**DEVELOPMENT OF MICROSATELLITE LOCI IN SCROPHULARIA INCISA (SCROPHULARIACEAE) AND CROSS-AMPLIFICATION IN CONGERIC SPECIES**

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- **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. *Tomiothyllum* 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. *Tomiothyllum* 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 *SspI* (TaKaRa Biotechnology Co., Dalian, China), and the restriction fragments were ligated to an unequal-length adapter using DNA Ligation fragment was ligated to an unequal-length adapter using DNA Ligation

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Kit version 2.0 (TaKaRa Biotechnology Co.). Second, DNA fragments flanked by a microsatellite at one end were amplified from both the Scin11 (AC)\textsuperscript{6} (AG)\textsuperscript{9} and Scin12 (AC)\textsuperscript{7} loci. Third, 12 compound SSR loci were developed from four populations (Manzhouli, Inner Mongolia, China [MZ]; Gendi, Qinghai, China [GD]; Zhangey, Gansu, China [ZY]; and Qilian, Qinghai, China [QL]) 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 Taskurung, 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\textsubscript{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 μL 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 GeneMarker software version 1.97 (SoftGenetics, State College, Pennsylvania, USA). Across these eight populations, 35 individuals of S. incisa from Xigaze, Tibet, China (RK), and Lhasa, Tibet, China (LS), and 40 individuals of S. kiriloviana from Wensu, Xinjiang, China (WS), and Taskurung, Xinjiang, China (TS), were analyzed for cross-species amplification tests. The voucher specimens were deposited in the Herbarium of Zhejiang University (HZU) (Appendix 1).
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 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   |