGGAGTTAAACC						
Acmi10	F: GCATATTGAGATGGTGGCAA	$(AG)_{14}$	151	60	PET (2)	1 : 39	KP825170
	R: GTTTCTTGGTTCACATTCTTCATCCTG						
Acmi11	F: CATTTGCACATCACCACCTC	$(TC)_{14}$	159	60	NED (1)	1 : 39	KP825171
	R: GTTTCTTGTGAGACATACATAACAGAGCAGA						
Acmi23	F: TAGACGGTTGGTGGTGATCC	$(AAT)_{12}$	226	60	HEX (2)	1 : 39	KP825172
	R: GTTTCTTAGAACACAACAAAGCCCAGG						
Acmi28	F: TTGCAAAGAATGTTAGTTTAGGATTG	$(GT)_{13}$	276	60	FAM (2)	1 : 19	KP825173
	R: GTTTCTTCCAACTGTGGTAATGCACC						
Acmi29	F: CAGATCCGTGCATTTCAATC	$(AG)_{16}$	282	60	HEX (2)	1 : 19	KP825174
	R: GTTTCTTTCCTCGTTCTCATCACTCA						
Acmi33	F: CCACAAATCTCCTCTGCCAT	$(TCT)_{11}$	111	60	FAM (1)	1 : 79	KP825175
	R: GTTTCTTGTAGAAACAAATTGGAACCCA						
Acmi38	F: TGAGAGAAGAGAGGAGGAGGG	$(AG)_{10}$	137	60	FAM (2)	1 : 1.5	KP825176
	R: GTTTCTTGCATAAATCTCCAGCAAATGG						
Acmi45	F: CATCTCTTGTGCATTGTTTGTG	$(AT)_{10}$	217	60	HEX (1)	1 : 4	KP825177
	R: GTTTCTTGTAATGAGATCGAGTGATCGG						
Acmi46	F: AACAGGTACACCATGTTTATGGC	$(AT)_{10}$	218	60	PET (1)	1 : 0.25	KP825178
	R: GTTTCTTGGGATAAAGGCCCTTAGGA						
Acmi53	F: TCTATGCATAAGCCAAGTCCC	$(AT)_8$	268	60	PET (2)	1 : 9	KP825179
	R: GTTTCTTAGGGTGGATTGTTGGTCAAT						

Note: T_a = annealing temperature used in PCR.

[a] Fluorescent label used for two sets of multiplex PCR.

[b] Ratio of fluorescent and unlabeled forward primers for multiplex PCR. See text for details.

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) by using 850 flows on Ion 314 Chip V2 (Life Technologies) according to the manufacturer's protocol. Single processing and base calling were performed with 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* f. *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

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

	Bibi (n = 22)				Kyouwa (n = 22)				Overall (n = 44)				
Locus	A	H_o[a]	H_e	Null	A	H_o[a]	H_e	Null	A	H_o[a]	H_e	Null	Allele size range (bp)
Acmi2	3	0.500	0.637	0.119	4	0.364	0.388	–0.001	5	0.432	0.560	0.134	110–122
Acmi8	4	0.909	0.754	–0.105	3	0.591	0.63	0.038	4	0.750	0.738	–0.013	134–149
Acmi10	4	0.818	0.698	–0.090	3	0.364	0.369	–0.012	5	0.591	0.581	–0.001	153–181
Acmi11	6	0.818	0.789	–0.034	5	0.455	0.508	0.061	8	0.636***	0.774	0.103	159–179
Acmi23	2	0.409	0.333	–0.113	2	0.409	0.511	0.099	2	0.409	0.468	0.062	225–228
Acmi28	2	0.364	0.406	0.044	3	0.409	0.443	0.085	3	0.386	0.557	0.184	274–284
Acmi29	3	0.682	0.524	–0.151	4	0.136	0.133	–0.026	4	0.409	0.364	–0.066	266–280
Acmi33	2	0.091	0.089	–0.014	1	0	0	—	2	0.045	0.045	–0.004	100–103
Acmi38	3	0.591	0.545	–0.052	4	0.864*	0.701	–0.131	4	0.727	0.673	–0.040	130–136
Acmi45	5	0.500*	0.682	0.131	6	0.810	0.769	–0.041	8	0.651*	0.790	0.080	211–229
Acmi46	4	0.864	0.687	–0.125	6	0.636	0.643	–0.004	7	0.750	0.681	–0.055	218–230
Acmi53	3	0.409	0.464	0.081	3	0.682	0.63	–0.063	3	0.545	0.565	0.009	269–273
Average	3.417	0.580	0.551	–0.026	3.667	0.477	0.477	0.000	4.583	0.528	0.566	0.033	

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).

[a] Asterisks indicate significant deviation from Hardy–Weinberg equilibrium after Bonferroni correction (*$P < 0.05$, **$P < 0.01$, ***$P \leq 0.001$). Note that there were no deviations at the $P < 0.01$ level.

TABLE 3. Cross-amplification of 12 microsatellite loci in species closely related to *Acer miyabei*.[a]

	A. campestre (n = 4)			*A. platanoides* (n = 4)			*A. pictum* (n = 4)		
Locus	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e
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	2	0.500	0.429	—	—	—	—	—	—
Acmi29	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	—	—	—	—	—	—
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_e = expected heterozygosity; H_o = observed heterozygosity.

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

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 Microsatellite 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. Eight of these primer pairs were newly labeled with PET or NED fluorescent dyes for performing two sets of multiplex-PCR reactions. PCR mixtures and the thermal cycler program were as described above. Multiplex amplification was successful under such conditions, and we finally tested DNA of 44 samples from two natural populations of *A. miyabei* subsp. *miyabei* f. *miyabei* at Bibi and Kyouwa in Chitose city, Hokkaido, Japan (Appendix 1). In these samples, nonspecific bands with three or more peaks were detected for six microsatellite loci. Thus, we consider the remaining 12 primer pairs (Table 1) as useful. No known genes were detected around the corresponding markers by BLAST searches with read sequence data.

For those markers, the mean number of alleles per locus was 3.42 in the Bibi population and 3.67 in the Kyouwa population (Table 2). For the Bibi population, the mean observed and expected heterozygosities per locus were 0.58 (range: 0.09–0.91) and 0.55 (0.09–0.79), respectively; for the Kyouwa population, the corresponding values were 0.48 (0.00–0.86) and 0.48 (0.00–0.77). For the two populations combined, the number of alleles per locus ranged from two to eight, whereas the observed and expected heterozygosities per locus were 0.05–0.75 and 0.05–0.79, respectively. These statistics were computed by CERVUS 3.0.7 (Marshall et al., 1998; Kalinowski et al., 2007). Deviations from Hardy–Weinberg equilibrium were tested with GENEPOP software (version 4.2; Raymond and Rousset, 1995). Significant deviations after Bonferroni correction ($P < 0.05$) were detected for the loci Acmi45 in Bibi and Acmi38 in Kyouwa (Table 2). Null allele frequencies estimated with CERVUS 3.0.7 (Marshall et al., 1998; Kalinowski et al., 2007) were nearly zero or negative except for Acmi2 and Acmi45 in the Bibi population. Cross-amplifications were carried out to test marker transferability to closely related taxa. All of the 12 loci were amplified with clear bands in a sample of *A. miyabei* subsp. *miaotaiense* (Appendix 1). Polymorphic variation was consistently detected in 10 microsatellite loci in *A. campestre*, five in *A. platanoides*, and four in *A. pictum* (Table 3). The result agrees with a morphological similarity between *A. miyabei* and *A. campestre* as demonstrated by Ogata (1967).

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]
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
A. miyabei subsp. *miyabei* f. *shibatae* (Nakai) K. Ogata	Sugadaira	Sugadaira, Ueda, Nagano, Japan	36.53°N, 138.31°E	1	IOS10184
A. miyabei subsp. *miaotaiense* (Tsoong) A. E. Murray	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 Koenigsbourg	Château du Haut Koenigsbourg, France	48.25°N, 7.34°E	1	IOS10191
	Stoffelberg	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.

[a]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.