DEVELOPMENT AND EVALUATION OF MICROSATELITE MARKERS FOR THE CRITICALLY ENDANGERED BIRCH Betula chichibuensis (Betulaceae)

YUJI IGARASHI2, HIROKI AIHARA3, YOSHIHITO HANADA3,5, HIROSHI KATSUMATA3,5, MASANORI FUJI4, KOICHIRO NAKANO3,5, AND TOSHIHIDE HIRAO2,6

2The University of Tokyo Chichibu Forest, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-49 Hinoda-machi, Chichibu, Saitama 368-0034, Japan; 3FASMAC Co. Ltd., 5-1-3 Midorigaoka, Atsugi, Kanagawa 243-0041, Japan; and 4Institute for Environmental Sciences, 1-7 Ienomae, Obuchi, Rokkasho, Kamikita, Aomori 039-3212, Japan

• Premise of the study: Microsatellite markers were developed and characterized for the critically endangered birch Betula chichibuensis (Betulaceae) to investigate the genetic structure of this species for conservation purposes.
• Methods and Results: Sixteen microsatellite markers with di-, tri-, and tetranucleotide repeat motifs were developed and optimized using MiSeq paired-end sequencing. Of these, 14 were polymorphic, with two to five alleles per locus, in 47 individuals from two newly discovered populations of B. chichibuensis in Japan. Observed and unbiased expected heterozygosities per locus ranged from 0.000 to 0.617 and from 0.000 to 0.629, respectively. These markers were tested for cross-species amplification in B. maximowicziana, B. platyphylla var. japonica, and B. schmidtii.
• Conclusions: This set of microsatellite markers, the first developed for B. chichibuensis, will help elucidate spatial patterns of gene flow and levels of inbreeding in this species to aid its conservation.

Key words: Betula chichibuensis; Betulaceae; conservation genetics; critically endangered species; microsatellites; MiSeq sequencing.

The genus Betula L. (Betulaceae) comprises approximately 60 tree species distributed in boreal and cool-temperate zones of the Northern Hemisphere (Furlow, 1990). Individuals of B. chichibuensis H. Hara (subgenus Aspera) are small trees endemic to Japan (Ashburner and McAllister, 2013). Partly because of its distribution, this species is narrowly confined to the Chichibu (McAllister, 1993; Igarashi and Yoshida, 2013) and Kitakami (Nagato and Shimai, 2007) mountains in central and northeastern Honshu, respectively. Because only a few small populations have been recorded in these locations, B. chichibuensis is listed as critically endangered on the IUCN Red List (Shaw et al., 2014). The small population sizes and restricted distribution of B. chichibuensis make this species susceptible to diseases and natural disasters, and seriously impede gene flow (Ministry of the Environment, 2015). Analysis of B. chichibuensis genetic structure and maintenance of its genetic diversity are therefore essential for both in situ and ex situ conservation.

In this study, we developed microsatellite markers for B. chichibuensis to investigate the current genetic status of the remaining populations. We also examined the transferability of these developed markers to three other Betula species: B. maximowicziana Regel (subgenus Acuminata), B. platyphylla Sukaczew var. japonica (Miq.) H. Hara (subgenus Betula), and B. schmidtii Regel (subgenus Aspera).

METHODS AND RESULTS

Plant material and DNA extraction—Plant materials of B. chichibuensis were collected from two newly discovered populations growing on limestone outcrops on western Futago Mountain (WF) and along the Oku-Chichibu Forest Road (OC) in the Chichibu Mountains of Japan (Appendix 1). Shoots of 23 and 24 individuals were collected from WF and OC, respectively. Genomic DNA was extracted from freeze-dried leaves and winter buds using a DNaseasy Plant Mini Kit (QIAGEN, Hilden, Germany). The concentration of genomic DNA was determined with a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, California, USA) and by gel electrophoresis.

Microsatellite marker development—A total of 400 ng of genomic DNA from an individual OC sample was sheared with NEBNext dsDNA Fragmentase (New England Biolabs, Ipswich, Massachusetts, USA). A paired-end library for MiSeq sequencing (Illumina, San Diego, California, USA) was generated using a NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs). A single 301-bp paired-end sequencing run yielded 20,746,148 reads (DNA Data Bank of Japan [DDBJ] Sequence Read Archive accession no.:...
DRA005642). Raw reads with quality scores less than 20 and lengths shorter than 20 bp were filtered using Sickle version 1.33 (Joshi and Fass, 2011). De novo assembling using Velvet version 1.2.10 (Zerbino and Birney, 2008) produced 204,911 contigs, where parameters were set as 4-mer as 91, auto coverage cut-off, and minimum contig length of 300. The data sets were collated and filtered in QDD version 3.1 (Meglécz et al., 2014) to generate sequences containing microsatellites and to design PCR primers. A total of 125 perfect microsatellite loci consisting of di-, tri-, tetra-, penta-, and hexanucleotide repeat motifs were identified according to the following parameters: GC content of 40–60%, a melting temperature of 57°C to 63°C, and a maximum difference of 2°C between forward and reverse primers.

**Microsatellite marker screening**—For initial screening, of the 125 loci identified, 56 were selected based on repeat number and fragment size. For these loci, PCR amplification and polymorphism were tested using 10 samples. Individual primer pairs were assayed in 10-μL reaction mixtures containing 4 ng of genomic DNA, 0.05 μM of M13–21-tagged (5'-TGTTAAAAAGCAGGCTCAG-3') forward primer, 0.2 μM of reverse primer, 0.2 μM of universal primer labeled with 6-FAM fluorescent dye (Applied Biosystems, Foster City, California, USA), 0.2 μL of PrimeSTAR GXL DNA polymerase (TaKaRa Bio Inc., Tokyo, Japan), 2 μL of 5× PrimeSTAR GXL Buffer, and 0.2 mM of dNTP mixture. Thermal cycling conditions consisted of 98°C for 5 min; followed by 38 cycles of 98°C for four cycles, 63°C for one cycle, and 50°C for 15 cycles for 90 s, and a final step of 68°C for 15 min. Finally, 1 μL of PCR product was mixed with 0.5 μL of GeneScan 600 LIZ Size Standard (Applied Biosystems) and 8.5 μL Hi-Di formamide (Applied Biosystems) and sequenced on an ABI 3730xl DNA Analyzer (Applied Biosystems). Genotypes were scored by analyzing fragment sizes using Peak Scanner version 2.0 (Applied Biosystems).

**Microsatellite marker evaluation**—Descriptive statistics were computed for the assayed markers using CERVUS version 3.0.7 (Kalinowski et al., 2007). Of the 16 loci tested, 14 were polymorphic, with two to five alleles per locus detected across 47 individuals from the WF and OC populations (Table 1). The mean number of alleles per locus was 2.43, with mean observed and unbiased expected heterozygosities per locus of 0.327 (0.000–0.617) and 0.350 (0.000–0.629), respectively. The mean number of alleles per locus was 2.250 in the WF population and 2.313 in the OC population. For the WF population, mean observed and unbiased expected heterozygosities were calculated using FSTAT (Goudet, 2001). The mean number of alleles per locus was 2.313 in the OC population. For the WF population, mean observed and unbiased expected heterozygosities were calculated using FSTAT (Goudet, 2001). The mean number of alleles per locus was 2.250 in the WF population and 2.313 in the OC population.

### Table 1. Characteristics of 16 microsatellite markers developed for *Betula chichibuensis*.

| Locus | Primer sequences (5’−3’) | Repeat motif | Allele size range (bp) | Fluorescent dyeb (Multiplex set no.) | GenBank accession no. |
|-------|--------------------------|--------------|-----------------------|-------------------------------------|----------------------|
| Bcc3  | F: CTTGTTCCCTATCAGCCTTG | (CT)₉ | 281–283 | PET2 (1) | LC214895 |
| Bcc4  | F: GCAATGGTGGTGATCATCTT | (CT)₉ | 243–245 | NED1 (1) | LC214896 |
| Bcc7  | F: GGAATGCCAACATCCACCT | (AG)₉ | 277–283 | VIC2 (1) | LC214897 |
| Bcc10 | F: AACCGGAGCGCATACTTAC | (AG)₉ | 230–234 | VIC1 (1) | LC214898 |
| Bcc11 | F: CATGCTATGAAAAAGCATCTA | (CT)₉ | 254–256 | PET1 (1) | LC214899 |
| Bcc13 | F: GGAGCCACTTTGAGATGCA | (TC)₉ | 234 | PET1 (2) | LC214900 |
| Bcc16 | F: CACACAGGGGCTATGCTT | (AG)₉ | 260–262 | VIC2 (2) | LC214901 |
| Bcc18 | F: GGATGCTGAACTGGAGAAAT | (AT)₉ | 260–276 | NED2 (2) | LC214902 |
| Bcc22 | F: GTGCCATAGCTCTGGGAGC | (CA)₉ | 302–305 | PET2 (2) | LC214903 |
| Bcc25 | F: ACCATCTTGTGTGGAAGCTT | (AG)₉ | 156–160 | FAM1 (2) | LC214904 |
| Bcc27 | F: AAAGCCTTAGATCTGTCTT | (CCA)₉ | 203–215 | NED1 (2) | LC214905 |
| Bcc30 | F: CAAGTACGGTCATTCCATA | (AA)₉ | 221–224 | FAM1 (1) | LC214906 |
| Bcc34 | F: GGATCAAGCGATCTGAGAC | (AA)₉ | 200–205 | VIC1 (2) | LC214907 |
| Bcc38 | F: AGCGTTTGCAATTTCTATG | (CTT)₉ | 241 | FAM2 (2) | LC214908 |
| Bcc46 | F: GACACAGCCACTTGGCTGAC | (GAA)₉ | 271–283 | VIC2 (1) | LC214909 |
| Bcc50 | F: TACAGGTTGTTGCGCAAT | (TTAT)₉ | 300–308 | NED2 (1) | LC214910 |

a Touchdown annealing temperatures (63°C to 57°C [−1°C every two cycles] for 14 cycles, 56°C for 15 cycles, 53°C to 51°C [−1°C every two cycles] for six cycles, and 50°C for 15 cycles) were used.

b Sequences of fluorescent labels were as follows: FAM1 = 5'-TGTTAAAAAGCAGGCTCAG-3' (M13–21), VIC1 = 5'-CGTATTACGTAGTCAGTAC-3' (CMV-Fw), NED1 = 5'-ATGCTTTGATTCTGCAG-3' (pBAD-F), PET1 = 5'-CAGTATTACGTAGTCAGTAC-3' (M13–P5), FAM2 = 5'-TGTTAAAAAGCAGGCTCAG-3' (modified M13–21), VIC2 = 5'-CGTATTACGTAGTAC-3' (modified CMV-Fw), NED2 = 5'-ATGCTTTGATTCTGCAG-3' (modified pBAD-F), PET2 = 5'-CAGTATTACGTAGTCAGTAC-3' (modified M13–P5).

http://www.bioone.org/loi/apps
Table 2. Genetic variation of 16 microsatellite loci in two natural populations of Betula chichibuensis in central Honshu, Japan.

| Locus | Western Futago Mountain (n = 23) | Oku-Chichibu Forest Road (n = 24) | Total (n = 47) |
|-------|----------------------------------|----------------------------------|---------------|
|       | A = number of alleles; $H_e$ = unbiased expected heterozygosity; $H_o$ = observed heterozygosity; $n$ = number of individuals sampled; Null = null allele frequency estimate. |       |       |
| Bcc3  | 2 0.478 0.414 −0.082 2 0.250 0.284 0.053 | 2 0.362 0.351 −0.021 |       |
| Bcc4  | 2 0.391 0.322 −0.107 2 0.458 0.510 0.043 | 2 0.426 0.454 0.027 |       |
| Bcc7  | 3 0.130 0.127 −0.024 3 0.542 0.513 −0.056 | 3 0.540 0.356 0.002 |       |
| Bcc10 | 3 0.391 0.492 0.101 3 0.542 0.536 −0.020 | 3 0.468 0.563 0.092 |       |
| Bcc11 | 1 0.000 0.000 1 0.250 0.223 −0.062 | 2 0.128 0.121 −0.024 |       |
| Bcc13 | 1 0.000 0.000 — 1 0.000 0.000 — | 1 0.000 0.000 — | 2 0.021 0.021 −0.001 |
| Bcc16 | 2 0.043 0.043 −0.004 1 0.000 0.000 — | 2 0.040 0.326 −0.111 |       |
| Bcc18 | 2 0.304 0.264 −0.079 2 0.500 0.383 −0.142 | 5 0.617 0.628 0.006 |       |
| Bcc22 | 3 0.348 0.414 0.066 3 0.167 0.159 −0.035 | 3 0.255 0.298 0.059 |       |
| Bcc25 | 3 0.304 0.425 0.167 3 0.708 0.681 −0.031 | 5 0.511 0.613 0.100 |       |
| Bcc27 | 4 0.565 0.641 0.052 4 0.667 0.593 −0.067 | 5 0.617 0.628 0.006 |       |
| Bcc30 | 2 — 0.391 0.414 0.018 2 0.417 0.496 0.077 | 2 0.404 0.461 0.060 |       |
| Bcc34 | 2 0.043 0.043 −0.004 3 0.500 0.465 −0.074 | 3 0.277 0.284 −0.009 |       |
| Bcc38 | 1 0.000 0.000 — 1 0.000 0.000 — | 1 0.000 0.000 — |       |
| Bcc46 | 2 0.522 0.487 −0.046 2 0.417 0.422 −0.004 | 2 0.468 0.500 0.027 |       |
| Bcc50 | 3 0.652 0.633 −0.033 3 0.458 0.606 0.153 | 3 0.553 0.629 0.058 |       |
| Average | 2.250 0.285 0.295 0.002 | 2.313 0.367 0.367 −0.014 | 2.438 0.327 0.350 0.019 |

Note: A = number of alleles; $H_e$ = unbiased expected heterozygosity; $H_o$ = observed heterozygosity; $n$ = number of individuals sampled; Null = null allele frequency estimate.

aVoucher and locality information are provided in Appendix 1.
bNo significant deviation from Hardy–Weinberg equilibrium was detected ($P < 0.05$).

CONCLUSIONS

We developed 16 microsatellite markers for the critically endangered birch B. chichibuensis using MiSeq paired-end sequencing. These markers will facilitate understanding of spatial patterns of gene flow and levels of inbreeding, information essential for the conservation of the small isolated populations of this species. Some of the markers were successfully transferred to closely related Betula species.

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### APPENDIX 1. Voucher information for species used in the development and evaluation of microsatellite markers for *Betula chichibuensis*.

| Taxon                  | Population                     | Location                  | Geographic coordinates\(^a\) | \(N\) | Voucher no.\(^b\) |
|------------------------|--------------------------------|---------------------------|-------------------------------|------|-------------------|
| *B. chichibuensis* H. Hara | Western Futago Mountain (WF)  | Ogano, Saitama, Japan     | 36°04′N, 138°51′E             | 23   | UTCFBC 00001–00015, 00017–00024 |
|                        | Oku-Chichibu Forest Rd. (OC)  | Chichibu, Saitama, Japan  | 35°57′N, 138°44′E             | 24   | UTCFBC 00073–00096 |
| *B. maximowicziana* Regel | Tochimoto                     | Chichibu, Saitama, Japan  | 35°57′N, 138°49′E             | 5    | UTCFFT 00039.1–00039.5 |
| *B. platyphylla* Sukaczew var. *japonica* (Miq.) H. Hara | Tochimoto                     | Chichibu, Saitama, Japan  | 35°56′N, 138°49′E             | 5    | UTCFFT 00040.1–00040.5 |
| *B. schmidtii* Regel    | Ochigawa                      | Chichibu, Saitama, Japan  | 35°54′N, 138°59′E             | 5    | UTCFFT 00041.1–00041.5 |

\(^a\) For conservation reasons, low-resolution geographic coordinates are given.

\(^b\) All vouchers are stored at the Herbarium of the University of Tokyo Chichibu Forest (UTC), The University of Tokyo, Saitama, Japan.

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**Note:** \(N\) = number of samples.