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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF POLYMORPHIC MICROSATELLITE LOCI FOR *SAXIFRAGA EGREGIA* (SAXIFRAGACEAE)[1]

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- *Premise of the study: Saxifraga egregia* (Saxifragaceae) is a perennial herb that is endemic to the Qinghai–Tibet Plateau. We developed 12 polymorphic microsatellite loci for *S. egregia* to investigate its population genetics.
- *Methods and Results:* Forty-eight pairs of microsatellite primers (including 36 monomorphic loci) were isolated and characterized by magnetic bead enrichment. Twelve of these markers showed polymorphism, and the number of alleles per locus ranged from four to 14 across 50 individuals from three populations of *S. egregia*. No linkage disequilibrium was detected in any pair of loci.
- *Conclusions:* These polymorphic markers are expected to be helpful in further studies on the systematics and phylogeography of *S. egregia* in the Qinghai–Tibet Plateau.

Key words: genetic diversity; microsatellite loci; Qinghai–Tibet Plateau; *Saxifraga egregia*; Saxifragaceae.

Saxifraga L., the largest genus in the Saxifragaceae, consists of approximately 450 species and is distributed in temperate to alpine regions of Eurasia and North and South America. Among the 216 species found in China, mainly in Sichuan and Yunnan provinces and Xizang (Tibet) Autonomous Region, 139 are endemic (Pan et al., 2001). *Saxifraga egregia* Engl. is a perennial herb that is endemic to the Qinghai–Tibet Plateau and mainly inhabits forests, forest understories, and scrubs, with an elevation of 2000–4600 m a.s.l. (Pan et al., 2001). *Saxifraga egregia* and its ca. 30 close relatives are of great importance in the fields of systematics and phylogeography to extend our knowledge of the patterns and processes of speciation and intraspecies diversification in alpine regions. They are also excellent organisms for investigating biotic responses to climate change (DeChaine et al., 2013). Microsatellites have become one of the most popular molecular markers because of their high polymorphism levels and the relative ease of scoring, and they have been used in systematic and phylogeographic applications (Zane et al., 2002). In this study, we isolated polymorphic microsatellite loci of *S. egregia* to facilitate our further investigations of systematics and phylogeography.

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METHODS AND RESULTS

Fifty *S. egregia* individuals from three populations (BM, DG, and CY) were sampled in Qinghai Province, Sichuan Province, and Xizang Autonomous Region (Appendix 1). Fresh leaves were collected and dried using silica gel.

Genomic DNA extraction, magnetic bead enrichment, and microsatellite-enriched library construction were performed according to published methods (Khan et al., 2014). Fragments from the microsatellite-enriched library were cloned into the pGEM-T Easy Vector (Promega Corporation, Madison, Wisconsin, USA), and then transfected into Trans5α Chemically Competent Cells (TransGen, Beijing, China).

A total of 2520 positive colonies were successfully screened using PCR with primer-probes $(AC)_{10}$/$(AG)_{10}$. The amplified PCR products showed two or more bands on agarose gel electrophoresis (Skinner and Denoya, 1992). Of these, 320 randomly selected positive colonies were sequenced using an ABI 3730xl DNA sequencer (Applied Biosystems, Foster City, California, USA) according to the manufacturer's instructions at the Key Laboratory of Adaptation and Evolution of Plateau Biota, Chinese Academy of Sciences. SSR Hunter software for the analysis of simple sequence repeats (SSR) was used to detect 1200 microsatellite motifs (Li and Wan, 2005). A total of 112 primers were designed using online software Primer3 version 4.0.0 (Rozen and Skaletsky, 1999; http://primer3.ut.ee/), with the minimum and maximum primer annealing temperature changed to 58°C and 60°C, respectively, based on a total of 112 randomly selected microsatellite motifs.

Loci polymorphism in the 50 *S. egregia* individuals was assessed by PCR with designed primer pairs. PCR was performed in 20-μL reaction volumes containing 10–100 ng of template DNA, 1× PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 200 nM of each primer, and 1 unit of *Taq* DNA polymerase (TaKaRa Biotechnology Co., Dalian, China). The PCR cycling profile included an initial step of 94°C for 5 min; followed by 35 cycles of 94°C for 45 s, primer annealing temperature (Table 1) for 30 s, and 72°C for 30 s; with a final extension step at 72°C for 7 min. All PCR products were analyzed by capillary electrophoresis using the QIAxcel DNA high-resolution kit (1200) in the QIAxcel Advanced system (QIAGEN, Hilden, Germany). Biocalculator QIAxcel software was used for data analysis and generation of a virtual gel image.

Preliminary population genetic analyses, including the number of alleles (A), observed (H_o) and expected (H_e) heterozygosities, deviations from

TABLE 1. Characteristics of 12 polymorphic microsatellite loci in *Saxifraga egregia*.

Locus	Primer sequences (5′–3′)	Repeat motif	Fragment size (bp)	T_a (°C)	*A*	GenBank accession no.
SE14	F: AAAGTGAATGGAGCAAAA	$(TGG)_5$	97	50	14	KP245861
	R: ACACTCCACCACTAACCA					
SE30	F: CCAAGGCATTTGCCTATA	$(TCACT)_4$	170	50	10	KP245862
	R: GTCGTTTTCTTCTTTTCTCC					
SE38	F: AATAGCTCCTTGGCGTGAT	$(TC)_{27}$	280	54	21	KP245863
	R: CTGGCAACCTAGAAGCAGAC					
SE43	F: TGAGGGCGATTGAGTGAT	$(GA)_{20}$	164	50	23	KP245864
	R: GAGTAAGGGCTAAAGGGT					
SE51	F: GTAACATGATCCGACCGG	$(GACCC)_3$	113	54	18	KP245865
	R: TGGCAGAGTGATGTGGTG					
SE63	F: TAAGGGAAGTCAACATGG	$(GT)_8$	107	50	15	KP245866
	R: CACAACCTAGGACTTCACTC					
SE68	F: GATGATTTTGTTGGTGTT	$(GAT)_4$	125	50	14	KP245867
	R: ACATCGTCATCAATAACC					
SE76	F: TATTGACGGGCTAAAATC	$(TG)_{10}$	161	50	16	KP245868
	R: CGTACAGAAAGCAAACACT					
SE102	F: CCGGTTGTGGTGAAGAAG	$(GTG)_4$	124	54	17	KP245869
	R: GGTATTTATAGAGTTGGGAATG					
SE105	F: TCTGACCTGGGATGATGC	$(TTGA)_3$	126	54	11	KP245870
	R: GTTCTCCCTCCCTCCGTA					
SE106	F: TCTGACCTGGGATGATGC	$(GGAG)_3$	155	54	6	KP245871
	R: AAGCTCAAATTCACAAAATCAC					
SE107	F: AATCGAAAGATTTAGGCG	$(TG)_8$	150	54	15	KP245872
	R: TGAGTGACCAGGCTCTGA					

Note: *A* = total number of alleles per locus; T_a = annealing temperature.

Hardy–Weinberg equilibrium (HWE), and linkage disequilibrium (LD) between all pairs of polymorphic loci, were calculated using GENEPOP version 4.2 (Raymond and Rousset, 1995; Rousset, 2008). Significance testing of the inbreeding coefficient (F_{IS}) at all loci was performed using FSTAT 2.9.3.2 (Goudet, 2002). MICRO-CHECKER (van Oosterhout et al., 2004) was used to detect null allele frequencies (*r*) for all loci.

Of the 112 primer pairs, 48 generated amplification products of expected sizes. Twelve of these displayed polymorphism, and their characteristics are shown in Table 1. Information on the 36 monomorphic primer pairs is listed in Appendix 2. Overall, *A* ranged from four to 14 per locus across 50 individuals (Table 2). H_o and H_e ranged from 0.421 to 1.000 and 0.622 to 0.939 per locus, respectively, which suggests that genetic diversity in this species is relatively high (Chen et al., 2009). This could be the result of interspecific hybridization between *S. egregia* and its closely related species with sympatric distribution ranges (e.g., *S. diversifolia* Wall. ex Ser.), considering their quite similar morphological features (Pan et al., 2001). Unfortunately, there is almost no research about the mating system of *Saxifraga*, and further study is needed before definite conclusions can be drawn. No linkage disequilibrium was detected in any pair of loci. Most loci (three, nine, and eight in populations DG, CY, and BM, respectively) showed a significant departure from HWE, consistent with the inbreeding coefficient. MICRO-CHECKER suggested that this may be affected by the presence of null alleles (Chapuis and Estoup, 2007).

CONCLUSIONS

In this study, we isolated 12 microsatellite loci, which displayed polymorphisms among populations of *S. egregia*. These polymorphic markers are expected to be helpful in further studies on the systematics and phylogeography of *S. egregia* in the Qinghai–Tibet Plateau.

TABLE 2. Initial primer screening in *Saxifraga egregia*.[a]

	Population DG (*N* = 14)					Population CY (*N* = 19)					Population BM (*N* = 17)				
Locus	*A*	H_o	H_e	*r*	F_{IS}	*A*	H_o	H_e	*r*	F_{IS}	*A*	H_o	H_e	*r*	F_{IS}
SE14	6	0.857	0.720*	0.011	−0.200	8	0.895	0.740*	0.063	−0.217	8	0.941	0.717*	0.000	−0.326
SE30	6	0.929	0.751	0.000	−0.247	7	0.895	0.774*	0.000	−0.391	8	1.000	0.825	0.000	−0.220
SE38	11	0.857	0.889	0.000	0.037	14	0.632	0.910	0.150	0.220	10	0.882	0.865*	0.000	−0.021
SE43	12	1.000	0.841*	0.000	−0.197	13	0.421	0.923*	0.237	0.465	12	0.765	0.927*	0.083	0.179
SE51	8	0.571	0.841	0.134	0.329	10	0.579	0.825*	0.131	0.247	13	0.706	0.913*	0.140	0.232
SE63	9	1.000	0.855	0.000	−0.178	12	1.000	0.900*	0.000	−0.114	10	1.000	0.806*	0.000	−0.251
SE68	9	0.786	0.831	0.000	0.056	9	0.684	0.805	0.000	0.002	9	0.529	0.702	0.048	0.252
SE76	10	0.786	0.902	0.051	0.133	12	0.632	0.873*	0.100	0.122	10	0.882	0.816	0.000	−0.084
SE102	9	0.714	0.889	0.081	0.202	12	0.579	0.882*	0.148	0.304	13	1.000	0.939	0.000	−0.067
SE105	9	0.929	0.862	0.000	−0.080	10	0.789	0.842*	0.008	0.064	5	1.000	0.622*	0.000	−0.639
SE106	4	0.929	0.664*	0.252	−0.420	5	0.947	0.687*	0.025	−0.394	4	0.941	0.658*	0.000	−0.450
SE107	9	1.000	0.855	0.000	−0.178	11	0.895	0.836	0.000	−0.158	9	1.000	0.807*	0.000	−0.248

Note: *A* = total number of alleles per locus; F_{IS} = inbreeding coefficient; H_e = expected heterozygosity; H_o = observed heterozygosity; *N* = sample size for each population; *r* = null allele frequency.

[a] See Appendix 1 for population locality information.

* Significant departure from Hardy–Weinberg equilibrium (HWE) at $P < 0.01$.

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Appendix 1. Locality information for populations of *Saxifraga egregia* used in the study.

Population code	Location	Sample size	Voucher no.[a]	Geographic coordinates	Altitude (m)
DG	Dege, Sichuan Province, China	14	Chen2007219	31°57′21″N, 98°51′48″E	4110
CY	Chaya, Xizang Autonomous Region, China	19	Chen2007193	30°41′11″N, 97°15′42″E	4090
BM	Banma, Qinghai Province, China	17	Chen03026	32°49′00″N, 100°5′22″E	3680

[a]The voucher specimens are deposited in the Herbarium of the Northwest Institute of Plateau Biology (HNWP), Xining, Qinghai Province, People's Republic of China.

Appendix 2. Characteristics of 36 monomorphic loci in *Saxifraga egregia.*

Locus	Primer sequences (5′–3′)	Repeat motif	Fragment size (bp)	T_a (°C)	GenBank accession no.
SE1	F: TGTGCCTTGTGAAATGAT R: ACACGAAACAAGACTCCC	$(AC)_6$	108	54	KR559317
SE9	F: TTGCTCAACTATAAATGC R: AGTAAGGAGATGCTGTCT	$(GT)_6$	183	50	KR559318
SE10	F: TCACAATGAAAGTGCAGGAA R: AGCCCGTTGAACGCTATA	$(AG)_{23}$	152	54	KR559319
SE12	F: CCTCGTTTACGTTTAGGA R: ATGAGTTCTTGGCACTAT	$(AG)_{40}$	215	54	KR559320
SE13	F: AGACGCTACAAACCTCCT R: AGCAAACCCAAGAATCCA	$(GA)_{29}$	201	54	KR559321
SE15	F: AGGCTCAAATAGTGTTCT R: GGATTGCTTGAAGGCTCT	$(AG)_{17}$	202	54	KR559322
SE16	F: ATCACATCACAACCAGCC R: GATCCCGACTTTCTTTCG	$(AAG)_5$	159	54	KR559323
SE22	F: GGGAAGGGTAGAGTGTTA R: ATCTCGCCCTCATTATTC	$(TTG)_4$	161	54	KR559324
SE28	F: GGTGCCTGCGAAAGTGAG R: CGAGCAAAGGGAACATAA	$(AGTTT)_3$	123	54	KR559325
SE31	F: AGACACGGATACCAACCA R: AGCACCCTCACCTCACTA	$(GTGA)_3$	111	54	KR559326
SE39	F: TAATTTGAAGCTTGAAAC R: AGTAACGAGCCTACATCC	$(GA)_{20}$	148	54	KR559327
SE40	F: AAACCTAATCAAAGCCACA R: ACAAGCAACCACCAACTC	$(GGT)_4$	120	54	KR559328
SE41	F: GGGCCTAACTAAATGAGC R: TTCCTCCACCCTTCCATC	$(GGT)_4$	145	54	KR559329
SE42	F: ATTGGTGAATGGTGGCTAT R: AGTAAGGCAAACGGGAAA	$(GA)_6$	131	54	KR559330
SE44	F: ACATCAGACTTCGAGGAG R: AGTAACATCACGTAGGGT	$(CT)_{32}$	215	54	KR559331

Appendix 2. Continued.

Locus	Primer sequences (5′–3′)	Repeat motif	Fragment size (bp)	T_a (°C)	GenBank accession no.
SE45	F: CATCAACTCCACCATCAAA	$(ACC)_4$	138	50	KR559332
	R: TGAGTAAGGGCCTAACTAAA				
SE47	F: GACCGACCAGCTTCCAGA	$(AG)_{34}$	161	54	KR559333
	R: CTCCTCCTCTTTTCTCCTCA				
SE50	F: TCAAAGCCTAACCAAAGA	$(AAG)_4$	137	54	KR559334
	R: TGAGTAACGTAGCTCCAA				
SE54	F: AAACATAACCAACCGAATT	$(TG)_5$	158	50	KR559335
	R: TTCATTACCCACAACCAG				
SE58	F: CCCACAAAGCCGAATCAA	$(GAA)_{12}$	122	54	KR559336
	R: CCCGCACGAGTACACGAA				
SE59	F: AGTAAGCATTGTCATAGA	$(TG)_{11}$	184	50	KR559337
	R: AAAAGGGAAACAGTGGAA				
SE65	F: ACCCTAAGCACAACAACC	$(AC)_{23}$	137	54	KR559338
	R: AGCAACTCTGGTCCCACC				
SE67	F: ACACGAAACAAGACTCCC	$(TG)_9$	118	54	KR559339
	R: TGTTTGTGCCTTGTGGAA				
SE70	F: CCTCACCAAACCCGAACA	$(CAA)_5$	126	60	KR559340
	R: GTAAGGTCAGATGCCAAA				
SE71	F: GTGATGGGTGTCGTAGGT	$(TG)_6$	145	54	KR559341
	R: CCTAAGCACAACAACCTG				
SE78	F: GAGCAACTTCAAGATAAA	$(AC)_7$	115	54	KR559342
	R: TAAGCAGCAGATGGTTTG				
SE80	F: TGTTTCCTACGTCAGTTG	$(GT)_8$	243	54	KR559343
	R: ACATAACATTGCTTGCTC				
SE83	F: CGACCGGCAGTTGATAAT	$(CA)_5$	193	54	KR559344
	R: GGTAGGACGAGACTTCCCT				
SE86	F: GAACGGAATCACATCTAT	$(AG)_{10}$	194	50	KR559345
	R: ACCCTAATTACAACAACC				
SE88	F: TGGTGAATAGTGAGCTAT	$(CT)_5$	124	54	KR559346
	R: CGGTAAATTACCTAAGAG				
SE90	F: TAAGCTCAAATTCACAAA	$(CTCC)_3$	160	50	KR559347
	R: AAGATCTGACCTAGGATG				
SE91	F: CATCATCGTTCTCCCTCC	$(TCAA)_3$	159	54	KR559348
	R: CCAATTCACGATTCAAAA				
SE94	F: CGTTGCTCGCTAAAGATA	$(GA)_6$	131	54	KR559349
	R: GCTCGCTGTAACACCTCT				
SE95	F: TGACCAGGCTCTGATACC	$(CA)_{10}$	151	54	KR559350
	R: AAATCGAAAGATTTAGGC				
SE96	F: CCACTGCTGCAATTTCTA	$(GA)_6$	174	54	KR559351
	R: GAACAACAACCGAGATTTA				
SE100	F: GAGTAGTTGTGCCTGACG	$(CTG)_4$	125	54	KR559352
	R: TCTTGTGAAGCATGGATT				

Note: T_a = annealing temperature.