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DEVELOPMENT OF MICROSATELLITE MARKERS USING ILLUMINA MiSEQ SEQUENCING TO CHARACTERIZE Ephedra gerardiana (Ephedraceae)¹

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• Premise of the study: Ephedra gerardiana (Ephedraceae), occurring in the Himalayan ranges, is an important plant species used in Tibetan medicine. Due to the lack of molecular markers to characterize genetic diversity, knowledge for conservation and uses of E. gerardiana resources is limited; we therefore developed microsatellite markers for use in this species.

• Methods and Results: Using Illumina MiSeq sequencing technology, we developed 29 polymorphic microsatellite loci suitable for E. gerardiana, of which 15 loci also showed polymorphisms in two related Ephedra species, E. saxatilis and E. monosperma. The average number of effective alleles per locus ranged from two to six. The observed and expected heterozygosity ranged from 0.23 to 0.83 and 0.44 to 0.86, respectively, in E. gerardiana populations.

• Conclusions: The developed 29 microsatellite markers are effective for the study of genetic structure and genetic diversity of E. gerardiana, and 15 of these markers are suitable for related Ephedra species.

Key words: conservation; Ephedra gerardiana; Ephedraceae; genetic diversity; next-generation sequencing; simple sequence repeat markers.

The genus Ephedra L. (Ephedraceae), also referred to as ma huang in Chinese, contains species that are sources of important Chinese traditional and Tibetan medicines (Konno et al., 1985). Ephedra gerardiana Wall. ex C. A. Mey. is distributed at altitudes above 3900 m in the Himalayan ranges (Editorial Committee of Chinese Flora, 1978). Because it is both a drought-resistant plant species (Shen, 1995) and an important Tibetan medicine (Pandey, 2006), E. gerardiana is useful for the study of the adaptive evolution and maintenance of genetic diversity in Ephedra. Relatively high genetic differentiation and variation are found for plants distributed on the Qinghai–Tibet Plateau due to its great geographical variability, in addition to frequent natural hybridization and polyploidization events (Wen et al., 2014). Chloroplast fingerprints revealed a high level of genetic differentiation as reflected by high levels of genetic diversity (FST) among populations of Ephedra species on the Qinghai–Tibet Plateau, such as in E. gerardiana (0.98) and E. saxatilis (Stapf) Royle ex Florin (0.86) (Qin et al., 2013).

Microsatellite (also referred to as simple sequence repeat [SSR]) markers are widely used in studies of plant population genetics and genetic diversity because of their high levels of polymorphism, stability, and codominance. However, traditional methods to develop microsatellite markers are time-consuming and complex. High-throughput and low-cost next-generation sequencing has accelerated the identification of large numbers of microsatellite markers (Rico et al., 2013). In this paper, we report the development of microsatellite markers for Ephedra species collected from the Qinghai–Tibet Plateau using Illumina MiSeq genome sequencing technology.

METHODS AND RESULTS

We collected a total of 106 individuals representing three E. gerardiana populations, two E. saxatilis populations, and one E. monosperma C. A. Mey. population from various locations on the Qinghai–Tibet Plateau. Vouchers of the sampled population materials were deposited in the Herbarium of Tibet University, Lhasa, China. The materials and their locality information are listed in Appendix 1.

The high-quality DNA sample for Illumina MiSeq sequencing (Illumina, San Diego, California, USA) was isolated from a randomly selected individual in an E. gerardiana population (Eg_QM, Appendix 1) using a DNA extraction kit (Tiangen Biotech, Beijing, China). DNA samples of all other Ephedra individuals used for the validation of the identified microsatellite markers were extracted using the cetyltrimethylammonium bromide (CTAB) method following Qin et al. (2013). The quality of extracted DNA samples was monitored on 1% agarose gels. The purity and concentration of the DNA samples were determined using the NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

For the development of microsatellite markers, an Illumina paired-end library was constructed using the TruSeq DNA Sample Prep Kit (Illumina) following the manufacturer’s instructions and sequenced using the Illumina MiSeq platform at Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China) to generate 250-bp paired-end reads. A total of 3,306,253,832 high-quality reads, consisting of 2,216,713,160,918 reads in 410,293 reads in 167,125 reads in 31,708 reads in 1,557 reads in 93 reads in 3 reads.

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Table 1. Characteristics of 29 microsatellite primer pairs developed in *Ephedra gerardiana*.

| Locus name | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | $T_a$ (°C) | Fluorescent dye | GenBank accession no. |
|------------|-------------------------|--------------|------------------------|------------|----------------|----------------------|
| EgSSR01*   | F: TGATGTATCATGAAATGATGTG | (AT)$_{20}$  | 140–172                | 56         | JOE            | KR073011             |
|            | R: GACAGATGAATGCTGTAAGA   | (TA)$_{18}$  |                        |            |                |                      |
| EgSSR02*   | F: CCAATGCACAAATTGCGGAG  | (GA)$_{17}$  | 210–246                | 59         | FAM            | KR073012             |
|            | R: TTCAATCAGCTGATTGTCCTC  | (GT)$_{15}$  |                        |            |                |                      |
| EgSSR03*   | F: CAGGCTGCAAATATGCCGCTT | (CT)$_{13}$  | 220–244                | 59         | JOE            | KR073013             |
|            | R: TTATGGCCCAACCACTAAAAC | (GC)$_{11}$  |                        |            |                |                      |
| EgSSR04*   | F: CAAATGAATGCCAAATCAGG   | (AG)$_{15}$  | 246–288                | 59         | FAM            | KR073014             |
|            | R: TCAGGCTGCAAATATGCCGCTT | (CT)$_{13}$  |                        |            |                |                      |
| EgSSR05*   | F: GTAATTGCTGAAAATGATGTG  | (AT)$_{14}$  | 146–164                | 56         | JOE            | KR073015             |
|            | R: TGGTTATGAAATGCGCAGG    | (CG)$_{12}$  |                        |            |                |                      |
| EgSSR06*   | F: TGTTGTGGAATGCAACATTTT | (AT)$_{12}$  | 222–243                | 54         | JOE            | KR073016             |
|            | R: TGTGGGAGATGACATTTTCTT  | (AT)$_{15}$  |                        |            |                |                      |
| EgSSR07*   | F: TATGTTGGAATGCAACATTTT | (AT)$_{12}$  | 140–240                | 58         | FAM            | KR073017             |
|            | R: GACAGATGAATGCTGTAAGA   | (TA)$_{15}$  |                        |            |                |                      |
| EgSSR08*   | F: CCAATGCACAAATTGCGGAG  | (GA)$_{17}$  | 188–200                | 58         | JOE            | KR073018             |
|            | R: TTCAATCAGCTGATTGTCCTC  | (GT)$_{15}$  |                        |            |                |                      |
| EgSSR09*   | F: CAGGCTGCAAATATGCCGCTT | (CT)$_{13}$  | 212–248                | 60         | JOE            | KR073019             |
|            | R: TTATGGCCCAACCACTAAAAC | (GC)$_{11}$  |                        |            |                |                      |
| EgSSR10*   | F: CAAATGAATGCCAAATCAGG   | (AG)$_{15}$  | 176–214                | 60         | JOE            | KR073020             |
|            | R: TCAGGCTGCAAATATGCCGCTT | (CT)$_{13}$  |                        |            |                |                      |
| EgSSR16    | F: TCACTGCAAGACCAATAGGAG | (GA)$_{12}$  | 285–295                | 58         | FAM            | KX077619             |
|            | R: CTTGTGAAATTGGTCTGAGCT  | (CT)$_{10}$  |                        |            |                |                      |
| EgSSR17    | F: CACAGCTGCAATATAAAAC    | (AT)$_{10}$  | 256–268                | 58         | FAM            | KX077620             |
|            | R: TCCATGCAATATAAAAC      | (AT)$_{10}$  |                        |            |                |                      |
| EgSSR18    | F: AACTCTATGAGGATGACCT    | (AT)$_{16}$  | 158–214                | 60         | JOE            | KX077621             |
|            | R: GGTTTGGATTGCTCAGTTCA   | (AT)$_{16}$  |                        |            |                |                      |
| EgSSR19    | F: TCACCTCAATATGACTTTTCCA | (AT)$_{15}$  | 280–290                | 60         | FAM            | KX077622             |
|            | R: TCTCATGCTAGGCTTCTGCA   | (AT)$_{15}$  |                        |            |                |                      |
| EgSSR21    | F: TTACATGCGATTGCTGTA     | (AT)$_{12}$  | 172–180                | 58         | JOE            | KX077624             |
|            | R: TGGCAATGCTGCTGCTGCTC   | (GT)$_{14}$  |                        |            |                |                      |
| EgSSR23*   | F: TTAGGGAGAGCCTTTTTTA   | (AT)$_{14}$  | 180–196                | 60         | JOE            | KX077626             |
|            | R: AACACACAAACACAAAGAAGAAACCTCC | (AT)$_{16}$  |                        |            |                |                      |
| EgSSR24    | F: TGAGCCAGGCCCAACAAATAT  | (TG)$_{2}$  | 241–250                | 60         | FAM            | KX077627             |
|            | R: ATAAACCACTGGTTGATATTCC | (AT)$_{13}$  |                        |            |                |                      |
| EgSSR29    | F: TGTCCTGGTCCTCGACTTCG  | (ATC)$_{3}$  | 232–238                | 59         | FAM            | KX077632             |
|            | R: TTTAGGAGATGCGTTAAGTAC  | (AT)$_{16}$  |                        |            |                |                      |
| EgSSR31    | F: TTAGGAGATGCGTTAAGTAC  | (AT)$_{16}$  | 150–169                | 60         | JOE            | KX077634             |
|            | R: GACATGCTGTTTATGTCGATTTC | (AT)$_{15}$  |                        |            |                |                      |
| EgSSR33*   | F: TGGCGTGGATGATGACATT  | (TG)$_{2}$  | 237–253                | 60         | FAM            | KX077636             |
|            | R: ATTCCCACAGAGCGCATTTT  | (AT)$_{15}$  |                        |            |                |                      |
| EgSSR35*   | F: GCATGCCTATGAGCCTAGCA  | (CAAC)$_{5}$ | 209–228                | 60         | JOE            | KX077638             |
|            | R: TGGAGAGAACGCTGCGTGCA  | (AT)$_{16}$  |                        |            |                |                      |
| EgSSR37    | F: TGACGTTTGGTGCTCCTGCC  | (AAAT)$_{3}$ | 236–262                | 60         | FAM            | KX077640             |
|            | R: TACTGTTGATGCTGCTGCT    | (ATA)$_{14}$ |                        |            |                |                      |
| EgSSR39    | F: CACGTCGCAAGAGACACAGAACA | (AC)$_{12}$  | 174–192                | 59         | JOE            | KX077642             |
| EgSSR40*   | F: CCTGGAATACTTCTCATCTCA | (AAT)$_{3}$  | 204–219                | 60         | JOE            | KX077643             |
|            | R: TGGAGAAACAAATGGGATTTAG | (AT)$_{14}$  |                        |            |                |                      |
| EgSSR41    | F: TGGAGAAACAAATGGGATTTAG | (AT)$_{14}$  | 246–256                | 58         | JOE            | KX077644             |
|            | R: GATGGATGATGATGATGCA   | (TA)$_{10}$  |                        |            |                |                      |
| EgSSR42    | F: TGGAGAAACAAATGGGATTTAG | (TA)$_{10}$  | 240–254                | 57         | FAM            | KX077645             |
|            | R: TTCAATGCTGCGACCTGTCT   | (AT)$_{10}$  |                        |            |                |                      |
| EgSSR43    | F: GTTTGGAATTGGTCGGGGGAT | (AT)$_{10}$  | 189–214                | 60         | JOE            | KX077646             |
| EgSSR44*   | F: AATTTACAGAAGGAGCAGGCCG | (AT)$_{10}$  | 112–216                | 60         | JOE            | KX077647             |
|            | R: AGAGGTTTGTACAGCAACAGG  | (AT)$_{10}$  |                        |            |                |                      |
| EgSSR45    | F: TACAGTGTGGACAAATCTTCA | (CCAAAG)$_{3}$ | 198–216                | 60         | JOE            | KX077648             |
|            | R: TTGCGTGAGAAGTGGCCCAA   | (AT)$_{16}$  |                        |            |                |                      |

Note: $T_a$ = annealing temperature.

*These 15 loci were screened in all samples of *E. gerardiana* and in two related species (*E. saxatilis* and *E. monosperma*) in the genus.
and Skaltsky, 1999), and 135 loci (mostly dinucleotide or pure nucleotide repeats) were determined suitable to use. Primer pairs were then synthesized at the 135 loci by Sangon Biotech Co. Ltd. (Shanghai, China), and we tested whether these primer pairs could produce PCR products in eight randomly selected individuals from two *E. gerardiana* populations (*Eg_Chd* and *Eg_Qm*: Appendix 1).

PCR amplifications were performed in a 10-μL volume with 10 ng of template DNA, 5 μL of 2× Taq PCR MasterMix (Tiangen Biotech), 0.4 μM of forward primers, 0.4 μM of reverse primers, and 3.7 μL of ddH2O following the protocol by Xu et al. (2014), except for the annealing temperatures as indicated in Table 1 for 30 s. PCR products were visualized on a 6% polyacrylamide gel electrophoresis (PAGE) gel with a 10-bp DNA ladder marker. Consequently, 45 primer pairs could produce PCR products with clear bands on PAGE, and only 29 of these primer pairs produced polymorphic bands among the eight *E. gerardiana* individuals. To confirm the reproducibility of polymorphisms of the 29 primer pairs in the eight *E. gerardiana* individuals, Fifteen randomly selected loci from the 29 confirmed microsatellite markers were used to test their polymorphisms and transferability in a larger sample set of *E. gerardiana* (59 individuals in three populations) and two related *Ephedra* species (47 individuals in *E. sataulis* and *E. monosperma*) (Appendix 1). The data matrices of the scored bands from all *Ephedra* species based on the 15 primer pairs were subject to analysis for genetic diversity estimates. Genetic parameters included the number of effective alleles per locus, observed heterozygosity, and Nei’s unbiased expected heterozygosity (Nei, 1978). All the analyses were conducted using GenAlEx software version 6.0 (Peakall and Smouse, 2012). Our results showed moderate to high polymorphisms of the 15 primer pairs among *E. gerardiana* populations, with effective alleles ranging from 2 to 6 per locus and observed and expected heterozygosity ranging from 0.23–0.83 and 0.44–0.86, respectively (Table 2). Results further indicated high polymorphisms of the 15 loci in the populations of *E. sataulis* and *E. monosperma* (Table 3). All the results suggest that these 15 microsatellite loci are suitable for

### Table 2. Allelic diversity of *Ephedra gerardiana* populations based on 15 microsatellite loci.

| Locus   | Total (n = 59) | *Eg_Chd* (n = 20) | *Eg_Qm* (n = 20) | *Eg_Nge* (n = 20) |
|---------|---------------|-------------------|------------------|------------------|
|         | *A* | *H* | *uH* | *A* | *H* | *uH* | *A* | *H* | *uH* |
| EgSSR01 | 2   | 0.689 | 0.572 | 2   | 0.684 | 0.575 | 3   | 0.632 | 0.642 |
| EgSSR02 | 6   | 0.250 | 0.859 | 5   | 0.400 | 0.828 | 6   | 0.000 | 0.842 |
| EgSSR03 | 3   | 0.466 | 0.644 | 2   | 0.211 | 0.508 | 3   | 0.632 | 0.629 |
| EgSSR05 | 3   | 0.575 | 0.655 | 3   | 1.000 | 0.701 | 2   | 0.526 | 0.539 |
| EgSSR06 | 2   | 0.829 | 0.641 | 3   | 0.737 | 0.565 | 3   | 1.000 | 0.606 |
| EgSSR07 | 2   | 0.233 | 0.438 | 1   | 0.000 | 0.097 | 2   | 0.000 | 0.569 |
| EgSSR08 | 2   | 0.722 | 0.488 | 2   | 0.750 | 0.501 | 2   | 0.667 | 0.457 |
| EgSSR09 | 3   | 0.314 | 0.808 | 4   | 0.176 | 0.758 | 5   | 0.211 | 0.805 |
| EgSSR10 | 6   | 0.484 | 0.768 | 7   | 0.421 | 0.886 | 2   | 0.421 | 0.522 |
| EgSSR23 | 3   | 0.516 | 0.654 | 3   | 0.217 | 0.622 | 2   | 0.538 | 0.575 |
| EgSSR33 | 4   | 0.816 | 0.774 | 3   | 0.647 | 0.765 | 2   | 0.800 | 0.839 |
| EgSSR35 | 3   | 0.827 | 0.638 | 4   | 0.750 | 0.738 | 3   | 0.842 | 0.647 |
| EgSSR40 | 4   | 0.642 | 0.646 | 3   | 0.643 | 0.690 | 2   | 0.615 | 0.443 |
| EgSSR44 | 3   | 0.617 | 0.569 | 1   | 0.263 | 0.235 | 3   | 0.882 | 0.697 |

*Note: A* = number of effective alleles; *H* = observed heterozygosity; *uH* = Nei’s unbiased expected heterozygosity (Nei, 1978).

### Table 3. Allelic diversity of populations of *Ephedra sataulis* and *E. monosperma* based on 15 microsatellite loci developed in *E. gerardiana.*

| Locus   | *Es_Tr* (n = 20) | *Es_Chd* (n = 19) | *Em_Cn* (n = 6) |
|---------|-----------------|------------------|----------------|
|         | *A* | *H* | *uH* | *A* | *H* | *uH* | *A* | *H* | *uH* |
| EgSSR01 | 3   | 0.895 | 0.716 | 2   | 0.778 | 0.606 | 3   | 0.625 | 0.725 |
| EgSSR02 | 3   | 0.200 | 0.677 | 3   | 0.789 | 0.679 | 2   | 0.800 | 0.644 |
| EgSSR03 | 6   | 1.000 | 0.863 | 5   | 0.842 | 0.824 | 4   | 0.125 | 0.792 |
| EgSSR04 | 2   | 0.200 | 0.354 | 2   | 0.684 | 0.491 | 3   | 0.800 | 0.778 |
| EgSSR05 | 2   | 0.350 | 0.550 | 1   | 0.368 | 0.309 | 1   | 1.000 | 0.000 |
| EgSSR06 | 2   | 0.450 | 0.396 | 2   | 1.000 | 0.539 | 3   | 0.333 | 0.697 |
| EgSSR07 | 1   | 0.000 | 0.097 | 2   | 0.053 | 0.366 | 3   | 0.625 | 0.725 |
| EgSSR08 | 2   | 0.450 | 0.442 | 2   | 0.316 | 0.548 | 2   | 0.125 | 0.592 |
| EgSSR09 | 4   | 0.250 | 0.737 | 2   | 0.316 | 0.508 | 3   | 1.000 | 0.758 |
| EgSSR10 | 4   | 0.474 | 0.767 | 5   | 0.895 | 0.839 | 3   | 0.750 | 0.783 |
| EgSSR23 | 2   | 0.050 | 0.529 | 2   | 0.357 | 0.542 | 4   | 0.750 | 0.783 |
| EgSSR33 | 2   | 0.267 | 0.570 | 3   | 1.000 | 0.742 | 2   | 0.750 | 0.542 |
| EgSSR35 | 3   | 0.600 | 0.633 | 3   | 0.294 | 0.483 | 1   | 0.000 | 0.000 |
| EgSSR44 | 2   | 0.556 | 0.527 | 3   | 0.278 | 0.648 | 2   | 0.500 | 0.433 |
| EgSSR44 | 2   | 0.450 | 0.376 | 2   | 0.000 | 0.398 | 1   | 0.375 | 0.325 |

*Note: A* = number of effective alleles; *H* = observed heterozygosity; *uH* = Nei’s unbiased expected heterozygosity (Nei, 1978).

*Locality and voucher information are provided in Appendix 1.*
use across species of the genus Ephedra in addition to the 14 loci that are suitable for use in *E. gerardiana* (Table 1).

**CONCLUSIONS**

We developed and confirmed polymorphic microsatellite markers at 29 loci based on *E. gerardiana* populations, using the Illumina MiSeq sequencing method. Fifteen of these microsatellite loci were highly transferable to two related species in the genus, *E. saxatilis* and *E. monosperma*. These microsatellite markers will be useful for the characterization of genetic diversity and analysis of genetic structure for *Ephedra* species.

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**APPENDIX 1.** Geographic and voucher information of *Ephedra* populations used in this study.

| Species                  | Population name | Population size | Locality information | GPS coordinates | Voucher ID |
|--------------------------|-----------------|-----------------|----------------------|-----------------|------------|
| *E. gerardiana* Wall. ex C. A. Mey. | Eg_Qm | 20 | Qomolangma | 28°9.891′N, 86°50.571′E | Eg-Qm01–20 |
| *E. gerardiana* | Eg_Chd | 19 | Chamdo | 30°19.147′N, 97°15.028′E | Eg-Chd01–19 |
| *E. gerardiana* | Eg_Ngz | 20 | Nagarze | 28°53.556′N, 90°17.224′E | Eg_Ng01–20 |
| *E. saxatilis* (Stapf) Royle ex Florin | Es_Tr | 19 | Chamdo | 30°07.647′N, 97°17.248′E | Eg-Chd01–19 |
| *E. monosperma* C. A. Mey. | Em_Cn | 8 | Cuona | 28°12.559′N, 86°49.368′E | Em-Cn01–08 |

*Collection localities are on the Qinghai–Tibet Plateau.

*Vouchers were deposited in the Herbarium of Tibet University, Lhasa, China.*