Applications in Plant Sciences

Botanical Society of America
leading scientists and educators since 1893

PRIMER NOTE

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

Ai-Li Zhang[1], Ya-Fang Gao[1], Guo-Dong Li[1,2], and Zi-Gang Qian[1,2]

Manuscript received 27 July 2018; revision accepted 3 October 2018.

[1] Faculty of Traditional Chinese Pharmacy, Yunnan University of Traditional Chinese Medicine, Kunming, Yunnan 650500, People's Republic of China

[2] Authors for correspondence: gammar116@163.com (G.-D.L.), qianzig@aliyun.com (Z.-G.Q.)

Citation: Zhang, A.-L., Y.-F. Gao, G.-D. Li, and Z.-G. Qian. 2018. Development, characterization, and cross-amplification of microsatellite markers for *Psammosilene tunicoides* (Caryophyllaceae). *Applications in Plant Sciences* 6(12): e1199.

doi:10.1002/aps3.1199

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*.

KEY WORDS Caryophyllaceae; cross-amplification; genetic diversity; microsatellite; *Psammosilene tunicoides*; *Silene*.

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 (Cavagnaro 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

TABLE 1. Characteristics of 11 polymorphic microsatellite loci developed for *Psammosilene tunicoides*.

Locus	Primer sequences (5′–3′)	Repeat motif	T_a (°C)	Allele size range (bp)	*A*	GenBank accession no.
E2	F: TCCCTCCATACTCATACA R: ATGCAAACCTTATTCTTC	$(GAA)_5$	45	278–284	4	KJ159956
E5	F: TCCGACGAAGGGAATGCT R: CGCCTGAAACTTCCACCA	$(GA)_{10}$	53	175–179	3	KJ159945
E7	F: GCGGCCTCCTAGTCACATT R: CACCACCTTTGCCTTCCTT	$(TCT)_9$	53	283–291	3	KJ159947
E10	F: CACCGTCACTCCTAACCA R: ATGCAGGAAAGGAAGTCG	$(TC)_9$	50	193–205	4	KJ159951
Z3	F: GTCGGAGAACTATCGAGAT R: GAGGAAGAGCGTGGAGGA	$(CT)_{11}$	53	123–135	3	KJ159953
Z5	F: ATATGTTTTACTTGGTGG R: CTTCCTCTTATTTGCTAG	$(AG)_{13}$	50	190–201	5	KJ159957
Z6	F: TCCCAATTTGCACTTTCA R: ACCCACCAACAACATAAGC	$(CTT)_9$	50	174–195	4	KJ159955
Z11	F: GGTTGTATGCCATCGTCG R: CCTTTCTGCCGTGATTTT	$(AG)_3AA(AG)_6$	50	201–208	2	KJ159952
Z12	F: ATTGTTTTCATCGCTCTA R: GGAGAAAGGTTGATAGGAG	$(TC)_{10}$	50	190–201	9	KJ159949
Z14	F: CAGGTGGTGGGCTGGTAAT R: CCTCGGTTCCGCCATTTGT	$(GA)_6$	56	170–180	3	KJ159941
Z16	F: CCCTCGGTTCCGCCATTT R: GGTGGTGGGCTGGTAATG	$(GT)_7$	52	155–170	2	KJ159937

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

(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 *Mse*I restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA). Then, using the universal adapter pair (F: TACTCAGGACTCAT, R: GACGATGAGTCCTGAG) 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 of 72°C for 8 min.

PCR product hybridization was performed with 5′-biotinylated $(AC)_{15}$/$(AG)_{15}$ probes, and hybridization products were enriched using magnetic beads. The collected enriched products were used as a template to conduct a PCR reaction according to the above program. After the amplified product was purified, the purified product was ligated into a pGM-T vector (Tiangen Biotech) and transformed using Trans1-T1 Phage Resistant Chemically Competent Cells (TransGen Biotech, Beijing, China) to carry out amplification and expression. Positive strains were selected using Luria–Bertani medium containing ampicillin. A total of 359 positive clones were selected and sequenced using the ABI PRISM 3730XL DNA sequencer (Applied Biosystems, Foster City, California, USA), including 217 sequences containing an SSR. Out of these 217 sequences, 46 successfully yielded clear bands; the others showed multi-banding patterns or no amplification.

Polymorphisms were validated using the 46 designed primers in 30 samples of *P. tunicoides* that were collected from three locations in China: Yunnan, Sichuan, and Guizhou (Appendix 1). PCR was performed in a 25-μL reaction volume, containing 1 μL of template DNA (5 ng/μL), 0.5 μL of reference primer, 1 unit of *Taq* polymerase (TaKaRa Biotechnology Co., Dalian, China), 2.5 μL of 10× PCR buffer, 2.0 μL of $MgCl_2$ (25 mM), 0.5 μL of dNTP mixture (10 mM each), and 17.8 μL ddH_2O. The amplification reaction was conducted using the following protocol: initial denaturation at 95°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 GeneMapper version 3.2 (Applied Biosystems). All sequences were deposited in 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]

	Lijiang population (*n* = 18)			Yanyuan population (*n* = 20)			Weining population (*n* = 20)		
Locus	*A*	H_o	H_e[b]	*A*	H_o	H_e[b]	*A*	H_o	H_e[b]
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_e = expected heterozygosity; H_o = observed heterozygosity; *n* = number of individuals.

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

[b]Significant deviation from Hardy–Weinberg equilibrium (*$P < 0.01$, **$P < 0.05$, ***$P < 0.001$).

TABLE 3. Cross-amplification of microsatellite loci developed for *Psammosilene tunicoides* in three related *Silene* species.[a]

	Silene gracilicaulis (*n* = 6)				*Silene huguettiae* (*n* = 5)				*Silene gonosperma* (*n* = 6)			
Locus	*A*	H_o	H_e	Allele size range (bp)	*A*	H_o	H_e	Allele size range (bp)	*A*	H_o	H_e	Allele size range (bp)
E2	—	—	—	—	—	—	—	—	—	—	—	—
E5	4	0.667	0.681	193–209	1	0.000	0.000	201–231	1	0.000	0.000	183–209
E7	—	—	—	—	—	—	—	—	—	—	—	—
E10	—	—	—	—	—	—	—	—	—	—	—	—
Z3	1	0.000	0.000	225	1	0.000	0.000	225	2	0.200	0.180	225
Z5	—	—	—	—	—	—	—	—	—	—	—	—
Z6	—	—	—	—	—	—	—	—	—	—	—	—
Z11	—	—	—	—	—	—	—	—	—	—	—	—
Z12	—	—	—	—	—	—	—	—	—	—	—	—
Z14	1	0.000	0.000	201	1	0.000	0.000	201–217	2	0.333	0.278	201–217
Z16	—	—	—	—	—	—	—	—	—	—	—	—

Note: — = unsuccessful amplification; *A* = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; *n* = number of individuals.
[a]Locality and voucher information are provided in Appendix 1.

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_o and H_e 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.

ACKNOWLEDGMENTS

The study was supported by the National Natural Science Foundation of China (grant no. 81560613 to G.-D.L.), the Yunnan Provincial Science and Technology Department–Applied Basic Research Joint Special Funds of Yunnan University of Traditional Chinese Medicine (grant no. 2015FB205(-015) to G.-D.L.), and the Key Laboratory Training Program in Yunnan (2017DG006).

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.

LITERATURE CITED

Cavagnaro, P. F., D. A. Senalik, L. Yang, P. W. Simon, T. T. Harkins, C. D. Kodira, Y. Weng, et al. 2010. Genome-wide characterization of simple sequence repeats in cucumber (*Cucumis sativus* L.). *BMC Genomics* 11(1): 569.

Dai, Z. B., C. C. Zhu, Z. G. Qian, and C. X. Pu. 2007. Genetic diversity analysis of germplasm resources of *Psammosilene tunicoides*. *Chinese Traditional and Herbal Drugs* 38(7): 1070–1073.

Fu, L. G., and C. M. Chin. 1992. China plant red data book: Rare and endangered plants. Science Press, Beijing, China.

Kalia, R. K., M. K. Rai, S. Kalia, R. Singh, and A. K. Dhawan. 2011. Microsatellite markers: An overview of the recent progress in plants. *Euphytica* 177: 309–334.

Li, J., M. Song, C. Xiong, B. Zhao, and W. Sun. 2016. Application of barcode high-resolution melting for rapid authentication of the medicinal plant *Psammosilene tunicoides*. *Biotechnology and Biotechnological Equipment* 30: 1–7.

Passos, M. A., V. O. de Cruz, F. L. Emediato, C. C. de Teixeira, V. C. Azevedo, A. C. Brasileiro, R. C. Togawa, et al. 2013. Analysis of the leaf transcriptome of *Musa acuminata* during interaction with *Mycosphaerella musicola*: Gene assembly, annotation and marker development. *BMC Genomics* 14(1): 78.

Peakall, R., and P. E. Smouse. 2012. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research–an update. *Bioinformatics* 28: 2537–2539.

Qu, Y., H. Yu, G. Wu, R.-F. Ma, and Y.-Y. Li. 2010. Genetic diversity and population structure of the endangered species *Psammosilene tunicoides* revealed by DALP analysis. *Biochemical Systematics and Ecology* 38: 880–887.

Qu, Y., H. Yu, and X. L. Zhou. 2011. Review on study advances on rare and endangered medicinal herb *Psammosilene tunicoides*. *China Journal of Traditional Chinese Medicine and Pharmacy* 26: 1795–1797.

Rousset, F. 2008. GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

Wei, W., X. Qi, L. Wang, Y. Zhang, W. Hua, D. Li, X. Zhang, et al. 2011. Characterization of the sesame (*Sesamum indicum* L.) global transcriptome using Illumina paired-end sequencing and development of EST-SSR markers. *BMC Genomics* 12: 451.

Zane, L., T. Patarnello, A. Ludwig, F. Fontana, and L. Congiu. 2002. Isolation and characterization of microsatellites in the Adriatic sturgeon (*Acipenser naccarii*). *Molecular Ecology Resources* 2: 586–588.

Zhang, Q. Y., Y. J. Zhao, and X. Gong. 2011. Genetic variation and phylogeography of *Psammosilene tunicoides* (Caryophyllaceae), a narrowly distributed and endemic species in south-western China. *Australian Journal of Botany* 59: 450–459.

APPENDIX 1. Voucher and locality information of three populations of *Psammosilene tunicoides* and three *Silene* species used in the study.[a]

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

Note: N = sample size for each population.
[a]Vouchers are stored in the herbarium of Yunnan University of Traditional Chinese Medicine, Kunming, Yunnan, China.
[b]LGD = Guodong Li; MS = Xiangguang Ma and Wenguang Sun.

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: CCCTTAGTTGTTACTTTCTC R: TTGATTACTTCTTCGCCAC	$(CA)_3A(CA)_5$	50	230	KJ159936
E3	F: ACTTCGAGCAGAACAGACT R: CAAATGGGACACTATAAATG	$(CA)_6$	50	122	KJ159939
E4	F: TTTCTATCCAAAAGGCACT R: CAAACATAAGCAACATTCA	$(CT)_4TT(CT)_6$	48	221	KJ159941
E6	F: TGGTCAAAGTAGGCAACA R: CCACGTACCCAATCAAAT	$(AG)_8$	52	117	KJ159942
E8	F: GCCATTGATTACTTCTTCG R: AGCCCTTAGTTGTTACTTTCTC	$(GT)_5T(GT)_3$	56	236	KJ159943
E9	F: AACGCAACGCAGTCCCTC R: ACCCAAGAATCCGTCCTA	$(TC)_6$	52	222	KJ159944
E11	F: CCACGTACCCAATCAAATA R: TGGTCAAAGTAGGCAACAC	$(CT)_7$	50	147	KJ159946
E12	F: GAGAATTGGAGGGTGTAG R: ACCTGAGAAAGATGGGAC	$(GT)_5$	48	147	KJ159948
Z1	F: GCCATTGATTACTTCTTCG R: AGCCCTTAGTTGTTACTTTCTC	$(TC)_6$	53	192	KJ159950
Z2	F: TCAATGCAATTTAGGAGGAA R: TGCTTGTTGAACCCTGTG	$(GA)_4$	50	238	KJ159954

Note: T_a = annealing temperature.