

# Development and characterization of EST-SSR markers for *Saxifraga fortunei* var. *incislobata* (Saxifragaceae)

Kana Magota<sup>1,2</sup> , Daiki Takahashi<sup>1</sup>, and Hiroaki Setoguchi<sup>1</sup>

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<sup>1</sup> Graduate School of Human and Environmental Studies, Kyoto University, Yoshida Nihonmatsu-cho, Sakyo-ku, Kyoto 606-8501, Japan

<sup>2</sup> Author for correspondence: magota.kana.47c@st.kyoto-u.ac.jp

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**PREMISE:** *Saxifraga fortunei* (Saxifragaceae) includes several infraspecific taxa that are ecologically and morphologically distinct. To investigate the evolutionary history of phenotypic polymorphisms in this species, we developed expressed sequence tag–simple sequence repeat (EST-SSR) markers for *S. fortunei*.

**METHODS AND RESULTS:** We developed 26 polymorphic markers based on transcriptome data obtained from Illumina HiSeq 2000. Within three populations of *S. fortunei* var. *incislobata*, the number of alleles ranged from four to 25, and the levels of observed and expected heterozygosity ranged from 0.200 to 0.847 and from 0.209 to 0.930, respectively. Furthermore, all 26 loci showed transferability for *S. fortunei* var. *obtusocuneata* and *S. fortunei* var. *suwoensis*, and 18 loci were also successfully amplified in *S. acerifolia*.

**CONCLUSIONS:** These newly developed EST-SSR markers will prove useful to infer the evolutionary history of *S. fortunei* var. *incislobata* and its relatives in population genetic studies.

**KEY WORDS** ecological polymorphisms; expressed sequence tag–simple sequence repeat (EST-SSR) markers; *Saxifraga fortunei*; Saxifragaceae; transcriptome.

*Saxifraga* L. is the largest genus in the Saxifragaceae family, with more than 440 species widely distributed throughout the Northern Hemisphere (Tkach et al., 2015). A recent phylogenetic study divided this genus into 13 sections and nine subsections, with section *Irregulares* Haw., a well characterized group with zygomorphic flowers, as the earliest diverged lineage (Tkach et al., 2015). This section comprises 15–20 species growing in moist temperate areas of East Asia, whereas most other sections of *Saxifraga* are widely distributed in boreal and/or alpine areas (Pan, 2001).

*Saxifraga fortunei* Hook., which belongs to sect. *Irregulares*, is a perennial herb distributed in East Asia, ranging from mainland China to Sakhalin and throughout the Japanese Archipelago. This species is ecologically and morphologically diverse and includes more than seven infraspecific ecotypic entities (Nakai, 1938; Ohba, 1982; Pan, 2001). *Saxifraga fortunei* var. *incislobata* (Engl. & Irmsch.) Nakai is the most widely distributed taxon, growing in shaded understory. *Saxifraga fortunei* var. *obtusocuneata* (Makino) Nakai is a riparian taxon with a cuneate leaf blade base, and *S. fortunei* var. *suwoensis* Nakai is also a riverbank taxon with deeply dissected leaf blades, and these two taxa have allopatric distributions in western Japan. There are other local endemics with specific characters, such as an alpine taxon that grows under direct sunlight, and an insular taxon with thick and deeply haired leaf blades. These intraspecific taxa are presumably adapted to specific

habitats, and these patterns of phenotypic variation provide an ideal model for the investigation of ecological adaptation and diversification. Magota et al. (2018) reported several chloroplast and nuclear microsatellite markers based on genomic DNA sequence data of *S. acerifolia* Wakabayashi & Satomi, an endangered plant species related to *S. fortunei* (Wakabayashi, 1973; Ministry of the Environment, Japan, 2019). However, only two of the previously identified nuclear markers showed polymorphisms in *S. fortunei*. Therefore, more polymorphic markers were needed to investigate the genetic structure and to infer the evolutionary history of *S. fortunei*. Expressed sequence tag–simple sequence repeat (EST-SSR) markers are valuable for their cross-transferability to related taxa in many plant species, and they are easier to develop at a lower cost than other types of nuclear markers (e.g., Takahashi et al., 2017). In this study, we developed EST-SSR markers of *S. fortunei* var. *incislobata* and examined their utility and transferability to related taxa.

## METHODS AND RESULTS

Fresh floral buds of *S. fortunei* var. *incislobata* (population F42, Appendix 1) were frozen in liquid nitrogen and total RNA was extracted using the Agilent Plant RNA Isolation Mini Kit (Agilent

**TABLE 1.** Characteristics of 26 polymorphic microsatellite loci developed for *Saxifraga fortunei* var. *incislobata*.<sup>a</sup>

| Locus  | Primer sequences (5'–3')                                                       | Repeat motif        | Allele size range (bp) | BLASTX top hit description                                                                   | E-value   | GenBank accession no. |
|--------|--------------------------------------------------------------------------------|---------------------|------------------------|----------------------------------------------------------------------------------------------|-----------|-----------------------|
| SF1037 | F: CGGAGAGCCGAGAGGTGCAGTTGCTCACCACAAGACC<br>R: GTTTCCTTCTCGTCTTCTCTGCGAACC     | (GAA) <sub>8</sub>  | 429–445                | PREDICTED: polynucleotide 3'-phosphatase ZDP [ <i>Vitis vinifera</i> ]                       | 6.08E-161 | LC465769              |
| SF1424 | F: CGGAGAGCCGAGAGGTGAAGCCACATTCCCTTTCCACC<br>R: GTTTCCTTATGAAGAGCCTCAGACCACC   | (CCA) <sub>11</sub> | 329–350                | Probable beta-D-xylosidase 2 isoform X1 [ <i>Olea europaea</i> var. <i>syvestris</i> ]       | 0.00      | LC465771              |
| SF816  | F: CGGAGAGCCGAGAGGTGCCCCACATTCTGGCATTGTG<br>R: GTTTCCTTAAAGAACAACATAGCCACGAC   | (GAT) <sub>9</sub>  | 317–344                | Uncharacterized protein LOC18994464 [ <i>Eutrema salsugineum</i> ]                           | 4.08E-24  | LC465773              |
| SF1057 | F: CTATAGGGCACGCGTGGTGACTTCCCATAGCTCCTCCG<br>R: GTTTCCTGGCGATCAGAACCACAATC     | (CT) <sub>11</sub>  | 303–335                | No significant hit                                                                           | —         | LC465774              |
| SF75   | F: TGTGGAATTGTGAGCGGATGGGACCAGCAGCATAAGG<br>R: GTTTCCTCGGAAGATCTGCATCAGCTC     | (AGG) <sub>8</sub>  | 340–359                | Ubiquitin carboxyl-terminal hydrolase 8 [ <i>Ziziphus jujuba</i> ]                           | 9.77E-07  | LC465775              |
| SF1016 | F: CTATAGGGCACGCGTGGTCTGTGGAACCTCACTTCTTG<br>R: GTTTCCTCTGTTTCTCGTCACAAACCG    | (AAG) <sub>8</sub>  | 252–279                | Uncharacterized protein LOC102620652 [ <i>Citrus sinensis</i> ]                              | 0.00      | LC465776              |
| SF166  | F: TGTGGAATTGTGAGCGGATGGTGGTGGTGATGACAAG<br>R: GTTTCCTGGCGATCTTCTCTCTCAAC      | (GA) <sub>12</sub>  | 221–234                | No significant hit                                                                           | —         | LC465777              |
| SF143  | F: CACGACGTTGTAAAACGACCCCTCGACATCAAGGTTACAC<br>R: GTTTCCTATTGGTTCCTTGCCTGTCC   | (CT) <sub>12</sub>  | 218–266                | PREDICTED: uncharacterized protein LOC100256691 [ <i>Vitis vinifera</i> ]                    | 0.00      | LC465780              |
| SF716  | F: CACGACGTTGTAAAACGACGAAGCCTTGAGTTGATTTCGC<br>R: GTTTCCTTTTCAGGCCTCCCATCACATG | (ATT) <sub>8</sub>  | 191–230                | No significant hit                                                                           | —         | LC465782              |
| SF319  | F: TGTGGAATTGTGAGCGGCGGAGGTTGAGATTGAAGGC<br>R: GTTTCCTTTTACCAACGCGCAGCATTC     | (TC) <sub>10</sub>  | 361–397                | Uncharacterized protein LOC110815042 [ <i>Carica papaya</i> ]                                | 3.75E-15  | LC465783              |
| SF1102 | F: CTATAGGGCACGCGTGGTCTCTTCTATCTCCTCGGCCG<br>R: GTTTCCTTTGGCATGTCAAAGCCATCTG   | (ATC) <sub>7</sub>  | 195–207                | Uncharacterized protein DDB_G0290685-like [ <i>Quercus suber</i> ]                           | 2.57E-06  | LC465784              |
| SF479  | F: TGTGGAATTGTGAGCGGGGAGATCCGCATGAAACACG<br>R: GTTTCCTTTCTATAAACGGCGATGAGTTGG  | (GAT) <sub>8</sub>  | 162–189                | PREDICTED: uncharacterized protein LOC104591093 [ <i>Nelumbo nucifera</i> ]                  | 2.97E-13  | LC465786              |
| SF314  | F: CGGAGAGCCGAGAGGTGGTGGTGTAGAAGGGTGAGGG<br>R: GTTTCCTTCAAAGCCTCTCCTATGGTGC    | (GTG) <sub>9</sub>  | 124–176                | PREDICTED: protein CURVATURE THYLAKOID 1D, chloroplastic [ <i>Vitis vinifera</i> ]           | 1.24E-60  | LC465788              |
| SF385  | F: TGTGGAATTGTGAGCGGACAGGAGGTGGTTTGTAGGG<br>R: GTTTCCTTGCCTTCACCTTCTCCACCC     | (GGT) <sub>8</sub>  | 105–186                | No significant hit                                                                           | —         | LC465790              |
| SF1135 | F: CTATAGGGCACGCGTGGTCATATTGCGCTCGCTGTCCAG<br>R: GTTTCCTTTGTGTTGGATTACGTGGGTG  | (CT) <sub>13</sub>  | 129–141                | No significant hit                                                                           | —         | LC465791              |
| SF1450 | F: CGGAGAGCCGAGAGGTGAGGCGCCGATTGTGTTGTC<br>R: GTTTCCTTTTCCCGTCACATCCGTACC      | (GA) <sub>12</sub>  | 119–133                | WD40 repeat-containing protein HOS15 [ <i>Momordica charantia</i> ]                          | 0.00      | LC465792              |
| SF941  | F: CACGACGTTGTAAAACGACGATCCGGCAACTGTTCAAGG<br>R: GTTTCCTTACTTCTTGCAACTCAACAGC  | (TGG) <sub>7</sub>  | 361–463                | Hypothetical protein CDL15_Pgr014496 [ <i>Punica granatum</i> ]                              | 1.39E-40  | LC465793              |
| SF1144 | F: TGTGGAATTGTGAGCGGGCCGAAGTAACAACACCACC<br>R: GTTTCCTTAGAGAGAGGTGGAAGTGTGC    | (TGC) <sub>7</sub>  | 395–479                | DEAD-box ATP-dependent RNA helicase 3, chloroplastic [ <i>Vitis vinifera</i> ]               | 0.00      | LC465794              |
| SF1529 | F: TGTGGAATTGTGAGCGGAGCTCTAAGAAACGGCGAAAC<br>R: GTTTCCTTGATGTGCTCTGTCCATGGC    | (ATC) <sub>7</sub>  | 317–365                | Homeobox-leucine zipper protein ATHB-13 [ <i>Ricinus communis</i> ]                          | 7.32E-75  | LC465799              |
| SF1116 | F: CGGAGAGCCGAGAGGTGAATGGCGGAGTTTACTTGC<br>R: GTTTCCTTAAGTTTGCCTCGTTCAACAG     | (AAC) <sub>7</sub>  | 275–304                | PREDICTED: proliferating cell nuclear antigen [ <i>Daucus carota</i> subsp. <i>sativus</i> ] | 2.50E-95  | LC465804              |
| SF1222 | F: CACGACGTTGTAAAACGACCTGGTCAGAGAGTGTGGAGG<br>R: GTTTCCTTCTCCAGAAACCCTAGGCTCC  | (GAT) <sub>7</sub>  | 246–270                | Unnamed protein product, partial [ <i>Vitis vinifera</i> ]                                   | 2.34E-136 | LC465805              |
| SF489  | F: CGGAGAGCCGAGAGGTGAAACTCACTTCGCCATGTGC<br>R: GTTTCCTTCCAGACGCCAGTTTCTCAC     | (CT) <sub>9</sub>   | 363–371                | Unnamed protein product, partial [ <i>Vitis vinifera</i> ]                                   | 1.73E-87  | LC465807              |
| SF644  | F: CACGACGTTGTAAAACGACAATTGCCCGGTGATGCATC<br>R: GTTTCCTCCCTACCAACAAAGTCGTACC   | (AG) <sub>10</sub>  | 168–210                | No significant hit                                                                           | —         | LC465813              |
| SF664  | F: CGGAGAGCCGAGAGGTGCTTACTGCCAGAACTCCAG<br>R: GTTTCCTTAATCACTCACACGGGAATACTC   | (AAT) <sub>7</sub>  | 145–171                | Zinc finger CCCH domain-containing protein 53 isoform X1 [ <i>Glycine max</i> ]              | 2.08E-172 | LC465814              |
| SF631  | F: CACGACGTTGTAAAACGACACTGAACAGATCTCCATGGC<br>R: GTTTCCTTGCACCATACTTACGAGGCC   | (TA) <sub>9</sub>   | 143–171                | No significant hit                                                                           | —         | LC465815              |
| SF519  | F: CTATAGGGCACGCGTGGTCACTCCCATGAACCTACCAAG<br>R: GTTTCCTTTCACACACAAGGAAAGCG    | (AAT) <sub>7</sub>  | 111–129                | Ferredoxin-3, chloroplastic [ <i>Vitis vinifera</i> ]                                        | 1.44E-83  | LC465816              |

<sup>a</sup>Annealing temperature is 58°C for all primer pairs.

Technologies, Santa Clara, California, USA) following the manufacturer's protocol. A cDNA library was constructed and sequenced using the Illumina HiSeq 2000 platform (Illumina, San Diego, California, USA; performed by BGI, Shenzhen, China). The raw

reads (paired-end 100 bp) are deposited in the DNA Data Bank of Japan (DDBJ; BioProject PRJDB8004). Low-quality reads were trimmed using Trimmomatic 0.38 (Bolger et al., 2014) with the following parameters: HEADCROP, 20 and SLIDINGWINDOW,

4:20. In all, 26,177,799 paired reads were obtained. We conducted de novo transcriptome assembly of these reads using Trinity v.2.8.4 (Haas et al., 2013), which produced 121,463 contigs (mean length 673 bp). Microsatellite regions ( $\geq 7$  dinucleotide or  $\geq 7$  trinucleotide repeats) were screened using MSATCOMMANDER (Faircloth, 2008). A total of 568 regions were obtained, and we selected 96 PCR primer pairs based on the repeat numbers of microsatellite motifs. For all loci, the forward primers were synthesized with one of four different M13 sequences (5'-CACGACGTTGTAAACGAC-3', 5'-TGTGGAATTGTGAGCGG-3', 5'-CTATAGGGCAGCGTG-3', or 5'-CGGAGAGCCGAGAGGTG-3'), and the reverse primers were tagged with a PIG-tail sequence (5'-GTTTCTT-3').

Twenty-four *S. fortunei* var. *incislobata* individuals from each of three populations (F05, F35, and F38; Appendix 1) were used to evaluate the polymorphisms of the target loci. Moreover, we used 24 individuals from each of three related taxa (*S. fortunei* var. *obtusocuneata*, *S. fortunei* var. *suwoensis*, and *S. acerifolia*) for cross-amplification. Genomic DNA for PCR was extracted from dried leaf materials using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987), after washing the leaf powder three times with HEPES buffer (pH = 8.0; Setoguchi and Ohba, 1995). The PCR was performed in a 5- $\mu$ L reaction volume, containing approximately 0.5 ng DNA, 2.5  $\mu$ L 2 $\times$  Multiplex PCR Master Mix (QIAGEN, Hilden, Germany), 0.01  $\mu$ M forward primer, 0.2  $\mu$ M reverse primer, and 0.1  $\mu$ M fluorescence-labeled M13 primer. The PCR thermal profile was set as follows: an initial denaturation at 95°C for 30 min; followed by 35 cycles of 95°C for 30 s, 58°C for 3 min, and 68°C for 1 min; and then a final extension at 68°C

for 20 min. Amplified products were loaded onto an ABI 3130xl Genetic Analyzer (Applied Biosystems, Carlsbad, California, USA) using the GeneScan 600 LIZ Size Standard (Applied Biosystems), POP7 polymer (Applied Biosystems), and a 36-cm capillary array. Fragment size was determined using GeneMapper software (Applied Biosystems). To evaluate the utility of the developed markers, genetic diversity indices (number of alleles, observed heterozygosity, and expected heterozygosity) were calculated using GenAlEx version 6.503 (Peakall and Smouse, 2006). Significant deviations from Hardy–Weinberg equilibrium and linkage disequilibrium were tested with 1000 randomizations using GENEPOP 4.2 (Raymond, 1995).

Of 96 primer pairs tested with an individual from population F42, 47 loci showed clear peaks. Of the 47 loci that were successfully amplified, 26 showed polymorphisms within each population of *S. fortunei* var. *incislobata* (Table 1) and 21 were monomorphic (Appendix 2). In total, from three populations (F05, F35, and F38), the number of alleles ranged from four to 25 and the levels of observed and expected heterozygosity ranged from 0.200 to 0.847 and from 0.209 to 0.930, respectively (Table 2). In all three populations, two loci (SF716 and SF314) significantly deviated from Hardy–Weinberg equilibrium ( $P < 0.01$ ), and significant linkage disequilibrium was detected between loci SF716 and SF166 ( $P = 0.00951$ ).

The results of cross-amplifications are shown in Table 3. In *S. fortunei* var. *obtusocuneata*, all 26 loci were successfully amplified and polymorphic. In *S. fortunei* var. *suwoensis*, all 26 loci were amplified, of which 22 showed polymorphisms. In *S. acerifolia*, 18 loci were amplified and 15 showed polymorphisms.

**TABLE 2.** Genetic diversity statistics for three populations of *Saxifraga fortunei* var. *incislobata* based on 26 newly developed EST-SSR markers.<sup>a</sup>

| Locus   | F05 (N = 24) |                             |                | F35 (N = 24) |                             |                | F38 (N = 24) |                             |                | Total (N = 72) |                |                |
|---------|--------------|-----------------------------|----------------|--------------|-----------------------------|----------------|--------------|-----------------------------|----------------|----------------|----------------|----------------|
|         | A            | H <sub>o</sub> <sup>b</sup> | H <sub>e</sub> | A            | H <sub>o</sub> <sup>b</sup> | H <sub>e</sub> | A            | H <sub>o</sub> <sup>b</sup> | H <sub>e</sub> | A              | H <sub>o</sub> | H <sub>e</sub> |
| SF1037  | 4            | 0.455                       | 0.415          | 7            | 0.778                       | 0.769          | 7            | 0.667                       | 0.675          | 8              | 0.625          | 0.718          |
| SF1424  | 7            | 0.583                       | 0.732          | 8            | 0.917                       | 0.793          | 9            | 0.750                       | 0.801          | 10             | 0.750          | 0.815          |
| SF816   | 7            | 0.750                       | 0.828          | 8            | 0.696                       | 0.841          | 9            | 0.833                       | 0.854          | 10             | 0.761          | 0.861          |
| SF1057  | 13           | 0.792                       | 0.885          | 11           | 0.875                       | 0.855          | 14           | 0.875                       | 0.890          | 17             | 0.847          | 0.899          |
| SF75    | 3            | 0.391                       | 0.373          | 3            | 0.095                       | 0.092          | 4            | 0.208                       | 0.261          | 7              | 0.235          | 0.265          |
| SF1016  | 6            | 0.583                       | 0.564          | 8            | 0.833                       | 0.834          | 8            | 0.667*                      | 0.780          | 12             | 0.694          | 0.811          |
| SF166   | 11           | 0.417*                      | 0.615          | 10           | 0.682                       | 0.808          | 9            | 0.583**                     | 0.829          | 15             | 0.557          | 0.783          |
| SF143   | 10           | 0.714                       | 0.883          | 12           | 0.650*                      | 0.871          | 20           | 0.875                       | 0.920          | 22             | 0.754          | 0.930          |
| SF716   | 10           | 0.391***                    | 0.836          | 9            | 0.391***                    | 0.780          | 9            | 0.458**                     | 0.773          | 15             | 0.414          | 0.834          |
| SF319   | 9            | 0.542**                     | 0.865          | 13           | 0.818                       | 0.882          | 11           | 0.750                       | 0.852          | 15             | 0.700          | 0.888          |
| SF1102  | 4            | 0.333**                     | 0.637          | 4            | 0.636                       | 0.657          | 5            | 0.333**                     | 0.607          | 5              | 0.429          | 0.687          |
| SF479   | 4            | 0.583                       | 0.559          | 7            | 0.583                       | 0.707          | 10           | 0.875                       | 0.852          | 10             | 0.681          | 0.787          |
| SF314   | 7            | 0.375***                    | 0.680          | 6            | 0.375***                    | 0.707          | 11           | 0.409***                    | 0.854          | 14             | 0.386          | 0.787          |
| SF385   | 16           | 0.609***                    | 0.869          | 14           | 0.714                       | 0.897          | 14           | 0.500***                    | 0.884          | 25             | 0.603          | 0.925          |
| SF1135  | 3            | 0.208                       | 0.254          | 6            | 0.292                       | 0.387          | 3            | 0.583                       | 0.424          | 7              | 0.361          | 0.375          |
| SF1450  | 5            | 0.500                       | 0.617          | 7            | 0.545                       | 0.727          | 9            | 0.625*                      | 0.788          | 10             | 0.559          | 0.742          |
| SF941   | 8            | 0.087***                    | 0.767          | 5            | 0.318**                     | 0.629          | 6            | 0.458                       | 0.574          | 10             | 0.290          | 0.751          |
| SF1144  | 8            | 0.739                       | 0.781          | 12           | 0.783                       | 0.830          | 10           | 0.833                       | 0.846          | 16             | 0.786          | 0.845          |
| SF1529  | 6            | 0.500                       | 0.549          | 6            | 0.875                       | 0.722          | 9            | 0.708                       | 0.809          | 9              | 0.694          | 0.751          |
| SF1116  | 2            | 0.048                       | 0.046          | 5            | 0.250*                      | 0.448          | 6            | 0.292                       | 0.362          | 8              | 0.200          | 0.304          |
| SF1222  | 3            | 0.125                       | 0.119          | 4            | 0.174                       | 0.164          | 4            | 0.375                       | 0.325          | 7              | 0.225          | 0.209          |
| SF489   | 2            | 0.042                       | 0.041          | 4            | 0.696                       | 0.695          | 4            | 0.375*                      | 0.574          | 4              | 0.366          | 0.509          |
| SF644   | 9            | 0.750                       | 0.724          | 16           | 0.833                       | 0.866          | 11           | 0.708                       | 0.759          | 20             | 0.764          | 0.825          |
| SF664   | 3            | 0.292                       | 0.254          | 3            | 0.217                       | 0.198          | 3            | 0.375                       | 0.398          | 4              | 0.296          | 0.296          |
| SF631   | 10           | 0.458***                    | 0.823          | 10           | 0.609**                     | 0.852          | 10           | 0.708                       | 0.790          | 13             | 0.592          | 0.864          |
| SF519   | 5            | 0.625                       | 0.734          | 4            | 0.652                       | 0.593          | 7            | 0.792                       | 0.734          | 8              | 0.690          | 0.725          |
| Average | 6.7          | 0.458                       | 0.603          | 7.6          | 0.588                       | 0.677          | 8.5          | 0.616                       | 0.701          | 11.6           | 0.548          | 0.700          |

Note: A = number of alleles; H<sub>o</sub> = expected heterozygosity; H<sub>e</sub> = observed heterozygosity; N = number of individuals sampled.

<sup>a</sup>Locality and voucher information are provided in Appendix 1.

<sup>b</sup>Asterisks indicate significant deviation from Hardy–Weinberg equilibrium after Bonferroni correction (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

**TABLE 3.** Cross-amplification and genetic diversity statistics of EST-SSR markers developed for *Saxifraga fortunei* var. *incislobata* in related taxa.<sup>a</sup>

| Locus   | <i>S. fortunei</i> var. <i>obtusocuneata</i> (N = 24) |                             |                | <i>S. fortunei</i> var. <i>suwoensis</i> (N = 24) |                             |                | <i>S. acerifolia</i> (N = 24) |                             |                |
|---------|-------------------------------------------------------|-----------------------------|----------------|---------------------------------------------------|-----------------------------|----------------|-------------------------------|-----------------------------|----------------|
|         | A                                                     | H <sub>o</sub> <sup>b</sup> | H <sub>e</sub> | A                                                 | H <sub>o</sub> <sup>b</sup> | H <sub>e</sub> | A                             | H <sub>o</sub> <sup>b</sup> | H <sub>e</sub> |
| SF1037  | 4                                                     | 0.650                       | 0.563          | 2                                                 | 0.125                       | 0.117          | 3                             | 0.091*                      | 0.206          |
| SF1424  | 3                                                     | 0.348                       | 0.396          | 3                                                 | 0.083                       | 0.081          | 4                             | 0.091*                      | 0.170          |
| SF816   | 5                                                     | 0.739                       | 0.707          | 6                                                 | 0.833                       | 0.799          | —                             | —                           | —              |
| SF1057  | 6                                                     | 0.565                       | 0.676          | 4                                                 | 0.250                       | 0.261          | 3                             | 0.091                       | 0.088          |
| SF75    | 2                                                     | 0.105                       | 0.100          | 3                                                 | 0.435                       | 0.468          | 2                             | 0.048                       | 0.046          |
| SF1016  | 3                                                     | 0.565                       | 0.662          | 4                                                 | 0.478                       | 0.472          | —                             | —                           | —              |
| SF166   | 5                                                     | 0.739*                      | 0.662          | 5                                                 | 0.667                       | 0.635          | —                             | —                           | —              |
| SF143   | 5                                                     | 0.750                       | 0.744          | 4                                                 | 0.583                       | 0.622          | 1                             | 0.000                       | 0.000          |
| SF716   | 8                                                     | 0.864                       | 0.846          | 5                                                 | 0.773                       | 0.743          | 6                             | 0.105***                    | 0.402          |
| SF319   | 6                                                     | 0.583                       | 0.642          | 3                                                 | 0.455                       | 0.430          | 3                             | 0.056***                    | 0.545          |
| SF1102  | 4                                                     | 0.458*                      | 0.548          | 2                                                 | 0.542                       | 0.457          | —                             | —                           | —              |
| SF479   | 3                                                     | 0.261                       | 0.334          | 2                                                 | 0.083                       | 0.080          | 6                             | 0.304                       | 0.345          |
| SF314   | 6                                                     | 0.708                       | 0.787          | 11                                                | 0.542***                    | 0.826          | 3                             | 0.125                       | 0.119          |
| SF385   | 6                                                     | 0.333***                    | 0.549          | 9                                                 | 0.500***                    | 0.788          | 4                             | 0.000***                    | 0.458          |
| SF1135  | 3                                                     | 0.042*                      | 0.119          | 3                                                 | 0.565**                     | 0.494          | 7                             | 0.667                       | 0.685          |
| SF1450  | 5                                                     | 0.455                       | 0.645          | 2                                                 | 0.042                       | 0.041          | 8                             | 0.542                       | 0.578          |
| SF941   | 5                                                     | 0.542                       | 0.739          | 1                                                 | 0.000                       | 0.000          | —                             | —                           | —              |
| SF1144  | 6                                                     | 0.636                       | 0.760          | 3                                                 | 0.625                       | 0.551          | —                             | —                           | —              |
| SF1529  | 7                                                     | 0.864                       | 0.746          | 4                                                 | 0.609                       | 0.632          | 1                             | 0.000                       | 0.000          |
| SF1116  | 5                                                     | 0.278***                    | 0.660          | 1                                                 | 0.000                       | 0.000          | 6                             | 0.167***                    | 0.608          |
| SF1222  | 3                                                     | 0.591                       | 0.574          | 2                                                 | 0.042                       | 0.041          | —                             | —                           | —              |
| SF489   | 4                                                     | 0.542*                      | 0.556          | 3                                                 | 0.667                       | 0.635          | 2                             | 0.043                       | 0.043          |
| SF644   | 7                                                     | 0.542                       | 0.753          | 7                                                 | 0.625*                      | 0.791          | 16                            | 0.696**                     | 0.881          |
| SF664   | 4                                                     | 0.652                       | 0.578          | 1                                                 | 0.000                       | 0.000          | 10                            | 0.762                       | 0.621          |
| SF631   | 5                                                     | 0.188***                    | 0.658          | 1                                                 | 0.000                       | 0.000          | 1                             | 0.000                       | 0.000          |
| SF519   | 3                                                     | 0.478                       | 0.532          | 2                                                 | 0.458                       | 0.430          | —                             | —                           | —              |
| Average | 4.7                                                   | 0.518                       | 0.598          | 3.6                                               | 0.384                       | 0.400          | 4.8                           | 0.210                       | 0.322          |

Note: A = number of alleles; H<sub>o</sub> = expected heterozygosity; H<sub>e</sub> = observed heterozygosity; N = number of individuals sampled.

<sup>a</sup>Locality and voucher information are provided in Appendix 1.

<sup>b</sup>Asterisks indicate significant deviation from Hardy–Weinberg equilibrium after Bonferroni correction (\*P < 0.05, \*\*P < 0.01, \*\*\*P < 0.001).

## CONCLUSIONS

We developed 26 novel polymorphic EST-SSR markers for *S. fortunei* var. *incislobata*. All loci were amplified in other infraspecific taxa of *S. fortunei*, and 18 of them were transferable to *S. acerifolia*. These markers will be useful for future studies to investigate the evolutionary histories of these species. In addition, they have also proved helpful in evaluating genetic diversity in *S. acerifolia*, an endangered species.

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## AUTHOR CONTRIBUTIONS

K.M. and H.S. conceived and designed the experiments. K.M., D.T., and H.S. contributed to sample collection. K.M. and D.T. conducted de novo transcriptome assembly. K.M. performed the molecular laboratory work, allele scoring, and analyses. K.M. drafted the manuscript and all authors participated in manuscript modifications and approved the final version for publication.

## DATA ACCESSIBILITY

Raw reads from the cDNA library sequenced by Illumina HiSeq 2000 have been deposited to the DNA Data Bank of Japan (DDBJ; BioProject PRJDB8004). Sequence information for the developed primers has been deposited to the National Center for Biotechnology Information (NCBI); GenBank accession numbers are shown in Table 1 and Appendix 2.

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#### APPENDIX 1. Sample information for *Saxifraga* species used in this study.

| Taxa                                                                            | Population | N  | Collection locality                           | Geographic coordinates | Voucher specimen accession no. <sup>a</sup> |
|---------------------------------------------------------------------------------|------------|----|-----------------------------------------------|------------------------|---------------------------------------------|
| <i>Saxifraga fortunei</i> Hook. var. <i>incislobata</i> (Engl. & Irmsch.) Nakai | F42        | 1  | Takahama-cho, Ohi-gun, Fukui Pref., Japan     | 35°30'N, 135°29'E      | KYO_00025612                                |
| <i>Saxifraga fortunei</i> var. <i>incislobata</i>                               | F05        | 24 | Oga City, Akita Pref., Japan                  | 39°53'N, 139°45'E      | KYO_00025344                                |
| <i>Saxifraga fortunei</i> var. <i>incislobata</i>                               | F35        | 24 | Hakusan City, Ishikawa Pref., Japan           | 36°11'N, 136°36'E      | KYO_00025616                                |
| <i>Saxifraga fortunei</i> var. <i>incislobata</i>                               | F38        | 24 | Sakai City, Fukui Pref., Japan                | 36°08'N, 136°22'E      | KYO_00025339                                |
| <i>Saxifraga fortunei</i> var. <i>obtusocuneata</i> (Makino) Nakai              | F67        | 24 | Niyodogawa-cho, Agawa-gun, Kochi Pref., Japan | 33°39'N, 133°08'E      | KYO_00025613                                |
| <i>Saxifraga fortunei</i> var. <i>suwoensis</i> Nakai                           | F75        | 24 | Imari City, Saga Pref., Japan                 | 33°13'N, 129°53'E      | KYO_00025356                                |
| <i>Saxifraga acerifolia</i> Wakabayashi & Satomi                                | SAF        | 24 | Sakai City, Fukui Pref., Japan                | 36°08'N, 136°22'E      | KYO_00025333                                |

Note: N = number of individuals.

<sup>a</sup>Vouchers are deposited at Kyoto University (KYO), Kyoto, Japan.

#### APPENDIX 2. Characteristics of 21 monomorphic microsatellite loci developed in *Saxifraga fortunei* var. *incislobata*.<sup>a</sup>

| Locus  | Primer sequences (5'–3')                                                       | Repeat motif       | Allele size range (bp) | BLASTX top hit description                                                                       | E-value   | GenBank accession no. |
|--------|--------------------------------------------------------------------------------|--------------------|------------------------|--------------------------------------------------------------------------------------------------|-----------|-----------------------|
| SF230  | F: CACGACGTTGTAAAACGACCTGATTGCGACGATGAGAGC<br>R: GTTTCCTTGTGCCTAACTTTCACCAACCC | (AT) <sub>14</sub> | 413                    | No significant hit                                                                               | —         | LC465770              |
| SF1095 | F: CTATAGGGCAGCGCTGGTTTGAACGCCCTTAAGACCGC<br>R: GTTTCCTTCGCTCGCCTTACTATAACCG   | (AT) <sub>18</sub> | 448                    | Probable E3 ubiquitin-protein ligase BAH1-like [ <i>Herrania umbratica</i> ]                     | 7.92E-41  | LC465772              |
| SF561  | F: CTATAGGGCAGCGCTGGTGATTTGGAGCCTCTTTGCCG<br>R: GTTTCCTTTGACACCAGCCCTCACTAG    | (AT) <sub>11</sub> | 317                    | No significant hit                                                                               | —         | LC465778              |
| SF293  | F: CTATAGGGCAGCGCTGGTAAACGAGACATGGCTGCTTG<br>R: GTTTCCTTCGGGTTTGGTCACAGAGAG    | (TTG) <sub>6</sub> | 215                    | No significant hit                                                                               | —         | LC465781              |
| SF112  | F: CGGAGAGCCGAGAGGTGTTTGAAGTGGCTGCCATC<br>R: GTTTCCTTCGTTGCTATGTGACTTGG        | (AT) <sub>12</sub> | 135                    | Sphinganine C4-monooxygenase [ <i>Actinidia chinensis</i> var. <i>chinensis</i> ]                | 1.66E-164 | LC465785              |
| SF1055 | F: CTATAGGGCAGCGCTGGTGAGTAAGAGGTGGTGAAACG<br>R: GTTTCCTTATGCAATCTCCTGGCAAGC    | (AG) <sub>12</sub> | 153                    | Stomatal closure-related actin-binding protein 1-like [ <i>Ziziphus jujuba</i> ]                 | 6.23E-08  | LC465787              |
| SF785  | F: CACGACGTTGTAAAACGACTCTTCTCAACGCTTGGTCTG<br>R: GTTTCCTTCGCGTGAGATCCAACATTG   | (ATC) <sub>8</sub> | 152                    | PREDICTED: probable acyl-activating enzyme 16, chloroplastic [ <i>Populus euphratica</i> ]       | 0.00      | LC465789              |
| SF1547 | F: CTATAGGGCAGCGCTGGTAGGCGACGTGTCAGAGTATC<br>R: GTTTCCTTGAAGAAGCTCGTGATCAGGC   | (AT) <sub>10</sub> | 454                    | Hypothetical protein CDL15_ Pgr014134 [ <i>Punica granatum</i> ]                                 | 5.14E-06  | LC465795              |
| SF1009 | F: CGGAGAGCCGAGAGGTGAACCATCTACTAGCAGGCG<br>R: GTTTCCTTGTGTGGCTGTACTTGTGGC      | (CAC) <sub>8</sub> | 445                    | Trihelix transcription factor GTL1 isoform X2 [ <i>Rosa chinensis</i> ]                          | 4.73E-95  | LC465796              |
| SF795  | F: CTATAGGGCAGCGCTGGTGACGCCCTTTACCTTGTTG<br>R: GTTTCCTTACAGAGAAGCATCCAGACCC    | (ATC) <sub>7</sub> | 435                    | Uncharacterized protein LOC110645629 [ <i>Hevea brasiliensis</i> ]                               | 1.26E-136 | LC465797              |
| SF1496 | F: CTATAGGGCAGCGCTGGTAGGCGCTAAGATTGAGGAG<br>R: GTTTCCTTGTGGTGGAGGAGGAGTACAC    | (AAG) <sub>7</sub> | 445                    | Dehydrin [ <i>Corchorus capsularis</i> ]                                                         | 4.00E-03  | LC465798              |
| SF145  | F: CTATAGGGCAGCGCTGGTTATCCCAAAGCAGCAGGAGG<br>R: GTTTCCTTAGGATTGGTTGAGGGAGACG   | (CCT) <sub>7</sub> | 318                    | PREDICTED: pentatricopeptide repeat-containing protein At4g38150-like [ <i>Camelina sativa</i> ] | 6.69E-29  | LC465800              |

(Continues)

## APPENDIX 2. (Continued)

| Locus  | Primer sequences (5′–3′)                                                      | Repeat motif       | Allele size range (bp) | BLASTX top hit description                                                                | E-value   | GenBank accession no. |
|--------|-------------------------------------------------------------------------------|--------------------|------------------------|-------------------------------------------------------------------------------------------|-----------|-----------------------|
| SF316  | F: CGGAGAGCCGAGAGGTGTGGGACGATACTTCACCGAC<br>R: GTTTCTTGCCATGGATGAGGTGAAAC     | (CT) <sub>10</sub> | 344                    | Lectin_legB domain-containing protein [ <i>Cephalotus follicularis</i> ]                  | 5.08E-51  | LC465801              |
| SF111  | F: CACGACGTTGTAAAACGACGCCAGTCCAATAAGTTCGGC<br>R: GTTTCTTCCTGCAATGGAGTGACTGAAC | (AT) <sub>11</sub> | 307                    | PREDICTED: protein NLRC3 [ <i>Prunus mume</i> ]                                           | 1.37E-101 | LC465802              |
| SF1530 | F: TGTGGAATTGTGAGCGGCGGTGAGAACGGAACAATGG<br>R: GTTTCTTTTGAGGATTCTGTGCCTCCG    | (ATC) <sub>7</sub> | 279                    | Zinc-finger homeodomain protein 5 [ <i>Jatropha curcas</i> ]                              | 3.15E-08  | LC465803              |
| SF1096 | F: CTATAGGGCACGCGTGGTTTCGACAGCAAACCGTTAGC<br>R: GTTTCTTATTATCCGGCTCCATCTCGG   | (TGG) <sub>8</sub> | 339                    | PREDICTED: formin-like protein 1 [ <i>Vitis vinifera</i> ]                                | 0.00      | LC465806              |
| SF553  | F: CACGACGTTGTAAAACGACGCCAGAGGAGTTTACGCTTG<br>R: GTTTCTTGTCATCGTCACAATCAACCAC | (TCG) <sub>7</sub> | 271                    | PREDICTED: calcium-dependent protein kinase 11-like [ <i>Juglans regia</i> ]              | 0.00      | LC465808              |
| SF1204 | F: TGTGGAATTGTGAGCGGACCACAAACGTATCTAGGCATG<br>R: GTTTCTTAACGACCCAAACAAGCACAG  | (AGC) <sub>7</sub> | 245                    | Beta-amylin 28-oxidase-like [ <i>Quercus suber</i> ]                                      | 5.67E-95  | LC465809              |
| SF76   | F: CGGAGAGCCGAGAGGTGCCATTCTCGCTCCAACATCG<br>R: GTTTCTTGCGCTGGAGGATTAGAATG     | (ACC) <sub>7</sub> | 286                    | SANT/Myb domain [ <i>Macleaya cordata</i> ]                                               | 2.19E-81  | LC465810              |
| SF1403 | F: CTATAGGGCACGCGTGGTCATCCGCCAGCATGTGATG<br>R: GTTTCTTGCGCTAGTGAAGTGATGGAG    | (TC) <sub>17</sub> | 169                    | PREDICTED: trigger factor-like protein TIG, Chloroplastic [ <i>Populus euphratica</i> ]   | 0.00      | LC465811              |
| SF1504 | F: CGGAGAGCCGAGAGGTGAGCGTGACCTTAACCTCCTC<br>R: GTTTCTTGTCGAGGAAGACGAAGGAC     | (ATC) <sub>7</sub> | 179                    | Membrane-associated kinase regulator [ <i>Actinidia chinensis</i> var. <i>chinensis</i> ] | 9.89E-46  | LC465812              |

\*Annealing temperature is 58°C for all primer pairs.