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Primer Note

# Development and characterization of EST-SSR markers in *Stipa breviflora* (Poaceae)[1]

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- *Premise of the study: Stipa breviflora* (Poaceae) is one of the dominant species of the desert steppe in the eastern Eurasian grasslands. Simple sequence repeat (SSR) markers were developed for use in genetic diversity studies of this species.
- *Methods and Results:* A total of 1954 potentially polymorphic loci were obtained by comparing transcriptome data of eight different *S. breviflora* individuals. We selected 81 loci to verify polymorphism and 63 loci amplified, of which 21 loci exhibited polymorphism. The number of alleles per locus varied from two to 24, the observed heterozygosity ranged from 0.083 to 0.958, and the expected heterozygosity ranged from 0.396 to 0.738.
- *Conclusions:* These newly identified SSR loci can be used for population genetic and landscape genetic studies of *S. breviflora*. In addition, 14 loci also amplified in six related *Stipa* species (*S. grandis*, *S. krylovii*, *S. bungeana*, *S. aliena*, *S. gobica*, and *S. purpurea*).

**Key words:** microsatellite markers; Poaceae; polymorphism; *Stipa breviflora*; transcriptome.

*Stipa breviflora* Griseb. (Poaceae) is one of the dominant species covering the desert steppe region of the eastern Eurasian grassland. In China, it is widely distributed in the southern Mongolia Plateau, western Ordos Plateau, northwestern Loess Plateau, Tibet Plateau, and Xinjiang Province (Zhang et al., 2009). *Stipa breviflora*, which is characterized by drought resistance, grazing tolerance, fine palatability, and early spring growth, serves as an indispensable forage resource for herbivores in dryland areas. Moreover, it plays an important role in both conserving soil and water and preventing desertification (Zhang et al., 2010). *Stipa breviflora* grassland, however, has been severely degraded by global warming and anthropogenic disturbances. Despite the ecological significance of *S. breviflora*, we know little about its population genetics and evolutionary biology using current technologies. The only study that has evaluated the genetic diversity of this species used RAPD markers (Zhang et al., 2012). Microsatellite (simple sequence repeat [SSR]) markers, which can be developed using genomes or transcriptomes, are powerful tools for examining population genetic diversity. SSRs are often presumed to be neutral, but can be subject to both positive and negative selection for a variety of reasons. Although we reported preliminary results on SSR development of *S. breviflora* (Zhao et al., 2016), a systematic and improved study is also required.

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## METHODS AND RESULTS

Transcriptome sequencing of eight *S. breviflora* individuals, collected from a wide geographic range, was conducted using Illumina HiSeq 4000 (Illumina, San Diego, California, USA). Approximately six million 150-bp reads of each individual were obtained and de novo assembled into 178,901 unigenes (>300 bp) using Trinity (Grabherr et al., 2011), with mean length of 1235 bp. SSRs were detected using MicroSAtellite Identification Tool (MISA; Thiel et al., 2003), with the criteria of 12, six, five, five, four, and four repeat units for mono-, di-, tri-, tetra-, penta-, and hexanucleotide motifs, respectively. The criteria were determined by the settings for minimum number of repeats according to the MISA software instructions. We adjusted the repeat numbers of pentanucleotide and hexanucleotide motifs on the basis of our search results for a higher number of SSR loci. A total of 29,817 SSRs were identified, with trinucleotide repeats (16,785, 62.76%) being the most common, followed by dinucleotide (7489, 28.00%), mononucleotide (3071, 10.30%), hexanucleotide (958, 3.58%), pentanucleotide (923, 3.45%), and tetranucleotide (591, 2.21%) repeats.

By comparing the transcriptome sequences, we obtained 1954 unigenes likely to contain polymorphic SSR loci. Then 81 loci demonstrating significant length variation among data for eight transcriptomes were selected. Primer3 (Untergasser et al., 2012) was used to design primer pairs with lengths of 18–21 bp amplifying product sizes ranging from 90–250 bp. We initially screened 81 primer pairs using 16 *S. breviflora* individuals; 63 of these loci were successfully amplified after PCR optimization. The polymorphism of these loci was tested using 24 samples from eight populations of *S. breviflora* (three individuals per population). Finally, we obtained 21 polymorphic expressed sequence tag–SSR (EST-SSR) markers, which were deposited into GenBank (Table 1). Polymorphism of these loci was assessed using 96 individuals from eight populations (12 individuals per population). *Stipa grandis* P. A. Smirn., *S. krylovii* Roshev., *S. bungeana* Trin., *S. aliena* Keng, *S. gobica* Roshev., and *S. purpurea* Griseb. (five individuals of each population) were used to test the cross-species amplification of polymorphic markers in *S. breviflora* (Appendix 1). Owing to lack of plant specimens, voucher specimens of these species could not be provided.

Genomic DNA was extracted from frozen leaf tissues using the Plant Genomic DNA Extraction Kit (Tiangen Biotech, Beijing, China). PCR amplification was performed in a reaction mixture (25 µL) containing 1 µL of template

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Table 1. Characteristics of 21 microsatellite primers developed for *Stipa breviflora*.

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size (bp) | $T_a$ (°C) | Fluorescent dye | GenBank accession no. | Putative function [Organism] | *E*-value |
|---|---|---|---|---|---|---|---|---|
| SB13 | F: CTTCTTGCGAGTACAGCGATTT<br>R: CAAACAGAGCTCAACATCACAAA | $(TC)_8$ | 137 | 57 | HEX | KY355614 | PREDICTED: plasminogen activator inhibitor 1 RNA-binding protein-like [*Oryza brachyantha*] | 4.00E-171 |
| SB16 | F: CAGTGGTTTTTGTTTAACAGCAG<br>R: GCCCGTACCATAATTTTCTTTTT | $(CAA)_6$ | 148 | 51 | TAMRA | KY355615 | Predicted protein [*Hordeum vulgare* subsp. *vulgare*] | 1.00E-83 |
| SB21 | F: ATGTACTTGGAAGAAACGAAGCA<br>R: TGCTGTTGTGATCTACAGGTTTG | $(CGG)_6$ | 94 | 57 | FAM | KY355616 | Predicted protein [*Hordeum vulgare* subsp. *vulgare*] | 6.00E-13 |
| SB32 | F: CAATTGTAGAGGGGTAACAACGA<br>R: AGTCAGTGTGCTGCTGTCAAATA | $(TTC)_5$ | 153 | 57 | TAMRA | KY355617 | Predicted protein [*Hordeum vulgare* subsp. *vulgare*] | 3.00E-102 |
| SB35 | F: CTACTGACATCCAACGTATTGAA<br>R: GAGATCAGGTTTACGAACCCC | $(G)_{16}$ | 153 | 57 | TAMRA | KY355618 | Uncharacterized protein LOC100845604 [*Brachypodium distachyon*] | 2.00E-34 |
| SB40 | F: GATCGCCATTGGTAGTATGTAAA<br>R: TTCCTTCTTCATCCTTCCACTTG | $(AG)_9$ | 160 | 57 | FAM | KY355619 | — | — |
| SB42 | F: TCCCTCAGAGAAAAATCAAAACA<br>R: ATCATCCTGTACACCGTCGTCTA | $(CT)_7$ | 138 | 56 | HEX | KY355620 | Uncharacterized protein LOC100826478 [*Brachypodium distachyon*] | 3.00E-24 |
| SB43 | F: AAATCCTTCCTCGCGCTC<br>R: CTCATCGATCTCCTCGCTTCT | $(ACC)_5$ | 152 | 60 | TAMRA | KY355621 | Uncharacterized protein LOC101756208 [*Setaria italica*] | 9.00E-100 |
| SB45 | F: CCGACACACACAAGATGAGC<br>R: GCTGGTGCAGGACCTCCC | $(GCG)_5$ | 111 | 51 | FAM | KY355622 | Uncharacterized protein LOC100823613 [*Brachypodium distachyon*] | 3.00E-53 |
| SB46 | F: TCCTTCTCTGTATATAAAGCCCG<br>R: ATGCATTTGCCTGGAATGTT | $(CT)_8$ | 136 | 56 | HEX | KY355623 | PREDICTED: NAC domain-containing protein 78 [*Brachypodium distachyon*] | 0 |
| SB49 | F: ACTCTCCTGCAACTCTGTGAAAG<br>R: TAATGCAAGCATTTGGCTATACA | $(GA)_9$ | 144 | 56 | TAMRA | KY355624 | — | — |
| SB50 | F: AGGAGCATCATCCTTGTCCTC<br>R: ACCGCACTTATCTCCTCTTTCTT | $(GAG)_6$ | 134 | 57 | HEX | KY355625 | PREDICTED: paramyosin-like isoform X1 [*Brachypodium distachyon*] | 2.00E-122 |
| SB52 | F: AGAAGAAGAGGAAGAAGAACCCA<br>R: AGATCCACCGCTCTTCCTAGT | $(GGC)_6$ | 146 | 57 | TAMRA | KY355626 | Uncharacterized protein LOC101770650 [*Setaria italica*] | 5.00E-78 |
| SB53 | F: GCAAAGGAACCTACGTCTTCC<br>R: GAGAGGCTCATATGGCTGAAC | $(GGC)_6$ | 133 | 59 | HEX | KY355627 | PREDICTED: cysteine-rich receptor-like protein kinase 10 [*Brachypodium distachyon*] | 2.00E-147 |
| SB54 | F: CACAAGGTACCGAAAAGGAAAG<br>R: ACCAACCCACTCTCTCTCTCTCT | $(GA)_6$ | 90 | 59 | FAM | KY355628 | Hypothetical protein EUGRSUZ_C01942, partial [*Eucalyptus grandis*] | 5.00E-11 |
| SB55 | F: AAATCTGCTCTCAGGTGGAATC<br>R: AAATCAATCGCACTCGCAAT | $(TGCGAT)_4$ | 131 | 54 | HEX | KY355629 | PREDICTED: heat shock protein 82 [*Brachypodium distachyon*] | 2.00E-13 |
| SB57 | F: AACTTGTGAAGGTTTGCAATGTC<br>R: AACCCAGTCACCTCTGACAACTA | $(AT)_{10}$ | 148 | 57 | TAMRA | KY355630 | Predicted protein [*Hordeum vulgare* subsp. *vulgare*] | 1.00E-37 |
| SB77 | F: ACTTTATTCCGCATGCTA<br>R: TTCGTTCTTTTGTCTGTG | $(AAG)_5$ | 125 | 56 | FAM | KY355631 | Predicted protein [*Hordeum vulgare* subsp. *vulgare*] | 0 |
| SB78 | F: TCACCATTACCCATTCGCTTCCT<br>R: TCATCTTCGGATCTCCTCCTCCC | $(GCG)_5TTG(GA)_{10}$ | 191 | 60 | FAM | KY355632 | PREDICTED: ATP synthase subunit d, mitochondrial-like [*Setaria italica*] | 6.00E-88 |
| SB79 | F: GATGGTCCACTCATCCAGGCTGT<br>R: GTGCGTGAGAAAGAAGCGGTCCT | $(TC)_9$ | 235 | 54 | FAM | KY355633 | RecName: Full = Thioredoxin H-type; Short = Trx-H; AltName: Full = TrxTa [*Triticum aestivum*] | 6.00E-54 |
| SB81 | F: CGCTCCACTACCTTTCGTATCAC<br>R: GGAATGAATGCCTTGAGTGAGTC | $(CT)_{11}(GT)_8$ | 194 | 60 | FAM | KY355634 | Uncharacterized protein LOC100845363 [*Brachypodium distachyon*] | 9.00E-137 |

*Note*: $T_a$ = annealing temperature.

TABLE 2. Results of initial screening of 21 polymorphic loci identified in eight populations of *Stipa breviflora*.[a]

| | Wulate ($N$ = 12) | | | Jungar ($N$ = 12) | | | Gaolan ($N$ = 12) | | | Hainan ($N$ = 12) | | | Alxa ($N$ = 12) | | | Chifeng ($N$ = 12) | | | Pulan ($N$ = 12) | | | Hejing ($N$ = 12) | | | Total | Mean | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Locus | $A$ | $H_o$ | $H_e$[b] | $A$ | $H_o$ | $H_e$[b] | $A$ | $H_o$ | $H_e$[b] | $A$ | $H_o$ | $H_e$[b] | $A$ | $H_o$ | $H_e$[b] | $A$ | $H_o$ | $H_e$[b] | $A$ | $H_o$ | $H_e$[b] | $A$ | $H_o$ | $H_e$[b] | $A$ | $H_o$ | $H_e$ |
| SB13 | 4 | 0.08 | 0.57*** | 3 | 0.08 | 0.54** | 4 | 0.17 | 0.68*** | 3 | 0.00 | 0.50*** | 4 | 0.167 | 0.413* | 2 | 0.083 | 0.080 | 1 | 0.000 | 0.000[M] | 3 | 0.083 | 0.434** | 7 | 0.083 | 0.401 |
| SB16 | 5 | 0.83 | 0.68 | 5 | 0.73 | 0.75 | 6 | 0.67 | 0.74 | 6 | 0.83 | 0.78 | 5 | 0.583 | 0.774 | 3 | 0.500 | 0.403 | 3 | 1.000 | 0.622** | 2 | 0.917 | 0.497* | 5 | 0.758 | 0.655 |
| SB21 | 3 | 0.75 | 0.66*** | 4 | 0.67 | 0.69*** | 4 | 0.75 | 0.69* | 4 | 0.55 | 0.67* | 4 | 0.667 | 0.705** | 5 | 0.833 | 0.694** | 2 | 1.000 | 0.500** | 3 | 1.000 | 0.594** | 2 | 0.777 | 0.649 |
| SB32 | 7 | 0.67 | 0.75 | 6 | 0.17 | 0.70*** | 8 | 0.75 | 0.75 | 11 | 0.67 | 0.81 | 5 | 0.333 | 0.722*** | 8 | 0.583 | 0.757* | 3 | 0.182 | 0.169 | 3 | 0.455 | 0.517 | 22 | 0.475 | 0.647 |
| SB35 | 4 | 0.17 | 0.66*** | 10 | 0.08 | 0.87*** | 8 | 0.33 | 0.81*** | 11 | 0.42 | 0.85*** | 9 | 0.455 | 0.855*** | 6 | 0.167 | 0.656*** | 3 | 0.167 | 0.156 | 5 | 0.083 | 0.698*** | 18 | 0.234 | 0.695 |
| SB40 | 7 | 0.33 | 0.72*** | 8 | 0.25 | 0.78*** | 4 | 0.25 | 0.52** | 4 | 0.00 | 0.65*** | 6 | 0.083 | 0.726*** | 6 | 0.273 | 0.653*** | 3 | 0.167 | 0.288 | 5 | 0.083 | 0.649*** | 13 | 0.180 | 0.623 |
| SB42 | 7 | 0.25 | 0.77*** | 6 | 0.00 | 0.78*** | 7 | 0.25 | 0.78*** | 5 | 0.17 | 0.72*** | 5 | 0.000 | 0.736*** | 5 | 0.417 | 0.476 | 2 | 0.091 | 0.087 | 5 | 0.333 | 0.747*** | 15 | 0.188 | 0.636 |
| SB43 | 3 | 0.25 | 0.34 | 6 | 0.33 | 0.48** | 6 | 0.83 | 0.70 | 7 | 0.75 | 0.62 | 3 | 0.167 | 0.156 | 2 | 0.333 | 0.278 | 2 | 0.083 | 0.080 | 6 | 0.583 | 0.569 | 12 | 0.417 | 0.403 |
| SB45 | 2 | 0.91 | 0.50* | 2 | 0.75 | 0.47 | 2 | 1.00 | 0.50** | 2 | 0.42 | 0.33 | 2 | 0.583 | 0.413 | 2 | 0.750 | 0.469 | 2 | 1.000 | 0.500** | 2 | 0.083 | 0.080 | 11 | 0.687 | 0.407 |
| SB46 | 11 | 0.67 | 0.88* | 9 | 0.50 | 0.80*** | 9 | 0.75 | 0.71 | 11 | 1.00 | 0.87 | 7 | 0.667 | 0.781 | 4 | 0.750 | 0.538 | 5 | 0.917 | 0.625 | 4 | 0.917 | 0.628 | 10 | 0.771 | 0.729 |
| SB49 | 8 | 0.33 | 0.82*** | 6 | 0.25 | 0.65*** | 5 | 0.36 | 0.74* | 8 | 0.42 | 0.84*** | 8 | 0.250 | 0.844*** | 4 | 0.250 | 0.538** | 3 | 0.250 | 0.226 | 3 | 0.167 | 0.635*** | 6 | 0.285 | 0.661 |
| SB50 | 7 | 0.73 | 0.79 | 7 | 0.92 | 0.69 | 5 | 0.92 | 0.76 | 6 | 0.75 | 0.73* | 9 | 0.750 | 0.712 | 5 | 0.833 | 0.642 | 3 | 0.833 | 0.542 | 4 | 1.000 | 0.677 | 8 | 0.841 | 0.692 |
| SB52 | 8 | 0.67 | 0.73 | 7 | 0.83 | 0.79 | 7 | 0.92 | 0.80 | 9 | 0.75 | 0.77 | 6 | 0.917 | 0.792 | 7 | 0.667 | 0.701 | 4 | 0.583 | 0.451 | 5 | 0.833 | 0.632 | 17 | 0.771 | 0.708 |
| SB53 | 7 | 0.92 | 0.81* | 4 | 0.83 | 0.64 | 5 | 0.92 | 0.74 | 6 | 0.82 | 0.76** | 6 | 0.818 | 0.814 | 6 | 0.917 | 0.819** | 3 | 0.333 | 0.288 | 7 | 0.917 | 0.799** | 20 | 0.809 | 0.709 |
| SB54 | 8 | 0.83 | 0.74 | 5 | 1.00 | 0.67 | 6 | 1.00 | 0.81*** | 6 | 1.00 | 0.82* | 8 | 0.833 | 0.774 | 7 | 1.000 | 0.722** | 5 | 1.000 | 0.628* | 2 | 1.000 | 0.500** | 16 | 0.958 | 0.708 |
| SB55 | 5 | 0.92 | 0.70 | 4 | 1.00 | 0.73 | 5 | 0.83 | 0.67 | 6 | 0.58 | 0.69 | 5 | 0.833 | 0.667 | 5 | 1.000 | 0.704 | 1 | 0.000 | 0.000[M] | 3 | 1.000 | 0.611** | 12 | 0.771 | 0.596 |
| SB57 | 9 | 0.75 | 0.82 | 7 | 0.42 | 0.48 | 9 | 0.67 | 0.84* | 9 | 0.73 | 0.85* | 8 | 1.000 | 0.726 | 8 | 0.583 | 0.698* | 4 | 0.333 | 0.295 | 7 | 0.500 | 0.663* | 11 | 0.622 | 0.671 |
| SB77 | 5 | 0.58 | 0.63 | 5 | 0.73 | 0.73 | 5 | 0.75 | 0.60 | 6 | 0.92 | 0.78 | 6 | 0.833 | 0.688 | 4 | 0.917 | 0.705 | 3 | 0.833 | 0.517 | 3 | 0.917 | 0.538* | 19 | 0.810 | 0.647 |
| SB78 | 4 | 0.27 | 0.68** | 5 | 0.08 | 0.57*** | 2 | 0.17 | 0.15 | 6 | 0.08 | 0.72*** | 4 | 0.000 | 0.514 | 4 | 0.083 | 0.295** | 2 | 0.083 | 0.080 | 2 | 0.000 | 0.153* | 11 | 0.097 | 0.396 |
| SB79 | 8 | 0.67 | 0.83 | 4 | 0.92 | 0.74 | 7 | 0.92 | 0.80 | 13 | 1.00 | 0.85 | 10 | 0.917 | 0.837 | 8 | 0.917 | 0.753* | 3 | 0.833 | 0.517 | 3 | 1.000 | 0.580* | 18 | 0.896 | 0.738 |
| SB81 | 6 | 0.42 | 0.64* | 8 | 0.33 | 0.82*** | 8 | 0.67 | 0.67 | 14 | 0.92 | 0.90 | 7 | 0.583 | 0.778 | 7 | 0.583 | 0.705 | 7 | 0.545 | 0.459 | 6 | 0.417 | 0.476 | 24 | 0.558 | 0.681 |

*Note*: $A$ = total number of alleles; $H_e$ = expected heterozygosity; $H_o$ = observed heterozygosity; $N$ = total number of samples analyzed.

[a] Locality information is provided in Appendix 1.

[b] Asterisks indicate significant deviation from Hardy–Weinberg equilibrium (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); M = monomorphic.

Table 3. Cross-species amplification results of 21 polymorphic EST-SSR loci developed for *Stipa breviflora* in six other *Stipa* species.[a]

| Locus | *S. grandis* | *S. aliena* | *S. bungeana* | *S. gobica* | *S. krylovii* | *S. purpurea* |
|---|---|---|---|---|---|---|
| SB13 | 1 | 1 | 1 | 1 | 1 | 1 |
| SB16 | 1 | 1 | 1 | 1 | 1 | 1 |
| SB21 | 1 | 1 | 1 | 1 | 1 | 1 |
| SB32 | 1 | 1 | 1 | 1 | 1 | 1 |
| SB35 | 0 | 0 | 0 | 1 | 0 | 0 |
| SB40 | 1 | 1 | 1 | 1 | 1 | 1 |
| SB42 | 1 | 1 | 1 | 1 | 1 | 1 |
| SB43 | 1 | 0 | 1 | 1 | 1 | 0 |
| SB45 | 0 | 0 | 1 | 0 | 0 | 1 |
| SB46 | 1 | 1 | 1 | 1 | 1 | 1 |
| SB49 | 1 | 1 | 1 | 1 | 1 | 1 |
| SB50 | 0 | 1 | 1 | 1 | 1 | 1 |
| SB52 | 0 | 0 | 1 | 0 | 0 | 0 |
| SB53 | 1 | 1 | 1 | 1 | 1 | 1 |
| SB54 | 1 | 1 | 1 | 1 | 1 | 1 |
| SB55 | 0 | 0 | 1 | 1 | 0 | 0 |
| SB57 | 1 | 1 | 1 | 1 | 0 | 0 |
| SB77 | 1 | 1 | 1 | 1 | 1 | 1 |
| SB78 | 1 | 1 | 1 | 1 | 1 | 1 |
| SB79 | 1 | 1 | 1 | 1 | 1 | 1 |
| SB81 | 1 | 1 | 1 | 1 | 1 | 1 |

*Note*: 1 = successful amplification; 0 = failed amplification.
[a] Locality information is provided in Appendix 1.

DNA (30–40 ng/μL), 0.5 μL (10 pM) of each primer, 12.5 μL of Premix *Taq* (TaKaRa Biotechnology Co., Dalian, Liaoning Province, China), and 10.5 μL of ddH$_2$O. Conditions for PCR amplification were as follows: 4 min at 94°C; 35 cycles of 30 s at 94°C, 30 s at a primer-specific annealing temperature (Table 1), 30 s at 72°C; and a final extension step at 72°C for 10 min. PCR products were first detected by 1.5% agarose gel electrophoresis to check for successful amplification. Forward primers for the 21 successfully amplified polymorphic loci were labeled with one of three different fluorescent dyes (FAM, HEX, or TAMRA) and used for amplifications with the same protocol. The labeled PCR products were analyzed on an ABI 3730 DNA Analyzer with a GeneScan 500 LIZ Size Standard (Applied Biosystems, Beijing, China). Allele sizes were called using GeneMarker version 2.6.0 (SoftGenetics, State College, Pennsylvania, USA). Number of alleles per locus, observed heterozygosity, and expected heterozygosity were calculated using GenAlEx version 6.5 (Peakall and Smouse, 2012). GENEPOP version 4.42 (Rousset, 2008) was used to measure the departure from Hardy–Weinberg equilibrium and linkage disequilibrium, and MICRO-CHECKER version 2.2.3 (van Oosterhout et al., 2004) was used to check the possibility of null alleles.

Among these 21 polymorphic loci, the total number of alleles per locus ranged from two to 24, observed heterozygosity ranged from 0.083 to 0.958, and expected heterozygosity ranged from 0.396 to 0.738. Only locus SB21 with null alleles consistently departed from Hardy–Weinberg equilibrium in all populations, and we did not detect linkage disequilibrium between any loci (Table 2). Cross-species amplification of the 21 polymorphic markers was tested in six related species, namely *S. grandis*, *S. krylovii*, *S. bungeana*, *S. aliena*, *S. gobica*, and *S. purpurea* (five individuals per population). Fourteen (66.67%) of the 21 loci successfully amplified in all tested species; the remaining loci were amplified in some species (Table 3).

## CONCLUSIONS

This is the first known report of 21 polymorphic EST-SSRs for *S. breviflora*. These SSRs will be used to evaluate impacts of isolation by distance and recent habitat fragmentation on the genetic diversity and structure of *S. breviflora* populations. These SSRs may also be used in investigations of genetic diversity of other *Stipa* L. species. Hodel et al. (2016) indicate that microsatellites generated from transcriptomes could likely be found in translated regions of the genome. The majority of loci favored in translated regions are trinucleotide repeats (Hodel et al., 2016). Markers occurring in or near coding regions are prone to selective pressures (Morgante et al., 2002), which casts doubt on the application of microsatellites in genetic diversity analysis. However, one study about *Glycine* Willd. and *Oenothera* L. demonstrates that many trinucleotide repeats linked closely to translated regions are not themselves within a translated region of a gene (Hodel et al., 2016); therefore, these loci are potentially useful. In our study, 21 SSRs were derived from transcriptome data, and a significant number of these loci (11/21) were or contained trinucleotide or hexanucleotide repeats. Therefore, we suggest that all 21 loci could be used to examine genetic diversity while neutrality is tested. Researchers investigating selection should use the 11 loci with trinucleotide repeats.

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APPENDIX 1. Location information for populations of *Stipa breviflora* and six other *Stipa* species used in this study.[a]

| Species | Collection locality | Altitude (m) | Geographic coordinates | *N* |
|---|---|---|---|---|
| *Stipa breviflora* Griseb. | Wulate Middle Banner, Inner Mongolia | 1321 | 41°29′28.63″N, 108°57′17.64″E | 12 |
| *S. breviflora* | Jungar, Inner Mongolia | 1160 | 39°46′8.43″N, 110°57′25.91″E | 12 |
| *S. breviflora* | Gaolan, Gansu | 1784 | 36°15′55.94″N, 103°48′44.18″E | 12 |
| *S. breviflora* | Hainan, Qinghai | 2956 | 36°18′59.32″N, 100°33′43.37″E | 12 |
| *S. breviflora* | Alxa, Inner Mongolia | 1461 | 39°50′39.65″N, 105°03′34.30″E | 12 |
| *S. breviflora* | Chifeng, Inner Mongolia | 576 | 42°38′22.48″N, 119°12′14.24″E | 12 |
| *S. breviflora* | Pulan, Tibet | 3863 | 30°16′33.28″N, 81°10′11.19″E | 12 |
| *S. breviflora* | Hejing, Xinjiang | 2141 | 42°54′05.90″N, 86°17′56.83″E | 12 |
| *S. aliena* Keng | Haibei, Qinghai | 3201 | 101°19′31.3″N, 37°36′38.9″E | 5 |
| *S. bungeana* Trin. | Hohhot, Inner Mongolia | 1040 | 40°47′24.11″N, 111°28′7.28″E | 5 |
| *S. gobica* Roshev. | Wuhai, Inner Mongolia | 1599 | 39°37′51.50″N, 106°53′43.77″E | 5 |
| *S. grandis* P. A. Smirn. | Manzhouli, Inner Mongolia | 659 | 49°33′01″N, 117°33′59″E | 5 |
| *S. krylovii* Roshev. | Hulunbuir, Inner Mongolia | 930 | 47°51′58″N, 115°46′48″E | 5 |
| *S. purpurea* Griseb. | Tianjun, Qinghai | 3195 | 37°12′40.2″N, 98.55′31.4″E | 5 |

*Note*: *N* = numbers of individuals sampled.
[a]Voucher specimens were not collected at the time of the study.