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Authors: Yin, Xin, Yang, Yunqiang, and Yang, Yongping

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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF 29 POLYMORPHIC EST-SSR MARKERS FOR *STIPA PURPUREA* (POACEAE)[1]

XIN YIN[2,3,4], YUNQIANG YANG[2,3,5], AND YONGPING YANG[2,3,5]

[2]Key Laboratory for Plant Diversity and Biogeography of East Asia, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, People's Republic of China; [3]Germplasm Bank of Wild Species, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, People's Republic of China; and [4]University of the Chinese Academy of Sciences, Beijing, People's Republic of China

- *Premise of the study:* Expressed sequence tag–simple sequence repeat (EST-SSR) markers were developed using Illumina sequencing for further genetic diversity studies of *Stipa purpurea* (Poaceae).
- *Methods and Results:* Twenty-nine polymorphic and eight monomorphic EST-SSR loci were developed and characterized in 90 individuals from nine *S. purpurea* populations. The number of alleles per locus ranged from two to 13, and heterozygosity within populations and total heterozygosity ranged from 0.04–0.76 and from 0.04–0.87, respectively. Of 37 loci, 12 showed interspecific transferability and polymorphism in a related species, *S. glareosa.*
- *Conclusions:* These newly developed EST-SSR primers provide a useful tool to investigate genetic diversity at the population level and to analyze the population structure of *S. purpurea*.

Key words: expressed sequence tag–simple sequence repeat (EST-SSR); Poaceae; polymorphism; *Stipa purpurea.*

Stipa purpurea Griseb. (Poaceae) is widely distributed along the precipitation gradient from the southeast to the northwest of the Qinghai–Tibet Plateau (Yue et al., 2011). It is a species endemic to the Qinghai–Tibet Plateau, and it plays a prominent role in protecting the ecological environment by acting as a windbreak, fixing sand, conserving water and soil, and preventing grassland degradation (Yue et al., 2008). *Stipa purpurea* is also important for the development of animal husbandry because of its high nutritional value and good palatability (Yue et al., 2008), but it has suffered natural and anthropogenic disturbances in recent years. Most studies on *S. purpurea* have focused on its biological characteristics (Li et al., 2015); however, little is known about its population genetic diversity (Yue and Peng, 2014).

To our knowledge, only a limited number of simple sequence repeat (SSR) markers (using the Fast Isolation by AFLP of Sequences COntaining repeats [FIASCO] protocol) (n = 15; Liu et al., 2011) and intersimple sequence repeat (ISSR) markers (n = 8; Liu et al., 2009) have been developed for use in *S. purpurea* to investigate its genetic diversity in different populations (Liu et al., 2009, 2011; Zhai, 2012). Furthermore, the development of microsatellite markers in *S. purpurea* has been very slow due to the lack of genome sequences. Thus, developing a greater number of microsatellite loci for this species is necessary for population genetic diversity studies of *S. purpurea* to progress. Next-generation sequencing allows for rapid development of a large number of SSR markers (Huang et al., 2014). Recently, we sequenced the *S. purpurea* transcriptome using the Illumina next-generation sequencing platform to understand drought tolerance (Yang et al., 2015). Here, we report the rapid and cost-effective development of 29 novel polymorphic expressed sequence tag (EST)–SSR markers for *S. purpurea*, which will be useful in future studies of population genetics in this species.

METHODS AND RESULTS

Transcriptome sequencing of *S. purpurea* was conducted using an Illumina Genome Analyzer (Illumina, San Diego, California, USA). Approximately 51 million 75-bp paired-end reads were obtained and assembled into 84,298 unigenes with mean sizes of 579 nucleotides (Yang et al., 2015). SSRs were detected using the MIcroSAtellite Identification Tool (MISA; Thiel et al., 2003), with the criteria of eight, five, five, five, and five repeat units for di-, tri-, tetra-, penta-, and hexanucleotide motifs, respectively. A total of 2105 SSRs were identified, with trinucleotide repeats (98.9%, 2081) being the most common, followed by dinucleotide (0.8%, 17), hexanucleotide (0.2%, 4), and tetranucleotide (0.1%, 3) repeats. Primer Premier 5 software (PREMIER Biosoft International, Palo Alto, California, USA) was used to design 50 primer pairs (GenBank accession numbers: KP729144–KP729174, KU987914–KU987932), 18–21 bp long, that amplified product sizes ranging from 100–600 bp.

Polymorphisms of these primer sets were assessed in 90 *S. purpurea* individuals from nine populations (10 individuals per population) in Tibet (Fig. 1, Appendix 1). We also chose *S. glareosa* P. A. Smirn. (Poaceae) (10 individuals; 32°39′47″N, 79°55′48″E) to test the cross-species amplification of polymorphic and monomorphic markers in *S. purpurea*. Voucher specimens (Appendix 1) were deposited in the herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN).

Genomic DNA was extracted from leaf tissues using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). PCR amplifications were performed in 20-μL reaction mixtures containing 0.5 units of *Taq* polymerase (TaKaRa Biotechnology Co., Dalian, China), 2 μL 10× PCR buffer (200 mM Tris-HCl [pH 8.8], 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 1% Triton X-100, 20 mM $MgSO_4$), 1.6 μL dNTPs (2.5 mM each), 0.5 μL each primer

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[5]Authors for correspondence: yangyunqiang@mail.kib.ac.cn, yangyp@mail.kib.ac.cn

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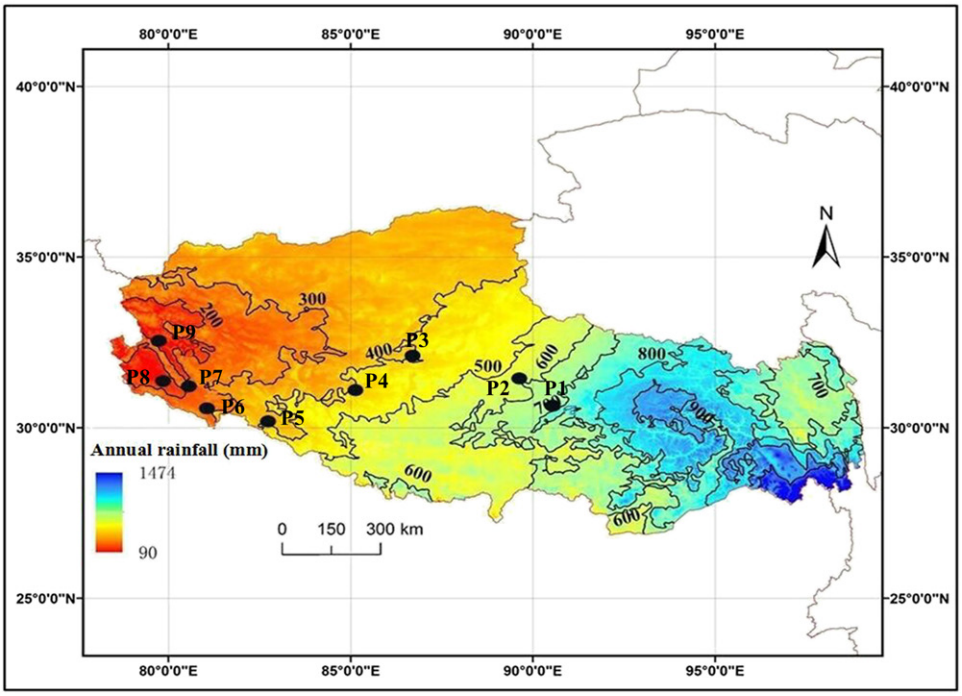

Fig. 1. Sampling locations of *Stipa purpurea* and *S. glareosa* in Tibet.

(10 µM), and 1 µL of genomic DNA (~50 ng/µL). PCR conditions comprised an initial denaturing step at 94°C for 4 min; followed by 35 cycles of 94°C for 30 s, appropriate annealing temperatures (Table 1) for 30 s, and 72°C for 35 s; and a final extension at 72°C for 5 min. PCR products were first detected using 1.0% agarose gel electrophoresis, then run on 6% denaturing polyacrylamide gels and silver stained. The band size was calculated by comparison with a 25-bp DNA ladder (Fermentas, Vilnius, Lithuania; Yan et al., 2011). *Stipa purpurea* is a tetraploid species ($2n = 4x = 44$) (Sheidai and Attaei, 2005), so traditional measures of genetic variability such as deviation from linkage disequilibrium and Hardy–Weinberg equilibrium (HWE) could not be determined. However, GenoDive v.2.0b20 software (Meirmans and Van Tienderen, 2004) enables the analysis of amplified polymorphic fragments (bands) from polyploids.

Of the 50 primer pairs, 29 (58%) amplified a polymorphism after excluding those that did not amplify (13, 26%) or were monomorphic (8, 16%) (Table 1), and this was assessed using 90 individuals from nine populations. The 29 polymorphic SSR markers were analyzed using GenoDive v.2.0b20 software, and the number of alleles per locus (A) according to Nei (1987) ranged from two to 13 alleles with a mean of 4.07 alleles per locus. The average heterozygosity within populations (H_s) and total heterozygosity (H_t) ranged from 0.044 to 0.756 and 0.044 to 0.868 with averages of 0.38 and 0.43, respectively, suggesting that genetic diversity was higher within than among populations. HWE ($P < 0.05$) tests showed that no loci significantly deviated from the equilibrium between locus pairs, suggesting that the nine populations investigated were in genetic equilibrium. All loci in the current study, including 29 polymorphic and eight monomorphic primers in *S. purpurea*, were also screened in cross-amplification tests of 10 *S. glareosa* individuals. Twelve of the 37 primers were successfully amplified and all revealed polymorphisms. A varied from two to three, and H_s and H_t ranged from 0.50 to 0.83 and 0.50 to 0.83 with averages of 0.58 and 0.58, respectively (Table 2).

CONCLUSIONS

This is the first known report of EST-SSRs developed for use in *S. purpurea*. Twenty-nine polymorphic and eight monomorphic primer sequences were described in this study. The markers described here appear to be highly reliable and will enable the investigation of genetic diversity at the population level and the analysis of population structure of *S. purpurea.* They may also contribute to studies of genetic diversity across other *Stipa* species.

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TABLE 1. Characteristics of 37 EST-SSR primers developed for use in *Stipa purpurea*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
ZH1	F: GCGGAACAAGGAGAACC R: ATAGACATTTCCGCCAGC	$(CGC)_8$	252–269	53	KP729144
ZH2	F: CGCCATCGCTCGCAGTA R: TACGACTGGAAGGGCGAG	$(CCG)_6$	45–65	53	KP729145
ZH3	F: AGCCAGACGACCAAGAACC R: TACAGCGACAACGACATGGAC	$(CCG)_6$	357–374	53	KP729146
ZH4	F: CAAGACCAAGCCAGTA R: AAAACGGGAACTGTGA	$(GAA)_7$	230–250	53	KP729147
ZH5	F: GCGAGTTCTGGCAGTTCA R: CAAGTCCATCGGGAGGC	$(CGG)_4$	379–390	50	KP729148
ZH6	F: AGACGATTGGGTGCTGTGC R: GCGAAGAGCGACGAGTAG	$(AAG)_3$	57–81	53	KP729149
ZH7	F: GCGTCCAACTCCAAGAA R: ACGAGGCAAAGAGTCCA	$(GTG)_8$	43–66	52	KP729150
ZH8	F: CCAGCACCGCCGATGTA R: TTGGACAGGCTGAGTAGG	$(CCA)_8$	71–94	53	KP729151
ZH9	F: TGAAGAAGACCACCATCGC R: CCTCCACAGCGTGTAATCC	$(CAG)_8$	153–176	53	KP729152
ZH10	F: CCTCCTCGCAGTTCTTCC R: CCACCTGTTCCCATCCTC	$(CCT)_5$	125	47	KP729153
ZH11	F: CGCCAATAACTGCGGCTTC R: CGGCGAGGAGGAATCAGAGG	$(CCT)_2$	342–348	51	KP729154
ZH12	F: TCCCCAGACTCCAATCCTTCC R: TCAATTGCGGGCTCATTGC	$(AGC)_7$	191–211	51	KP729155
ZH13	F: TAGCATCAGCGGCACCTC R: GCGGCTTGCTTGTTTCCT	$(CCG)_6$	70–87	51	KP729156
ZH14	F: CCTCCAGTGAGCAACCCA R: GACGGCAGACGACTCCTT	$(TGC)_4$	193–204	50	KP729157
ZH15	F: AGGCTCAGCAGCAAAGA R: CAGAAGTGGACGCAAAC	$(CCA)_5$	456–464	50	KP729158
ZH16	F: GGTGAAGGAGGGTTGCG R: TGTCGGTGCCGTTGCTG	$(CCA)_2$	303–308	52	KP729159
ZH17	F: CCAAAAACCAAGCGAACCGA R: TTTGTTGGCCTCATCCTCGT	$(CCG)_6$	252–269	51	KP729160
ZH18	F: ACACTCCCAGTTCAGCCATC R: CGTGGTACCATCTGGCCTTG	$(CAG)_7$	542–562	51	KP729161
ZH19	F: CTGTGGCTACTCGTGAT R: CGATAAAGGCAGATAGTAAA	$(CAG)_7$	416	53	KP729162
ZH20	F: CCCACTTCGGCGGCATCAT R: AGCATCGGTCGCAGGGAGGA	$(CAG)_6$	380–397	51	KP729163
ZH21	F: AGGCTCCATCCATCTTTACT R: TTTCAGATAACCACCAGATT	$(TGC)_6$	42–59	50	KP729164
ZH22	F: CCCTCATCGCCATCTTTG R: GCACTCCTGCCACTCCAT	$(AGC)_4$	161–172	50	KP729165
ZH23	F: GCATCCATCCCTACCTCA R: CAGCGTCACCATTAGCAG	$(AAG)_7$	198–218	50	KP729166
ZH24	F: GCTGCTCCTCATCGTCGTCT R: GCCTTCACCTTCTTGCCCT	$(CCT)_4$	80–91	51	KP729167
ZH25	F: CTCGCGTGATTTCCAAACCC R: CGCAACCCTAGCTAACAACA	$(CCG)_2$	484–489	51	KP729168
ZH26	F: TCATCAAGCTCTTCCTGCCG R: GCCGCCATTTCCATTTCCAT	$(CCG)_3$	50–58	51	KP729169
ZH27	F: GCGGATGAGGAAGTAGAGG R: GCAGAAGGTCCATCAACA	$(AAG)_2$	53–58	51	KP729170
ZH28	F: GCTCCCTACCGTCCTCCTC R: TGGGTTGGGTGGTGGCTC	$(CT)_{13}$	53–78	55	KP729171
ZH29	F: CGGCGAGCGAACTGTCCAT R: CGCTTGAGGGTAGCCAGATGA	$(AAC)_5$	172–186	55	KP729172
ZH30	F: AGCACTTGGCAACCTGAA R: GCATCAAGCCTCACAAACCAT	$(GA)_5$	571–580	53	KP729173
ZH31	F: TACTGCCATTGCCACCTT R: TCGCCGCATTCGTTGT	$(AGC)_8$	515–538	50	KP729174
ZH32	F: GTCGCCGCATTGTCATCAG R: TATCGGTGCTGGAGGAGGC	$(CCA)_6$	282	53	KU987914
ZH33	F: GCCCAGTTCTTGGCTATCTTAC R: CTGCTGAGAAACGGTGGGT	$(CAC)_5$	282	53	KU987915
ZH34	F: CAACTCGCTGGTATCGTGC R: TCTCGGCTATGTCAGGTGC	$(CAC)_7$	154	55	KU987916

TABLE 1. Continued.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
ZH35	F: ACCGTGGCACAGGCTCCGTC R: AGGCATCCCATTGCGTAG	$(GCA)_7$	507	55	KU987917
ZH36	F: CACAAGGGTTACTGGGATA R: AACGGTGGGTGCGGATG	$(CCA)_6$	291	55	KU987918
ZH37	F: TGGCACAGGCTCCGTC R: TCCCATTGCGTAGGTAG	$(GCA)_7$	435	53	KU987919

Note: T_a = annealing temperature.

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TABLE 2. Results of initial primer screening for the 29 polymorphic loci in *Stipa purpurea* and cross-species amplification in *S. glareosa*.

	Stipa purpurea																												*Stipa glareosa*		
	Nagqu (N = 10)			Bangor (N = 10)			Nyima (N = 10)			Tsochen (N = 10)			Tradom (N = 10)			Burang (N = 10)			Menshi (N = 10)			Zanda (N = 10)			Shiquanhe (N = 10)			Total (N = 90)			Shiquanhe (N = 10)		
Locus	A	H_s	H_t	A	H_s	H_t	A	H_s	H_t	A	H_s	H_t	A	H_s	H_t	A	H_s	H_t	A	H_s	H_t	A	H_s	H_t	A	H_s	H_t	A	H_s	H_t	A	H_s	H_t
ZH1	2	0.533	0.533	2	0.356	0.356	2	0.533	0.533	2	0.356	0.356	2	0.533	0.533	2	0.356	0.356	2	0.356	0.356	2	0.533	0.533	2	0.356	0.356	3	0.444	0.451	—	—	—
ZH2	2	0.356	0.356	3	0.622	0.622	3	0.622	0.622	2	0.533	0.533	1	0.000	0.000	2	0.356	0.356	2	0.533	0.533	2	0.356	0.356	2	0.356	0.356	3	0.489	0.621	—	—	—
ZH3	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	2	0.000	0.000	1	0.000	0.000	2	0.044	0.044	2	0.500	0.500
ZH4	1	0.000	0.000	2	0.356	0.356	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.533	0.533	1	0.000	0.000	2	0.044	0.232	—	—	—
ZH5	3	0.622	0.622	1	0.000	0.000	2	0.533	0.533	2	0.533	0.533	2	0.356	0.356	2	0.356	0.356	2	0.356	0.356	2	0.711	0.711	2	0.356	0.356	3	0.456	0.531	2	0.667	0.667
ZH6	2	0.533	0.533	2	0.356	0.356	3	0.711	0.711	3	0.622	0.622	3	0.711	0.711	2	0.356	0.356	4	0.800	0.800	3	0.571	0.571	2	0.356	0.356	4	0.644	0.644	—	—	—
ZH7	4	0.800	0.800	3	0.622	0.622	2	0.533	0.533	5	0.889	0.889	3	0.711	0.711	2	0.356	0.356	4	0.800	0.800	2	0.711	0.711	4	0.800	0.800	13	0.756	0.868	3	0.833	0.833
ZH8	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	3	0.622	0.622	2	0.356	0.356	2	0.356	0.356	1	0.000	0.000	3	0.356	0.356	3	0.622	0.622	5	0.333	0.356	—	—	—
ZH9	2	0.356	0.356	1	0.000	0.000	2	0.533	0.533	3	0.711	0.711	2	0.356	0.356	2	0.356	0.356	2	0.533	0.533	2	0.000	0.000	2	0.356	0.356	4	0.444	0.572	2	0.500	0.500
ZH11	2	0.356	0.356	2	0.533	0.533	1	0.000	0.000	2	0.356	0.356	2	0.356	0.356	2	0.356	0.356	2	0.356	0.356	1	0.533	0.533	1	0.000	0.000	5	0.378	0.473	—	—	—
ZH12	1	0.000	0.000	2	0.356	0.356	1	0.000	0.000	3	0.714	0.714	1	0.000	0.000	2	0.356	0.356	2	0.533	0.533	2	0.000	0.000	3	0.622	0.622	4	0.222	0.222	—	—	—
ZH13	1	0.000	0.000	2	0.356	0.356	2	0.356	0.356	1	0.000	0.000	2	0.356	0.356	1	0.000	0.000	2	0.356	0.356	1	0.356	0.356	2	0.356	0.356	4	0.178	0.178	2	0.500	0.500
ZH14	1	0.000	0.000	2	0.356	0.356	1	0.000	0.000	2	0.356	0.356	1	0.000	0.000	1	0.000	0.000	2	0.356	0.356	2	0.356	0.356	1	0.000	0.000	5	0.600	0.588	2	0.667	0.667
ZH15	3	0.622	0.622	2	0.356	0.356	2	0.533	0.533	3	0.622	0.622	3	0.711	0.711	3	0.711	0.711	2	0.533	0.533	2	0.000	0.000	2	0.356	0.356	2	0.222	0.202	—	—	—
ZH16	1	0.000	0.000	1	0.000	0.000	2	0.356	0.356	2	0.356	0.356	2	0.356	0.356	2	0.356	0.356	1	0.000	0.000	1	0.356	0.356	2	0.356	0.356	3	0.467	0.469	2	0.500	0.500
ZH17	2	0.533	0.533	2	0.356	0.356	2	0.533	0.533	2	0.356	0.356	2	0.533	0.533	2	0.356	0.356	2	0.356	0.356	2	0.800	0.800	2	0.356	0.356	6	0.489	0.635	2	0.667	0.667
ZH18	2	0.533	0.533	3	0.711	0.711	3	0.711	0.711	1	0.000	0.000	3	0.622	0.622	1	0.000	0.000	1	0.000	0.000	4	0.533	0.533	3	0.711	0.711	4	0.189	0.21	2	0.500	0.500
ZH20	4	0.857	0.857	2	0.667	0.667	3	0.622	0.622	2	0.356	0.356	2	0.429	0.429	3	0.714	0.714	2	0.533	0.533	2	0.711	0.711	1	0.000	0.000	5	0.756	0.77	—	—	—
ZH21	4	0.8	0.8	3	0.622	0.622	4	0.800	0.800	3	0.622	0.622	3	0.711	0.711	3	0.622	0.622	3	0.622	0.622	3	0.000	0.000	2	0.533	0.533	4	0.211	0.211	—	—	—
ZH22	1	0.000	0.000	2	0.356	0.356	3	0.622	0.622	1	0.000	0.000	1	0.000	0.000	2	0.356	0.356	1	0.000	0.000	1	0.356	0.356	2	0.356	0.356	2	0.178	0.267	2	0.500	0.500
ZH23	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	2	0.356	0.356	2	0.356	0.356	1	0.000	0.000	2	0.000	0.000	2	0.356	0.356	5	0.389	0.39	2	0.500	0.500
ZH24	2	0.533	0.533	2	0.356	0.356	2	0.356	0.356	2	0.356	0.356	2	0.356	0.356	3	0.622	0.622	1	0.000	0.000	1	0.000	0.000	2	0.533	0.533	2	0.111	0.127	—	—	—
ZH25	2	0.429	0.429	2	0.356	0.356	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.533	0.533	1	0.000	0.000	3	0.456	0.487	2	0.596	0.596
ZH26	1	0.000	0.000	1	0.000	0.000	2	0.571	0.571	2	0.356	0.356	2	0.533	0.533	3	0.622	0.622	2	0.429	0.429	2	0.356	0.356	3	0.711	0.711	2	0.044	0.044	—	—	—
ZH27	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	1	0.000	0.000	2	0.356	0.356	1	0.000	0.000	9	0.633	0.794	—	—	—
ZH28	2	0.356	0.356	4	0.800	0.800	3	0.711	0.711	4	0.800	0.800	2	0.356	0.356	1	0.000	0.000	5	0.889	0.889	2	0.533	0.533	4	0.800	0.800	3	0.478	0.555	—	—	—
ZH29	1	0.000	0.000	2	0.533	0.533	3	0.622	0.622	2	0.533	0.533	3	0.711	0.711	1	0.000	0.000	2	0.533	0.533	2	0.714	0.714	2	0.356	0.356	5	0.733	0.733	—	—	—
ZH30	2	0.571	0.571	3	0.622	0.622	3	0.800	0.800	3	0.622	0.622	2	0.533	0.533	3	0.711	0.711	3	0.800	0.800	3	0.000	0.000	4	0.800	0.800	3	0.289	0.453	—	—	—
ZH31	1	0.000	0.000	2	0.533	0.533	2	0.356	0.356	2	0.356	0.356	2	0.356	0.356	2	0.356	0.356	2	0.356	0.356	1	0.356	0.356	1	0.000	0.000	3	0.289	0.453	—	—	—

Note: A = number of alleles; H_s = heterozygosity within populations; H_t = total heterozygosity.

Appendix 1. Locality and accession information of the nine *Stipa purpurea* populations and a single *S. glareosa* population of the Qinghai–Tibet Plateau used in this study. All specimens are deposited in the herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences (KUN), Kunming, Yunnan, China.

Population code/Species	Collection locality	Voucher specimen accession no.	Altitude (m a.s.l.)	Geographic coordinates	*N*
P1/*S. purpurea* Griseb.	Nagqu, Tibet	1270416	4690	30°50′52″N, 90°37′15″E	10
P2/*S. purpurea*	Bangor, Tibet	1270417	4636	31°36′58″N, 89°32′14″E	10
P3/*S. purpurea*	Nyima, Tibet	1270411	4538	32°00′05″N, 86°50′54″E	10
P4/*S. purpurea*	Tsochen, Tibet	1270414	4794	31°03′06″N, 85°05′42″E	10
P5/*S. purpurea*	Tradom, Tibet	1270412	4651	30°14′54″N, 82°58′31″E	10
P6/*S. purpurea*	Burang, Tibet	1270415	4639	30°42′57″N, 81°21′39″E	10
P7/*S. purpurea*	Menshi, Tibet	1270408	4495	31°10′57″N, 80°47′22″E	10
P8/*S. purpurea*	Zanda, Tibet	1270409, 1270410	4584	31°31′43″N, 79°58′42″E	10
P9/*S. purpurea*	Shiquanhe, Tibet	1270406	4682	32°39′47″N, 79°55′48″E	10
P9/*S. glareosa* P. A. Smirn.	Shiquanhe, Tibet	1270424	4682	32°39′47″N, 79°55′48″E	10

Note: a.s.l. = above sea level; *N* = number of individuals.