Development of Microsatellite Loci in Scrophularia incisa (Scrophulariaceae) and Cross-Amplification in Congeneric Species

Authors: Wang, Rui-Hong, Chen, Chuan, Ma, Qing, Li, Pan, and Fu, Cheng-Xin

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Primer Note

Development of microsatellite loci in *Scrophularia incisa* (Scrophulariaceae) and cross-amplification in congeneric species[1]

Rui-Hong Wang[2], Chuan Chen[3], Qing Ma[2], Pan Li[2], and Cheng-Xin Fu[2,4,5]

[2]Key Laboratory of Conservation Biology for Endangered Wildlife of the Ministry of Education, College of Life Sciences, Zhejiang University, Hangzhou 310058, People's Republic of China; [3]Hangzhou Botanical Garden, Hangzhou 310013, People's Republic of China; and [4]Key Laboratory of Biological Resources and Conservation and Application, Talimu University, Xinjiang, People's Republic of China

- *Premise of the study:* To elucidate the population genetics and phylogeography of *Scrophularia incisa*, microsatellite primers were developed. We also applied these microsatellite markers to its closely related species *S. dentata* and *S. kiriloviana*.
- *Methods and Results:* Using the compound microsatellite marker technique, 12 microsatellite primers were identified in *S. incisa*. The number of alleles ranged from 14 to 26 when assessed in 78 individuals from four populations. With high cross-species transferability, these primers also amplified in *S. dentata* and *S. kiriloviana*.
- *Conclusions:* These results indicate that these microsatellite markers are adequate for detecting and characterizing population genetic structure in the Chinese species of sect. *Tomiophyllum* at fine and range-wide geographical scales.

Key words: genetic diversity; medicinal herb; microsatellite; Qinghai–Tibet Plateau; *Scrophularia dentata*; *Scrophularia kiriloviana*.

Scrophularia incisa Weinm. (Scrophulariaceae) is a perennial plant inhabiting floodplains, grasslands, and mountain valleys at altitudes between 600 and 3600 m. It presents a belt-like distribution primarily in northern China stretching westward to Central Asia and eastward to Siberia, Russia (Ma et al., 1980; Hong et al., 1998). This species is a traditional Mongolian medicinal herb applied in the treatment of measles, smallpox, chickenpox, and scarlet fever (Ma et al., 1980). According to our field investigations, its current population number and size appears limited, possibly as a consequence of over-exploitation and habitat loss. Therefore, population genetic analyses of *S. incisa* will be necessary to infer its evolutionary processes and to determine appropriate conservation strategies. Nuclear microsatellites (simple sequence repeats [SSRs]) are highly polymorphic, codominant markers that have been widely applied in assessing population genetic structure and gene flow (Liu et al., 2009). There are hitherto no microsatellite loci available for *S. incisa*. Hence, development of polymorphic markers is needed. Furthermore, researchers increasingly require universal markers that can readily be transferred between species. Such transferable markers facilitate comparisons among closely related taxa for addressing the mechanisms involved in population divergence and speciation (Noor and Feder, 2006). *Scrophularia incisa*, *S. dentata* Royle ex Benth., and *S. kiriloviana* Schischk. constitute sect. *Tomiophyllum* of *Scrophularia* in China. *Scrophularia incisa* and its allies are morphologically similar and geographically largely separated, presenting a roughly circular geographic pattern on the Qinghai–Tibet Plateau. *Scrophularia dentata* is distributed in southern and western Tibet, while *S. kiriloviana* occurs in northern Xinjiang extending to Central Asia (Hong et al., 1998). Thus, transferable markers are critical for comparative studies, even if they only allow investigations in related species. In this sense, they can be used to address whether and which heterogeneous evolutionary processes acted in the same geological time frame in the Qinghai–Tibet Plateau and adjacent regions.

In the current study, we aim to identify polymorphic compound microsatellite markers for *S. incisa* using a recently developed isolation technique (Lian et al., 2006) to characterize genetic variation of *S. incisa* populations, and to test their transferability to its close allies, *S. dentata* and *S. kiriloviana*. Our developed universal markers should be valuable and robust to address these purposes.

METHODS AND RESULTS

The compound microsatellite marker technique based on a dual-suppression PCR method was applied to develop SSR markers for *S. incisa* according to Zhai et al. (2010). DNA was isolated from silica gel–dried leaf materials using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle, 1991). First, total DNA of two individuals from a population in Gandi, Qinghai Province, China (population code: GD), were digested by the restriction enzymes *Hae*III and *Ssp*I (TaKaRa Biotechnology Co., Dalian, China), and the restriction fragments were ligated to an unequal-length adapter using DNA Ligation

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[5]Author for correspondence: cxfu@zju.edu.cn

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Table 1. Characteristics of 12 compound microsatellite loci developed for *Scrophularia incisa*.

Locus	Repeat motif	Primer sequences (5′–3′)	Allele size range (bp)	T_a (°C)	*A*	GenBank accession no.
Scin1	$(AC)_6(AG)_{19}$	F: $(AC)_6(AG)_5$ R: TGAAGACGGAAGAAGAAGG	109–128	54	23	JQ773338
Scin2	$(AC)_6(AG)_8$	F: $(AC)_6(AG)_5$ R: ACTTGTATGGCGGGCTTG	140–158	55	20	JQ773339
Scin3	$(AC)_6(AG)_5$	F: $(AC)_6(AG)_5$ R: TTGCAGCATTTTGTTTCC	144–162	55	18	JQ773340
Scin4	$(AC)_6(AG)_{14}$	F: $(AC)_6(AG)_5$ R: GTTTCCCGATGACAGACG	225–243	55	26	JQ773341
Scin5	$(AC)_6(AG)_{15}$	F: $(AC)_6(AG)_5$ R: GAATGAAGTTGTTGGAGC	291–309	54	19	JQ773342
Scin6	$(AC)_6(AG)_{14}$	F: $(AC)_6(AG)_5$ R: CATGGCCTGCTTAAATTAC	113–132	54	21	JQ773343
Scin7	$(AC)_6(AG)_{14}$	F: $(AC)_6(AG)_5$ R: TGGTCCGAGGCTTTACAT	183–201	56	25	JQ773344
Scin8	$(AC)_6(AG)_{10}$	F: $(AC)_6(AG)_5$ R: TATCATGGGAGAAAGTCGA	107–126	56	19	JQ773345
Scin9	$(AC)_6(AG)_{10}$	F: $(AC)_6(AG)_5$ R: CGAGAAACCCAAGGAAAG	110–128	55	14	JQ773346
Scin10	$(AC)_6(AG)_{16}$	F: $(AC)_6(AG)_5$ R: TCAGGAATTGGATCAGAAAC	144–164	54	15	JQ773347
Scin11	$(AC)_6(AG)_9$	F: $(AC)_6(AG)_5$ R: AGTTGTTGGAGCATTGTTTTC	273–294	55	15	JQ773348
Scin12	$(AC)_6(AG)_9$	F: $(AC)_6(AG)_5$ R: AACAATGGTGGAGAAAGGTA	132–162	54	22	JQ773349

Note: *A* = number of alleles per locus; F = forward primer; R = reverse primer; T_a = optimized annealing temperature.

Kit version 2.0 (TaKaRa Biotechnology Co.). Second, DNA fragments flanked by a microsatellite at one end were amplified from both the *Hae*III and *Ssp*I libraries using the compound SSR primer $(AC)_6(AG)_5$ or $(TC)_6(AC)_5$ and an adapter primer AP2 (5′-CTATAGGGCACGCGTGGT-3′). PCR products of 400–1000 bp were purified, inserted, and ligated into PMD18-T vector (TaKaRa Biotechnology Co.) to form a recombinant DNA. Third, the recombinant DNA was transformed into DH5α competent cells (TaKaRa Biotechnology Co.) for culturing, and the clone cells were amplified by an M13 primer to detect the positive clones. Finally, a total of 190 positive clones were obtained and sequenced on an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems, Carlsbad, California, USA).

One hundred and ten sequences were found to contain $(AC)_6(AG)_n$ or $(TC)_6(AC)_n$ compound SSR motifs, of which 56 fragments possessed sufficient flanking regions for designing specific primers. Sixteen primers were designed using PRIMER version 5.0 (Clarke and Gorley, 2001) following the criteria of Zheng et al. (2012). A total of 78 samples of *S. incisa* from four populations (Manzhouli, Inner Mongolia, China [MZ]; Gandi, Qinghai, China [GD]; Zhangye, Gansu, China [ZY]; and Qilian, Qinghai, China [QL]) were used to estimate polymorphism. Thirty-five individuals of *S. dentata* from Xigaze, Tibet, China (RK), and Lhasa, Tibet, China (LS), and 40 individuals of *S. kiriloviana* from Wensu, Xinjiang, China (WS), and Tashkurgan, Xinjiang, China (TS), were analyzed for cross-species amplification tests. The voucher specimens were deposited in the Herbarium of Zhejiang University (HZU) (Appendix 1).

PCRs were conducted in a 15-μL reaction mixture containing 1.5 μL of 10× PCR buffer with $MgCl_2$, 0.75 μL of dNTPs (2.5 mM each), 0.38 μL of each primer (10 μM), 60–100 ng of genomic DNA, 0.5 U of *Taq* polymerase (TaKaRa Biotechnology Co.), and 0.1 μL of bovine serum albumin (BSA; TaKaRa Biotechnology Co.). PCR amplification conditions were as follows: initial denaturation at 94°C for 5 min, followed by 38 cycles of 30 s at 94°C, 45 s at the optimal annealing temperature (Table 1), 90 s of elongation at 72°C, ending with a 10-min extension at 72°C. PCR amplification products were analyzed on a MegaBACE 1000 autosequencer (GE Healthcare Biosciences, Pittsburgh, Pennsylvania, USA), and alleles were scored by GeneMaker software version 1.97 (SoftGenetics, State College, Pennsylvania, USA). Across these eight

Table 2. Results of initial primer screening in four populations of *Scrophularia incisa*.[a]

	Population MZ (*N* = 20)				Population GD (*N* = 20)				Population ZY (*N* = 18)				Population QL (*N* = 20)			
Locus	*A*	H_o	H_e	HWE[b]	*A*	H_o	H_e	HWE[b]	*A*	H_o	H_e	HWE[b]	*A*	H_o	H_e	HWE[b]
Scin1	8	0.950	0.874	0.0069**	7	0.800	0.695	0.2973	9	0.944	0.789	0.026*	8	0.750	0.787	0.1041
Scin2	6	0.850	0.806	0.1555	7	0.900	0.803	0.9065	6	0.389	0.738	0.000**	4	0.800	0.746	0.0517
Scin3	7	0.850	0.836	0.7407	10	0.950	0.903	0.8081	8	0.889	0.827	0.690	4	0.450	0.642	0.1139
Scin4	11	0.900	0.872	0.0262*	12	0.950	0.921	0.8109	8	0.833	0.840	0.309	11	0.850	0.897	0.0532
Scin5	10	0.850	0.812	0.7471	11	0.900	0.786	0.5517	6	0.833	0.787	0.186	7	0.800	0.646	0.9496
Scin6	9	0.950	0.867	0.8221	9	0.950	0.821	0.9622	9	0.944	0.873	0.180	8	0.800	0.873	0.4536
Scin7	6	0.800	0.782	0.6407	12	0.800	0.901	0.0473*	7	0.611	0.757	0.075	6	0.550	0.786	0.0065**
Scin8	7	0.400	0.812	0.0000***	8	0.850	0.854	0.7488	8	0.944	0.817	0.673	8	0.800	0.785	0.3367
Scin9	9	0.800	0.879	0.1291	5	0.850	0.741	0.1276	7	0.833	0.783	0.861	7	0.850	0.821	0.0013**
Scin10	7	0.750	0.829	0.3720	7	0.900	0.813	0.9964	9	0.778	0.879	0.157	7	0.750	0.735	0.4054
Scin11	6	0.750	0.755	0.5172	7	0.750	0.717	0.3867	6	0.722	0.743	0.193	7	0.650	0.626	0.4729
Scin12	7	0.900	0.827	0.0946	9	0.850	0.877	0.2666	10	0.833	0.851	0.837	10	0.850	0.897	0.1581
Mean	7.75	0.813	0.829		8.50	0.871	0.819		7.75	0.742	0.770		7.25	0.779	0.782	

Note: *A* = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; *N* = sample size for each population.

[a] Locality and voucher information is provided in Appendix 1.

[b] Significant deviations from Hardy–Weinberg equilibrium at $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, respectively.

Table 3. Results of primer cross-species amplification in *Scrophularia dentata* and *S. kiriloviana*.[a]

	S. dentata								*S. kiriloviana*							
	Population RK (N = 15)				Population LS (N = 10)				Population WS (N = 20)				Population TS (N = 20)			
Locus	A	H_o	H_e	HWE[b]	A	H_o	H_e	HWE[b]	A	H_o	H_e	HWE[b]	A	H_o	H_e	HWE[b]
Scin1	7	1.000	0.848	0.0014**	5	0.600	0.800	0.2646	9	0.850	0.777	0.7851	9	0.900	0.803	0.0178*
Scin2	4	0.933	0.609	0.0008***	2	0.200	0.337	0.3065	6	0.850	0.818	0.1466	13	0.550	0.873	0.0000***
Scin3	5	0.400	0.743	0.0000***	2	0.400	0.505	0.5732	8	0.600	0.662	0.1540	5	0.550	0.574	0.1363
Scin4	6	0.800	0.763	0.9335	4	0.500	0.695	0.2801	9	0.800	0.878	0.5637	12	0.650	0.914	0.0076**
Scin5	6	0.867	0.807	0.5949	4	0.700	0.753	0.2933	9	0.850	0.895	0.0414*	11	0.500	0.823	0.0003***
Scin6	8	0.933	0.832	0.1842	3	0.400	0.689	0.0850	11	0.800	0.894	0.5252	9	0.800	0.835	0.4918
Scin7	6	0.800	0.699	0.0861	3	0.700	0.679	1.0000	6	0.700	0.773	0.0337*	12	0.500	0.922	0.0000***
Scin8	8	0.867	0.818	0.0001***	5	0.800	0.816	0.0998	10	0.900	0.796	0.9847	8	0.550	0.854	0.0053**
Scin9	8	0.933	0.832	0.0083**	3	0.800	0.700	0.1293	7	0.800	0.744	0.7117	8	0.700	0.838	0.3850
Scin10	6	0.800	0.699	0.7845	3	0.600	0.674	0.8448	8	0.550	0.565	0.6567	6	0.350	0.653	0.0016**
Scin11	7	0.867	0.818	0.6091	4	0.700	0.774	0.8559	8	0.900	0.842	0.0963	8	0.400	0.873	0.0000***
Scin12	8	0.867	0.802	0.8971	4	0.600	0.726	0.5635	6	0.750	0.742	0.9295	14	0.650	0.906	0.0001***
Mean	6.58	0.839	0.773		3.50	0.583	0.679		8.08	0.779	0.782		9.58	0.592	0.822	

Note: A = number of alleles per locus; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; N = sample size for each population.

[a] Locality and voucher information is provided in Appendix 1.

[b] Significant deviations from Hardy–Weinberg equilibrium at $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, respectively.

populations, the number of observed alleles per locus, as well as observed and expected heterozygosities, were calculated using CERVUS version 3.0.3 (Kalinowski et al., 2007). Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between all these primer pairs were tested using GENEPOP version 4.0.7 (Rousset, 2008).

Twelve loci could be amplified repeatedly and demonstrated polymorphism, and the remaining four loci could not be amplified reliably. The statistics reported are from the 12 polymorphic loci that could be reliably scored. The mean number of alleles was 19.75 (range: 14–26) for the four *S. incisa* populations (Table 1); 7.75 (range: 6–11), 8.50 (range: 5–12), 7.75 (range: 6–10), and 7.25 (range: 4–11) for populations MZ, GD, ZY, and QL, respectively (Table 2). The four populations exhibit comparable levels of microsatellite diversity (Table 2). The 12 microsatellite loci developed for *S. incisa* were successfully transferred in the other two species of sect. *Tomiophyllum*, *S. dentata* and *S. kiriloviana*. All of the SSR markers developed from *S. incisa* are codominant in *S. dentata* and *S. kiriloviana*. Their overall mean numbers of alleles were 5.18 (range: 2–8) and 8.83 (range: 5–13) per locus for *S. dentata* and *S. kiriloviana*, and they also exhibit comparable levels of microsatellite diversity (Table 3). We detected deviation from HWE ($P < 0.05$) at some of the microsatellite loci as a result of heterozygote excess, e.g., three (Scin1, 4, 8), one (Scin7), two (Scin1, 2), and two (Scin7, 9) loci for populations MZ, GD, ZY, and QL, respectively (Table 2); five (Scin1, 2, 3, 8, 9), two (Scin5, 7), and nine (Scin1, 2, 4, 5, 7, 8, 10, 11, 12) loci for populations RK, WS, and TS, respectively (Table 3). No significant LD signal ($P < 0.01$) was detected for each locus pair across all populations.

CONCLUSIONS

The application of these 12 polymorphic microsatellite markers in combination with chloroplast DNA sequences should be robust to reveal geographic patterns of molecular variation in *S. incisa*, *S. dentata*, and *S. kiriloviana* at the population level and across the species ranges in China. From a perspective of comparative phylogeography, these data from such a study system will be substantially valuable to address roles of different evolutionary processes in plants inhabiting the Qinghai–Tibet Plateau and adjacent regions, and to guide appropriate conservation action in the vulnerable ecosystems.

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Appendix 1. Information on representative voucher specimens deposited at the Herbarium of Zhejiang University (HZU), Hangzhou, Zhejiang Province, China.

Taxon	Population code	Location	Altitude (m)	Geographic coordinates	Voucher no.
Scrophularia incisa	MZ	Manzhouli, Inner Mongolia, China	650	49°05′40.07″N, 117°30′36.34″E	CXF100704
	GD	Gandi, Qinghai Province, China	3066	36°22′37.1″N, 100°22′16.9″E	WRH110703
	ZY	Zhangye, Gansu Province, China	2753	38°32′32.46″N, 100°15′00.39″E	LP1109069
	QL	Qilian, Qinghai Province, China	2985	38°10′04.17″N, 100°00′58.06″E	LP1109068
Scrophularia dentata	RK	Xigaze, Tibet, China	3807	29°20′35.47″N, 89°38′01.45″E	LP0907045
	LS	Lhasa, Tibet, China	3768	29°42′32.47″N, 91°09′42.52″E	LP0907046
Scrophularia kiriloviana	WS	Wensu, Xinjiang, China	2458	42°55′23.00″N, 83°39′12.09″E	WRH13070
	TS	Tashkurgan, Xinjiang, China	3106	37°47′12.54″N, 75°13′08.89″E	WRH130706