Development, characterization, and cross-amplification of microsatellite markers for *Psammosilene tunicoides* (Caryophyllaceae)

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**PREMISE OF THE STUDY:** *Psammosilene tunicoides* (Caryophyllaceae) is a narrowly distributed and endemic plant species in southwestern China. The overexploitation of natural *P. tunicoides* has led to the destruction of many populations. Population and genetic studies will provide crucial data for the protection and management of *P. tunicoides*. In this study, we develop simple sequence repeat markers of *P. tunicoides* to analyze population diversity.

**METHODS AND RESULTS:** Microsatellite loci of *P. tunicoides* were isolated with FIASCO. Eleven polymorphic and 10 monomorphic primers were developed. The 11 polymorphic primers were tested in three *P. tunicoides* populations, yielding two to nine alleles per locus. Levels of observed heterozygosity varied from 0.000 to 1.000, and levels of expected heterozygosity ranged from 0.000 to 0.615. In addition, three of these loci were successfully amplified, and showed polymorphism, in three *Silene* species.

**CONCLUSIONS:** These microsatellite markers can be valuable tools to investigate the genetic diversity and population structure of *P. tunicoides*.

**KEYWORDS:** Caryophyllaceae; cross-amplification; genetic diversity; microsatellite; *Psammosilene tunicoides*; *Silene*.

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*Psammosilene tunicoides* W. C. Wu & C. Y. Wu (Caryophyllaceae), a perennial and monotypic herb endemic to southwestern China, was described more than 500 years ago and is highly valued in traditional Chinese medicine for pain relief, coagulative effects, and promoting blood circulation (Qu *et al.*, 2011). However, population sizes of this species have been declining dramatically in recent years due to overharvesting, and it is currently listed in the *China Plant Red Data Book* as a rare and endangered species (Fu and Chin, 1992). This species urgently requires protection. Previous genetic diversity analysis developed for *P. tunicoides* conservation strategies were mostly dependent on molecular markers, including amplified fragment length polymorphisms (AFLP) (Dai *et al.*, 2007), direct amplification of length polymorphisms (DALP) (Qu *et al.*, 2010; Li *et al.*, 2016), and DNA sequencing (Zhang *et al.*, 2011). The DALP and AFLP markers are often composed of multiple fragments in large genome templates, which complicates their use for genetic analysis.

As molecular markers, microsatellites (also known as simple sequence repeats [SSRs]) are DNA motifs composed of one to six nucleotides, which have gained considerable importance in plant genetics analysis and breeding due to their many desirable attributes, including codominant inheritance, stability, extensive genome coverage, and amenability to automation. SSRs have been found ubiquitously in genetic diversity research, genome evolution, species conservation, and marker-assisted selection breeding (Cavagnarò *et al.*, 2010; Kalia *et al.*, 2011; Wei *et al.*, 2011; Passos *et al.*, 2013). Because *P. tunicoides* is an endangered species and cultivated herb, it is necessary to develop SSR markers for both conservation strategies and marker-assisted selection breeding. However, the National Center for Biotechnology Information (NCBI) database contains no SSR sequences for *P. tunicoides* based on Sanger sequencing data. In this study, we report the development and characterization of 11 novel polymorphic genomic SSR markers for *P. tunicoides*. Additionally, we cross-amplified these loci in three species of the genus *Silene* L.: *S. gracilicaulis* C. L. Tang, *S. huguettiae* Bocquet, and *S. gonosperma* (Rupr.) Bocquet.

**METHODS AND RESULTS**

Total genomic DNA was extracted from silica gel–dried leaf tissue from seven samples of *P. tunicoides* from different populations
(Appendix 1) using the DNeasy Plant Mini Kit (Tiangen Biotech, Beijing, China). These microsatellite markers were developed using the Fast Isolation by AFLP of Sequences Containing repeats protocol (FIASCO) with modifications (Zane et al., 2002). Approximately 500 ng of genomic DNA was digested with MseI restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA). Then, using the universal adapter pair (F: TACTCAGGACTCAT; R: GACGATGAGTCCTGAAG) combined with its fragment, the digested product was placed at 37°C. This mixture was amplified by PCR using a reaction program containing an initial denaturation of 94°C for 3 min; followed by 20 cycles of 94°C for 30 s, 55°C for 60 s, and 72°C for 60 s; with a final extension at 72°C for 8 min.

PCR product hybridization was performed with 5′-biotinylated (AC)15/(AG)15 probes, and hybridization products were enriched and digested product was placed at 37°C. This mixture was amplified by PCR using a reaction program containing an initial denaturation of 94°C for 3 min; followed by 30 cycles of 95°C for 30 s, 45–56°C for 1 min (Table 1), and 72°C for 30 s; and a final extension at 72°C for 10 min. After PCR amplification, PCR products were separated and visualized using an ABI 3730 automated sequencer (ABI 3730XL, Applied Biosystems), and the size of the alleles at each locus was scored by GenBank (Table 1).

Polymorphisms were validated using the 46 designed primers (Table 2), and 72°C for 30 s; and a final extension at 72°C for 10 min. After PCR amplification, PCR products were separated and visualized using an ABI 3730 automated sequencer (ABI 3730XL, Applied Biosystems), and the size of the alleles at each locus was scored by GenBank (Table 1).

Numbers of alleles per locus, observed heterozygosity (\(H_o\)), and expected heterozygosity (\(H_e\)) were calculated by GenAlEx 6.5 (Peakall and Smouse, 2012); linkage disequilibrium and deviations from Hardy–Weinberg equilibrium were estimated using Table 2. Genetic properties of 11 newly developed polymorphic microsatellite markers for Psammosilene tunicoides.a

| Locus | Primer sequences (5′-3′) | Repeat motif | \(T_a\) (°C) | Allele size range (bp) | A | GenBank accession no. |
|-------|-------------------------|--------------|--------------|-----------------------|---|-----------------------|
| E2    | F: TCCCTCCATACCTCATACA  | (GAA)\(_{15}\) | 45           | 278–284               | 4 | KJ159956              |
| E5    | F: TCCGACGAAGGAGTCTGCT  | (GA)\(_{15}\) | 53           | 175–179               | 3 | KJ159945              |
| E7    | F: GCAGGCTTCTAGTGACATT  | (TCT)\(_{15}\) | 53           | 283–291               | 3 | KJ159946              |
| E10   | F: CACCGTACCTCAACAA     | (TC)\(_{15}\) | 50           | 193–205               | 4 | KJ159951              |
| Z3    | F: GTGGAGAAATCATGGAG    | (CT)\(_{15}\) | 53           | 123–135               | 3 | KJ159953              |
| Z5    | F: ATATGTTTACTCTGTTG    | (AG)\(_{15}\) | 50           | 190–201               | 5 | KJ159957              |
| Z6    | F: TCCCAATTGGCATTCA     | (CTT)\(_{15}\) | 50           | 174–195               | 4 | KJ159955              |
| Z11   | F: GGTGTATGCTACCTGTC    | (AG)\(_{15}\) | 50           | 201–208               | 2 | KJ159952              |
| Z12   | F: ATGTTTCTATGCTCTAA    | (TC)\(_{15}\) | 50           | 190–201               | 9 | KJ159949              |
| Z14   | F: CAGTGGGGCTGGCTGTAAT  | (GA)\(_{15}\) | 56           | 170–180               | 3 | KJ159941              |
| Z16   | F: CCCCCGTCCTGGCACT     | (GT)\(_{15}\) | 52           | 155–170               | 2 | KJ159937              |

Note: A = number of alleles; \(T_a\) = annealing temperature.

TABLE 2. Genetic properties of 11 newly developed polymorphic microsatellite markers for Psammosilene tunicoides.a

| Locus | Lijiang population (\(n = 18\)) | Yanyuan population (\(n = 20\)) | Weining population (\(n = 20\)) |
|-------|---------------------------------|---------------------------------|---------------------------------|
|       | \(A\)  | \(H_o\)  | \(H_e\)  | \(A\)  | \(H_o\)  | \(H_e\)  | \(A\)  | \(H_o\)  | \(H_e\)  |
| E2    | 2      | 0.200   | 0.180   | 2      | 0.100   | 0.095   | 2      | 0.200   | 0.180   |
| E5    | 3      | 0.200   | 0.185   | 1      | 0.000   | 0.000   | 3      | 0.500   | 0.395   |
| E7    | 2      | 0.400   | 0.420   | 1      | 0.000   | 0.000   | 2      | 0.000   | 0.420** |
| E10   | 3      | 1.000   | 0.545** | 4      | 1.000   | 0.615   | 3      | 1.000   | 0.545** |
| Z3    | 1      | 0.000   | 0.000   | 2      | 0.100   | 0.095   | 2      | 0.100   | 0.095   |
| Z5    | 1      | 0.000   | 0.000   | 3      | 0.100   | 0.485** | 2      | 0.100   | 0.095   |
| Z6    | 3      | 0.100   | 0.185** | 2      | 0.100   | 0.095   | 3      | 0.300   | 0.615   |
| Z11   | 2      | 0.900   | 0.495** | 2      | 1.000   | 0.500** | 2      | 1.000   | 0.500** |
| Z12   | 5      | 0.600   | 0.720   | 3      | 0.400   | 0.595   | 4      | 0.600   | 0.595** |
| Z14   | 2      | 0.000   | 0.180** | 2      | 0.000   | 0.180** | 2      | 0.100   | 0.095   |
| Z16   | 2      | 0.000   | 0.180** | 2      | 0.100   | 0.255   | 2      | 0.100   | 0.095   |

Note: A = number of alleles; \(H_o\) = observed heterozygosity; \(H_e\) = expected heterozygosity; \(n\) = number of individuals.

aLocality and voucher information are provided in Appendix 1.

**Significant deviation from Hardy–Weinberg equilibrium (*P < 0.01, **P < 0.05, ***P < 0.001).
GENEPOP version 3.4 (Rousset, 2008). Only 11 primer pairs displayed polymorphism among these three populations (Table 1), and the amplification products were within the expected size range. The number of alleles per locus ranged from two to nine, with an average of 3.81 alleles. The average levels of $H_e$ and $H_o$ in all three populations were 0.31 ± 0.06 and 0.29 ± 0.04, respectively (Table 2). Five loci (E10, Z6, Z11, Z14, Z16) in the Lijiang population, three loci (Z5, Z11, Z14) in the Yanyuan population, and four loci (E7, E10, Z11, Z12) in the Weining population showed significant deviations from Hardy–Weinberg equilibrium, indicating heterozygote deficiencies. In addition to the 11 polymorphic loci, 10 monomorphic microsatellite loci were obtained and the sequence information was deposited to NCBI (Appendix 2).

We also tested 11 primer pairs in three species of the related genus Silene: *S. gracilicaulis*, *S. huguettiae*, and *S. gonosperma*. The results revealed that only three primers (E5, Z3, and Z14) show amplified bands, with lower $H_o$ and $H_e$ in these loci (Table 3).

**CONCLUSIONS**

This is the first study to characterize microsatellite markers specifically for *P. tunicoides*. The 11 polymorphic markers developed here will enable further studies investigating the population genetic structure, the development of conservation strategies, and marker-assisted selection breeding of this species. However, the cross-species amplification of these markers indicates that they may be less useful in related genera, such as *Silene*, because of the distant phylogenetic relationships between *P. tunicoides* and other species in Caryophyllaceae.

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**DATA ACCESSIBILITY**

Sequence information for the developed primers has been deposited to the National Center for Biotechnology Information (NCBI); GenBank accession numbers are provided in Table 1.

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APPENDIX 1. Voucher and locality information of three populations of Psammosilene tunicoides and three Silene species used in the study.4

| Taxon                  | Population code | Voucher specimen accession no. | N     | Locality                  | Geographic coordinates        | Altitude (m) |
|------------------------|-----------------|---------------------------------|-------|---------------------------|------------------------------|--------------|
| Psammosilene tunicoides| LJ              | LGD2016005                      | 18    | Lijiang, Yunnan Province  | 26°16′06″N, 100°16′16″E       | 2381         |
|                        | YY              | LGD2016018                      | 20    | Yanyuan, Sichuan Province | 27°06′37″N, 100°42′09″E       | 2650         |
| P. tunicoides           | WN              | LGD2016020                      | 20    | Weining, Guizhou Province | 27°06′58″N, 104°07′27″E        | 2400         |
| S. gracilicaulis C. L. | XGLL            | MS2017230                      | 6     | Xianggeli, Yunnan Province | 27°32′18″N, 99°43′08″E        | 3240         |
| Tang                   | JJ              | MS2017506                      | 5     | Xiaojin, Sichuan Province | 30°54′42″N, 102°53′49″E        | 5040         |
| S. huguettiae Bocquet   | JL              | MS2017536                      | 6     | Jiuulong, Sichuan Province | 28°22′39″N, 101°37′32″E        | 2135         |
| S. gonosperma (Ruopr.) |                |                                 |       |                           |                              |              |

Note: N = sample size for each population.

APPENDIX 2. Characteristics of 10 monomorphic microsatellite loci developed in Psammosilene tunicoides.

| Locus | Primer sequences (5′–3′) | Repeat motif   | T_a (°C) | Allele size (bp) | GenBank accession no. |
|-------|--------------------------|----------------|----------|-----------------|-----------------------|
| E1    | F: CCCTTAGTTGTTACTTTTCTC | (CA)_n (CA)_n  | 50       | 230             | KJ159936              |
|       | R: TTGATTTCTTTGACCACT    |                |          |                 |                       |
| E3    | F: ACTTCGAGCAGAAGCAGACT  | (CA)_n         | 50       | 122             | KJ159939              |
|       | R: CAAATGGGACACTATAAATG  |                |          |                 |                       |
| E4    | F: TTTCTATCCAAGGCACACT  | (CT)_n (CT)_n  | 48       | 221             | KJ159941              |
|       | R: CAAACATAAGCAACATC     |                |          |                 |                       |
| E6    | F: TGGTCAAGTGAAGGCACA   | (AG)_n         | 52       | 117             | KJ159942              |
|       | R: CAGCCTACCAAATCAAT     |                |          |                 |                       |
| E8    | F: GCCATTGATTACTCTTTCG   | (GT)_m (GT)_m  | 56       | 236             | KJ159943              |
|       | R: AGCCCTTGTTGCTTACTTTCTC|                |          |                 |                       |
| E9    | F: AAGCCAAAGCAGTCCCTCT  | (TC)_n         | 52       | 222             | KJ159944              |
|       | R: ACCCAAGATCGTCTCTA     |                |          |                 |                       |
| E11   | F: CCACGTCCCACTCAAATA   | (CT)_m         | 50       | 147             | KJ159946              |
|       | R: TGCTCAAGTACGCAAACAC  |                |          |                 |                       |
| E12   | F: GAGAAATTGGAGGTACGAG  | (GT)_m         | 48       | 147             | KJ159948              |
|       | R: ACCTGAGAAAGATGAGGAC  |                |          |                 |                       |
| Z1    | F: GCCATTGATTACTCTTCG   | (TC)_n         | 53       | 192             | KJ159950              |
|       | R: AGCCCTTGTTGCTTACTTTCTC|                |          |                 |                       |
| Z2    | F: TCAATGCAATTAGGGAGGAA | (GA)_n         | 50       | 238             | KJ159954              |
|       | R: TGCTTTGGAACCTTGTG     |                |          |                 |                       |

Note: T_a = annealing temperature.