

DEVELOPMENT AND EVALUATION OF MICROSATELLITE MARKERS FOR *ACER MIYABEI* (SAPINDACEAE), A THREATENED MAPLE SPECIES IN EAST ASIA¹

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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,

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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: TCACATCCACTCTCTTTTCACA R: GTTTCTTCAGCAGCAACAACAACA | (CT) ₁₅ | 108 | 60 | HEX (1) | 1:39 | KP825168 |
| Acmi8 | F: CGCAGTTGACTGGTGTCTC R: GTTTCTTCGACGTTGTTGGAGTTAAACC | (AG) ₁₆ | 147 | 60 | HEX (2) | 1:39 | KP825169 |
| Acmi10 | F: GCATATTGAGATGGTGGCAA R: GTTTCTTGGTTCACATCTTTCATCCTG | (AG) ₁₄ | 151 | 60 | PET (2) | 1:39 | KP825170 |
| Acmi11 | F: CATTTCACATCACCACCTC R: GTTTCTTGTGAGACATACATAACAGAGCAGA | (TC) ₁₄ | 159 | 60 | NED (1) | 1:39 | KP825171 |
| Acmi23 | F: TAGACGGTGGTGGTGATCC R: GTTTCTTAGAACACACAACAAGCCCAGG | (AAT) ₁₂ | 226 | 60 | HEX (2) | 1:39 | KP825172 |
| Acmi28 | F: TTGCAAAGAATGTTAGTTTAGGATTG R: GTTTCTTCCAACTGGTAAATGCACC | (GT) ₁₃ | 276 | 60 | FAM (2) | 1:19 | KP825173 |
| Acmi29 | F: CAGATCCGTGCATTTCAATC R: GTTTCTTTCCTCGTTCTCACTCA | (AG) ₁₆ | 282 | 60 | HEX (2) | 1:19 | KP825174 |
| Acmi33 | F: CCACAAATCTCCTCTGCCAT R: GTTTCTTGTAGAAACAATGGAAACCCA | (TCT) ₁₁ | 111 | 60 | FAM (1) | 1:79 | KP825175 |
| Acmi38 | F: TGAGAGAAGAGAGGAGGAGGG R: GTTTCTTGCATAAAATCTCCAGCAATGG | (AG) ₁₀ | 137 | 60 | FAM (2) | 1:1.5 | KP825176 |
| Acmi45 | F: CATCTCTGTGCATTTGTTGTG R: GTTTCTTGTAAATGAGATCGAGTGATCGG | (AT) ₁₀ | 217 | 60 | HEX (1) | 1:4 | KP825177 |
| Acmi46 | F: AACAGGTACACCATGTTTATGGC R: GTTTCTTGGGATAAAGGCCCTTAGGA | (AT) ₁₀ | 218 | 60 | PET (1) | 1:0.25 | KP825178 |
| Acmi53 | F: TCTATGCATAAGCCAAGTCCC R: GTTTCTTAGGTTGGATTGTTGGTCAAT | (AT) ₈ | 268 | 60 | PET (2) | 1:9 | KP825179 |

Note: T_a = annealing temperature used in PCR.

^aFluorescent label used for two sets of multiplex PCR.

^bRatio 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 (Megléc 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.

| Locus | Bibi (n = 22) | | | | Kyouwa (n = 22) | | | | Overall (n = 44) | | | | |
|---------|---------------|-----------------------------|----------------|--------|-----------------|-----------------------------|----------------|--------|------------------|-----------------------------|----------------|--------|------------------------|
| | 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).

^aAsterisks 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.

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

| Locus | <i>A. campestre</i> (n = 4) | | | <i>A. platanoides</i> (n = 4) | | | <i>A. pictum</i> (n = 4) | | |
|---------|-----------------------------|----------------|----------------|-------------------------------|----------------|----------------|--------------------------|----------------|----------------|
| | 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.

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

primers were PIG-tailed by adding GTTTCCTT 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. *miao-taiense* (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*.

LITERATURE CITED

- BROWNSTEIN, M. J., J. D. CARPTEN, AND J. R. SMITH. 1996. Modulation of non-templated nucleotide addition by *Taq* DNA polymerase: Primer modifications that facilitate genotyping. *BioTechniques* 20: 1004–1006, 1008–1010.
- GRIMM, G. W., AND T. DENK. 2014. The Colchic region as refuge for relict tree lineages: Cryptic speciation in field maples. *Turkish Journal of Botany* 38: 1050–1066.
- HOTTA, M. 2004. Effects of habitat fragmentation on reproductive success and genetic diversity of an endangered maple, *Acer miyabei*. Master's Thesis, Hokkaido University, Sapporo, Japan.
- IUCN (INTERNATIONAL UNION FOR CONSERVATION OF NATURE). 2014. The IUCN Red List of Threatened Species, Version 2014.3. Website <http://www.iucnredlist.org/> [accessed 25 March 2015].
- KALINOWSKI, S. T., M. L. TAPER, AND T. C. MARSHALL. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* 16: 1099–1106.
- KENTA, T., J. GRATTEN, N. S. HAIGH, G. N. HINTEN, J. SLATE, R. K. BUTLIN, AND T. BURKE. 2008. Multiplex SNP-SCALE: A cost-effective medium-throughput single nucleotide polymorphism genotyping method. *Molecular Ecology Resources* 8: 1230–1238.
- MARSHALL, T. C., J. SLATE, L. E. B. KRUK, AND J. M. PEMBERTON. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology* 7: 639.
- MEGLÉCZ, E., C. COSTEDOAT, V. DUBUT, A. GILLES, T. MALAUSA, N. PECH, AND J.-F. MARTIN. 2010. QDD: A user-friendly program to select microsatellite markers and design primers from large sequencing projects. *Bioinformatics* 26: 403–404.

- MINISTRY OF THE ENVIRONMENT, GOVERNMENT OF JAPAN. 2012. Red list of vascular plants in Japan. Website <https://www.env.go.jp/press/15619.html> [accessed 26 March 2015] (in Japanese).
- NAGAMITSU, T., S. KIKUCHI, M. HOTTA, T. KENTA, AND T. HIURA. 2014. Effects of population size, forest fragmentation, and urbanization on seed production and gene flow in an endangered maple (*Acer miyabei*). *American Midland Naturalist* 172: 303–316.
- OGATA, K. 1965. A dendrological study on the Japanese Aceraceae, with special reference to the geographical distribution. *Bulletin of the Tokyo University Forests* 60: 1–99.
- OGATA, K. 1967. A systematic study of the genus *Acer*. *Bulletin of the Tokyo University Forests* 63: 89–206.
- RAYMOND, M., AND F. ROUSSET. 1995. GENEPOP (version 1.2): Population genetics software for exact tests and ecumenicism. *Journal of Heredity* 86: 248.
- RENNER, S. S., L. BEENKEN, G. W. GRIMM, A. KOCYAN, AND R. E. RICKLEFS. 2007. The evolution of dioecy, heterodichogamy, and labile sex expression in *Acer*. *Evolution* 61: 2701–2719.
- SUYAMA, Y. 2012. SSR genotyping method. In Y. Tsumura and Y. Suyama [eds.], *Molecular ecology in forest ecosystems*, 291–323. Bun-ichi Sogo Shuppan, Tokyo, Japan (in Japanese).
- TSOONG, P. C. 1954. A new *Acer* from China. *Kew Bulletin* 9: 83.
- VAN GELDEREN, D. M., P. C. DE JONG, AND H. J. OTERDOM. 1994. *Maples of the world*. Timber Press, Portland, Oregon, USA.

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 |
|---|------------------------------|---|------------------------|----|--------------------------|
| <i>A. miyabei</i> Maxim. subsp. <i>miyabei</i> f. <i>miyabei</i> | 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 |
| <i>A. miyabei</i> subsp. <i>miyabei</i> f. <i>shibatae</i> (Nakai) K. Ogata | Sugadaira | Sugadaira, Ueda, Nagano, Japan | 36.52°N, 138.34°E | 1 | IOS10183 |
| | Sugadaira | Sugadaira, Ueda, Nagano, Japan | 36.53°N, 138.31°E | 1 | IOS10184 |
| <i>A. miyabei</i> subsp. <i>miaotaiense</i> (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 |
| <i>A. campestre</i> 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 |
| <i>A. platanoides</i> 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 |
| <i>A. pictum</i> Thunb. | Stoffelberg | Stoffelberg, Germany | — | 1 | IOS10192 |
| | 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.

^aAll 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.