Development of EST-SSR markers in Saxifraga sinomontana (Saxifragaceae) and cross-amplification in three related species

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PREMISE: Saxifraga sinomontana (Saxifragaceae) is a widespread alpine species in the Qinghai–Tibetan Plateau and its flanking mountains. We developed a set of expressed sequence tag–simple sequence repeat (EST-SSR) markers to investigate the genetic diversity and evolutionary history of the species.

METHODS AND RESULTS: We initially designed 50 EST-SSR markers based on transcriptome data of S. sinomontana. Nineteen of 50 loci (38%) were successfully amplified, 13 of which were polymorphic. These were tested on 71 individuals from four populations. Three to 18 alleles per locus were detected, and the levels of observed and expected heterozygosity ranged from 0.2817 to 0.9155 and 0.2585 to 0.8495, respectively. In addition, cross-amplification was successful for all 13 loci in three congeneric species, S. tangutica, S. heleonastes, and S. congestiflora.

CONCLUSIONS: These EST-SSR markers will be useful for studying the genetic diversity of S. sinomontana and disentangling the phylogenetic relationships of related species.

KEYWORDS: EST-SSR markers; Saxifraga congestiflora; Saxifraga heleonastes; Saxifraga sinomontana; Saxifraga tangutica; transcriptome.

The genus Saxifraga L. (Saxifragaceae) consists of approximately 500 species that are mainly distributed in the mountainous regions of Europe and Asia (Pan et al., 2001; Gao et al., 2015; Tkach et al., 2015), including the Qinghai–Tibetan Plateau (QTP) and Hengduan Mountains region (HDM), which is a biodiversity hotspot of Saxifraga (Pan et al., 2001). Saxifraga sinomontana J. T. Pan & Gornall is a widespread perennial herb in the QTP and its peripheral regions. It prefers scrub, alpine/marshy meadows, or calcareous crevices at elevations of 2700–5300 m (Pan et al., 2001). Diagnostic features of the species are pedicels with sparsely brown crisped villi and erect sepals that are covered by crisped villi marginally and abaxially (Pan et al., 2001). However, S. sinomontana is an extraordinarily variable species in morphology, as described in the Flora of China (Pan et al., 2001), as well as according to our long-term field surveys, which have demonstrated that gradations in traits are common in the species (e.g., plant height, number of flowers). Saxifraga sinomontana has been the focus of recent systematics research (Gao et al., 2015; Tkach et al., 2015), and Li et al. (2018) revealed that this species possesses a high level of genetic diversity, which may be the result of Quaternary climatic oscillations.

Microsatellite markers for S. sinomontana and its closely related species are not available at present, which limits the development of genetic studies. Due to the advantages of codominance, high polymorphism, and widespread distribution throughout the genome (Bouck and Vision, 2007), expressed sequence tag–simple sequence repeat (EST-SSR) markers are widely applied in genetic diversity research. Moreover, EST-SSR markers are relatively easy and inexpensive to develop, and more transferable among closely related species than genomic SSRs (Bouck and Vision, 2007; Ellis and Burke, 2007). In this study, we developed 13 EST-SSR markers for further population genetic studies of S. sinomontana. Additionally, we evaluated the transferability of these markers in the three sympatric and congeneric species S. tangutica Engl., S. heleonastes Harry Sm., and S. congestiflora Engl. & Irmsch.

METHODS AND RESULTS

Fresh leaf tissue of S. sinomontana was collected from Yushu, Qinghai Province, China (Appendix 1), and was frozen in liquid nitrogen before storage at −80°C. Total RNA was extracted using
### TABLE 1. Characteristics of the 19 EST-SSR markers developed for Saxifraga sinomontana.

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | T<sub>a</sub> (°C) | Fluorescent label | GenBank accession no. | BLASTX top hit description | E-value | GenBank accession no. of BLASTX top hit |
|-------|---------------------------|--------------|------------------------|-----------------|------------------|----------------------|-----------------------------|--------|--------------------------------------|
| SS1   | F: CGGAATCTGATGGCTGCTCT   | (GA)<sub>6</sub> | 244–298                | 53.5            | FAM              | MK348907             | No significant similarity found     |        |                                     |
|       | R: AAAAACCTCTCCACAACGACA  |              |                       |                 |                  |                      |                              |        |                                     |
| SS2   | F: TCCACCCATAGGACAACAA   | (AG)<sub>6</sub> | 264–294                | 62              | FAM              | MK348908             | No significant similarity found     |        |                                     |
|       | R: GATCTGCAAGGGAATATGA    |              |                       |                 |                  |                      |                              |        |                                     |
| SS8   | F: TTTTCTGCTTGCGTCGTCGCT | (TC)<sub>6</sub> | 245–263                | 52              | FAM              | MK348910             | Transcription factor TCP20 [Cucumis sativus]  | 5E-12  | XP_004148022.1                       |
|       | R: GTGAGCCAATTCTCTCTCTGA  |              |                       |                 |                  |                      |                              |        |                                     |
| SS9   | F: CCTCGTCTATAGGCTCCTGC   | (TTG)<sub>6</sub> | 189–198                | 53.5            | FAM              | MK348911             | Trihelix transcription factor ASIL1-like [Lupinus angustifolius]  | 8E-05  | XP_019455555.1                       |
|       | R: GTGCCGCCATTGTCAGAGA    |              |                       |                 |                  |                      |                              |        |                                     |
| SS10  | F: CCGAGAATGCGTTACGAAA   | (TTT)<sub>6</sub> | 264–275                | 53.5            | HEX              | MK348912             | No significant similarity found     |        |                                     |
|       | R: TTGTTCGCAAAACGATGCT    |              |                       |                 |                  |                      |                              |        |                                     |
| SS11  | F: TGAATAGGGGCGAGATGCT    | (GTG)<sub>6</sub> | 92–125                 | 53.5            | HEX              | MK348913             | No significant similarity found     |        |                                     |
|       | R: AGAAGTTGGGGCTGTTACGT   |              |                       |                 |                  |                      |                              |        |                                     |
| SS16  | F: AGCCAAAGGTAGGAGGAGTG   | (AAT)<sub>6</sub> | 244–286                | 53.5            | HEX              | MK348914             | Cyclin P/U [Corchorus capsularis]    | 3E-12  | OMO60173.1                          |
|       | R: AGTCCATTTCCTAGAGTGGTG  |              |                       |                 |                  |                      |                              |        |                                     |
| SS32  | F: TCTCAGCTTTGGAAATAGGCT  | (GTG)<sub>6</sub> | 170–185                | 53.5            | HEX              | MK348920             | No significant similarity found     |        |                                     |
|       | R: GCTCCGCCCGCTCTAAATTA   |              |                       |                 |                  |                      |                              |        |                                     |
| SS35  | F: GGGGAAAGGAAATGCGTGGC   | (ATT)<sub>G</sub> | 250–339                | 56.5            | ROX              | MK348921             | Hypothetical protein               | 3.9    | XP_021878789.1                       |
|       | R: AGGAGGCTCCGAAACACATT   |              |                       |                 |                  |                      |                              |        |                                     |
| SS40  | F: TCGGATAGGCCCATGGGGG    | (AAGCC)<sub>3</sub> | 221–241                | 53.5            | ROX              | MK348922             | No significant similarity found     |        |                                     |
|       | R: ATCGGGGTGTAAGTCCAGCCCT |              |                       |                 |                  |                      |                              |        |                                     |
| SS44  | F: CCGCTATGCTGGGCAACTAT   | (CAAGA)<sub>2</sub> | 109–130                | 53.5            | ROX              | MK348923             | No significant similarity found     |        |                                     |
|       | R: TGTCTCTACACAAACCACAGGT |              |                       |                 |                  |                      |                              |        |                                     |
| SS46  | F: ACAATGCGGCGACTGTGGA    | (TTGCC)<sub>6</sub> | 328–402                | 56.5            | ROX              | MK348924             | Phosphoglycerate mutase family protein [Artemisia annua] | 2E-16  | PWA00260.1                          |
|       | R: AGGCTTCTCTCACATCGTCTTG  |              |                       |                 |                  |                      |                              |        |                                     |
| SS47  | F: CCACCTCGTCGGGAGAAAC    | (GACCA)<sub>2</sub> | 187–251                | 53.5            | FAM              | MK348925             | No significant similarity found     |        |                                     |
|       | R: TTTGATCCTGCTGCTGCTAGG  |              |                       |                 |                  |                      |                              |        |                                     |
| SS56  | F: AGCTCACTCCATGCAATGCTCA | (TA)<sub>6</sub> | 233                    | 53.5            | —                | MK348909             | No significant similarity found     |        |                                     |
|       | R: CCAAGACGGTTGCTCCTCCTCT |              |                       |                 |                  |                      |                              |        |                                     |
| SS24  | F: AGTCCCTGCTCCAAAAGTACTA |              |                       |                 |                  |                      |                              |        |                                     |
|       | R: TCCCGGACCTCCATTTCACTTACG |             |                       |                 |                  |                      |                              |        |                                     |
| SS27  | F: AGCAATGTCCGTCTGGCATATCC |              |                       |                 |                  |                      |                              |        |                                     |
|       | R: GCCAGAGATTGGTCTTCAGATCC |              |                       |                 |                  |                      |                              |        |                                     |
| SS28  | F: ACATTTTCTACATCTACAGGTTG | (CAGT)<sub>6</sub> | 215                    | 56.5            | —                | MK348917             | No significant similarity found     |        |                                     |
|       | R: TGGAGATGTAGGTAGATTGTAG  |              |                       |                 |                  |                      |                              |        |                                     |
| SS29  | F: CTGCTGCTGCTGGATAGGGA   | (TTG)<sub>G</sub> | 114                    | 53.5            | —                | MK348918             | 60S ribosomal protein like [Actinidia chinensis var. chinensis] | 0.001  | PSS30694.1                          |
|       | R: TCCAGGAAACGAAATGTGCTG  |              |                       |                 |                  |                      |                              |        |                                     |
| SS31  | F: TCGAGGCTGTGAACTGCCGAGT | (GACA)<sub>6</sub> | 140                    | 56.5            | —                | MK348919             | DUF4153 domain-containing protein [Stenotrophomonas maltophilia] | 5.7    | WP_100463508.1                       |

Note: T<sub>a</sub> = annealing temperature.

*Monomorphic loci.*
the protocol described by Kumar and Singh (2012). The mRNA was then purified from total RNA using poly-T oligo-attached magnetic beads and fragmented into short fragments. cDNA libraries were prepared for 150–200 bp paired-end sequencing following the Illumina protocol (Illumina version 3, San Diego, California, USA). Sequencing was performed by Novogene Biotechnology Company (Tianjin, China) on an Illumina HiSeq 2000 platform (Illumina), yielding 94,855,756 raw reads. All raw reads have been deposited into the National Center for Biotechnology Information’s (NCBI) Sequence Read Archive (SRA; BioProject accession number: SRR8365238). Total genomic DNA was extracted from silica-dried leaves using the modified cetyltrimethylammonium bromide (CTAB) method of Doyle and Doyle (1987). PCR reactions were carried out in a total volume of 25 μL containing 2.0 μL of total genomic DNA (10–20 ng), 0.3 μL of each primer (10 μM), 0.3 μL of 10× PCR buffer (with Mg++), and 2.5 μL of 10 mM dNTPs. The PCR profile included an initial pretreatment of 10 min at 94°C; followed by 35 cycles of 1 min denaturation at 94°C, 50 s at locus-specific annealing temperatures (Table 1), and 1 min elongation at 72°C; and a final extension at 72°C for 10 min. The PCR products were screened using 1% agarose electrophoresis to determine whether amplifications were successful for the expected sizes and then separated on 6% polyacrylamide gels. Overall, 19 of 50 (38%) EST-SSR primer pairs produced clear, unique amplification products of the expected size. Of these, 13 loci were polymorphic across populations. Characteristics of all 19 loci are listed in Table 1. For all 13 polymorphic SSR loci, the 5′ end of each forward primer was labeled with one of three fluorescent dyes (FAM, HEX, or ROX; Table 1). PCR amplifications were then performed using 71 individuals from four populations of S. sinomontana with the same protocol described above (Table 2). The fluorescently tagged PCR products were analyzed on an ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, California, USA) with a GeneScan 500 LIZ Size Standard (Applied Biosystems), and allele sizes were scored with GeneMapper version 3.2 (Applied Biosystems). Number of alleles per locus (A), observed heterozygosity, and expected heterozygosity were calculated with POPGENE version 1.32 (Yeh et al., 1999). Hardy–Weinberg equilibrium was tested for each population using GENEPOP version 4.2 (Rousset, 2008). Using MICRO-CHECKER version 2.2 (van Oosterhout et al., 2004), we found no evidence of null alleles across all loci.

Among the 13 polymorphic loci, A ranged from three to 18 (mean = 8). The Dingri population had the lowest mean values (A = 2) among the four populations. Levels of observed and expected heterozygosity varied from 0.2817 to 0.9155 and 0.2585 to 0.8495, respectively, which indicates that genetic diversity is relatively high in this species. Additionally, a few loci showed significant deviations from Hardy–Weinberg equilibrium: three in the Aba population, two in the Changdu population, and seven in the Dingri population (P < 0.05; Table 2).

All 13 EST-SSR markers also amplified successfully in S. tanguitica, S. heleonastes, and S. congestiflora, using the same PCR protocol as for S. sinomontana (Table 3).

### TABLE 2. Genetic diversity of the 13 polymorphic loci across four Saxifraga sinomontana populations.

| Locus | Aba (n = 8) | Changdu (n = 28) | Chengdu (n = 18) | Dingri (n = 17) | Total (n = 71) |
|-------|------------|-----------------|-----------------|----------------|---------------|
|       | A | H_e | H_o | A | H_e | H_o | A | H_e | H_o | A | H_e | H_o |
| S51   | 5 | 0.6250 | 0.6083 | 5 | 0.7500 | 0.7110 | 7 | 0.8333 | 0.6984* | 2 | 1.0000 | 0.5152* |
| S52   | 5 | 0.6250 | 0.7667 | 8 | 0.7500 | 0.8247 | 10 | 0.7222 | 0.8571 | 2 | 0.1176 | 0.1141 |
| S55   | 5 | 0.8750 | 0.7750* | 6 | 0.7500 | 0.6117 | 5 | 0.8899 | 0.7444 | 2 | 1.0000 | 0.5152* |
| S59   | 3 | 0.3750 | 0.4917 | 3 | 0.4643 | 0.4643 | 4 | 0.6111 | 0.5603 | 3 | 1.0000 | 0.6078* |
| S510  | 2 | 0.1250 | 0.3250 | 2 | 0.3214 | 0.2747 | 3 | 0.7222 | 0.5222 | 1 | 0.0000 | 0.0000 |
| S511  | 7 | 0.6250 | 0.7417 | 5 | 0.6429 | 0.7188 | 4 | 0.5556 | 0.4968 | 3 | 0.1176 | 0.1159 |
| S516  | 3 | 0.5000 | 0.4250 | 2 | 0.2143 | 0.2494 | 5 | 0.7778 | 0.5841 | 3 | 1.0000 | 0.5473* |
| S532  | 3 | 0.2500 | 0.5667* | 4 | 0.4643 | 0.5591 | 2 | 0.6111 | 0.6270 | 2 | 1.0000 | 0.5152* |
| S535  | 4 | 1.0000 | 0.6417 | 3 | 0.2500 | 0.2305 | 3 | 0.2778 | 0.2524 | 1 | 0.0000 | 0.0000 |
| S540  | 3 | 0.6250 | 0.7570 | 4 | 0.3571 | 0.4227 | 2 | 0.4444 | 0.3556 | 2 | 0.2353 | 0.2139 |
| S544  | 3 | 1.0000 | 0.5917* | 3 | 0.7857 | 0.6370 | 3 | 1.0000 | 0.6413* | 3 | 1.0000 | 0.6505* |
| S546  | 13 | 0.8750 | 0.9750 | 5 | 0.7500 | 0.7221 | 8 | 0.8333 | 0.7413 | 2 | 0.0588 | 0.0588 |
| S547  | 7 | 0.7500 | 0.8417 | 8 | 0.8929 | 0.8587 | 6 | 0.7778 | 0.7365 | 2 | 1.0000 | 0.5152* |
| Mean  | 5 | 0.6363 | 0.6932 | 5 | 0.6587 | 0.5586 | 6 | 0.6966 | 0.5889 | 2 | 0.5792 | 0.8034 |

Note: A = total number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled.

*Population and voucher information are provided in Appendix 1.

*Significant departure from Hardy–Weinberg equilibrium at P < 0.05.
TABLE 3. Genetic diversity in three congeneric species based on the 13 polymorphic microsatellite loci developed for Saxifraga sinomontana.*

| Locus | Cuona (n = 8) | Xinghai (n = 15) | Chengdu (n = 7) | Luozha (n = 4) | Dege (n = 7) | Shiqu (n = 6) |
|-------|--------------|-----------------|----------------|---------------|-------------|--------------|
|       | H_1          | H_2             | H_1            | H_2           | H_1         | H_2          |
| S51   | 5.0000       | 0.6667          | 6.0000         | 0.9333        | 4.0000      | 0.8214       |
| S52   | 4.6250       | 0.6583          | 6.0000         | 0.8299        | 4.0000      | 0.7500       |
| S58   | 4.0000       | 1.0000          | 3.0000         | 0.5770*       | 2.0000      | 0.5385*      |
| S59   | 5.7500       | 0.6083          | 3.0000         | 0.6897        | 2.0000      | 0.7149       |
| S510  | 12.0000      | 0.9583          | 7.0000         | 0.5103        | 1.0000      | 0.2637       |
| S511  | 5.3750       | 0.6083*         | 4.0000         | 0.5973*       | 2.0000      | 0.4945       |
| S516  | 2.1250       | 0.1250          | 2.0000         | 0.5149*       | 3.0000      | 0.5824       |
| S532  | 3.3750       | 0.3417          | 4.0000         | 0.6345        | 10.0000     | 0.9333       |
| S555  | 2.0000       | 0.2333          | 4.0000         | 0.3954        | 2.0000      | 0.1429       |
| S540  | 2.8750       | 0.5250          | 3.0000         | 0.4759        | 3.0000      | 0.5714       |
| S544  | 1.0000       | 0.0000          | 5.0000         | 0.3333        | 7.0000      | 0.4115*      |
| S546  | 3.3333       | 0.6000          | 9.0000         | 0.4167        | 2.0000      | 0.5000       |
| S547  | 7.0000       | 0.8833          | 10.0000        | 0.8529        | 3.0000      | 0.7143       |

Note: A = total number of alleles per locus; H_1 = expected heterozygosity; H_2 = observed heterozygosity; n = number of individuals sampled.

*Significant departure from Hardy–Weinberg equilibrium at P < 0.05.

CONCLUSIONS

The 13 EST-SSR markers developed here showed high polymorphism in S. sinomontana and high cross-species amplification success. Hence, these are valuable loci for investigating genetic diversity, population structure, and evolutionary history in S. sinomontana and throughout Saxifraga.

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DATA ACCESSIBILITY

Raw sequencing reads were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (BioProject SRX8365238). Sequence information for the developed primers has been deposited in NCBI’s GenBank, and accession numbers are provided in Table 1.

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APPENDIX 1. Locality and voucher information for the populations of *Saxifraga* used in this study.

| Species                        | Voucher no. | Collection locality | Geographic coordinates | Elevation (m) | n  |
|--------------------------------|-------------|---------------------|-------------------------|---------------|----|
| *Saxifraga sinomontana* J. T. Pan & Gornall | Chen 2014558 | Yushu, Qinghai       | 32°34′20.7″N, 97°12′41.6″E      | 4880          | 1  |
|                                | Chen 2012317 | Aba, Sichuan         | 32°46′02″N, 101°40′01″E         | 3450          | 8  |
|                                | Chen 2014282 | Changdu, Tibet       | 31°04′48″N, 96°56′59″E          | 4610          | 28 |
|                                | Chen 2012347 | Chengduo, Qinghai    | 33°12′02″N, 97°28′13″E          | 4450          | 18 |
|                                | Chen 2007078 | Dingri, Tibet        | 28°55′58″N, 87°26′24″E          | 5160          | 17 |
| *Saxifraga tangutica* Engl.     | Chen 2014409 | Cuona, Tibet         | 28°19′23.4″N, 91°55′08.5″E      | 4770          | 8  |
|                                | Chen 2007004 | Xinghai, Qinghai     | 35°36′50″N, 99°32′05″E          | 3980          | 15 |
| *Saxifraga heleonastes* Harry Sm. | Chen 2006024 | Chengduo, Qinghai    | 34°07.457′N, 97°39.411′E        | 4850          | 7  |
|                                | Chen 2014483 | Luozha, Tibet        | 28°24′39.2″N, 90°34′31.4″E      | 5110          | 4  |
| *Saxifraga congestiflora* Engl. & Irmsch. | Chen 2007226 | Dege, Sichuan        | 31°57′25″N, 98°52′43″E          | 4180          | 7  |
|                                | Chen 2007250 | Shiqu, Sichuan       | 32°29′33″N, 98°27′17″E          | 4380          | 6  |

Note: n = number of individuals sampled.
Voucher specimens deposited at the herbarium of the Northwest Institute of Plateau Biology (HNWP), Xining, Qinghai, China.