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Source: Applications in Plant Sciences, 5(10)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1700028

Applications in Plant Sciences 2017 5(10): 1700028

PRIMER NOTE

MICROSATELLITES FOR *PHYTOLACCA ACINOSA* (PHYTOLACCACEAE), A TRADITIONAL MEDICINAL HERB[1]

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- *Premise of the study: Phytolacca acinosa* (Phytolaccaceae) is a traditional Chinese herb with multiple medicinal uses and is an important pigment source. Due to excessive human harvesting, the population numbers and sizes have decreased dramatically.
- *Methods and Results:* Using an enriched genomic library, we developed and characterized 15 microsatellite primers for *P. acinosa*, 13 of which were polymorphic. The number of alleles varied from two to seven. The observed heterozygosity and expected heterozygosity per locus ranged from 0.267 to 1.000 and 0.331 to 0.743, respectively. All of the primers that were developed were also successfully applied in *P. americana*.
- *Conclusions:* These markers should be useful in probing the genetic diversity, genetic structure, and mating systems of *P. acinosa*, which could provide information about protecting and sustainably harvesting this species.

Key words: genetic conservation; microsatellite; *Phytolacca acinosa*; *Phytolacca americana*; Phytolaccaceae.

Phytolacca acinosa Roxb., belonging to the family Phytolaccaceae, is a perennial herb native to East Asia (Zheng et al., 2002). Its large, fleshy roots contain polysaccharides, proteins, and triterpenoid saponin and serve as a traditional Chinese herb with multiple medicinal applications, including antibacterial, anti-inflammatory, antiviral, anticancer, and immunity-enhancing uses (Zhang et al., 1990; Gao et al., 2009). Among these, the anti-inflammatory and immunity-enhancing properties are prominent, suggesting that this plant could potentially be used to develop a drug to target autoimmune diseases in the future (Li and Yao, 2011). In addition, some previous findings have shown that it could be used as a resource for red color in wool fabric dyeing or as a food additive (Zhao et al., 2014; Wu et al., 2016). For these reasons, *P. acinosa* has been overharvested in recent years in China, which has led to dramatic decreases in population numbers and sizes. To protect and sustainably harvest this valuable plant resource, we need information about genetic diversity, genetic structure, and mating systems of the species based on molecular markers. In a previous study, microsatellite markers were developed for the congener *P. americana* L.; however, these primers were of very limited use in *P. acinosa* (Bentley et al., 2015). In this study, we developed a set of microsatellite (simple sequence repeat [SSR]) loci for *P. acinosa*, which could be used to describe patterns of its genetic diversity. Cross-species amplification was also tested in one population of *P. americana*.

[1]Manuscript received 27 May 2017; revision accepted 16 August 2017.

This work was supported by the National Natural Science Foundation of China (31670389).

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doi:10.3732/apps.1700028

METHODS AND RESULTS

Leaf samples of *P. acinosa* were collected in Zhumadian (ZMD), Xinmi (XM), and Beijing (BJ), China (Appendix 1). Genomic DNA was extracted from silica gel–dried leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Fang et al., 2009). One of the genomic DNA samples was selected to construct a microsatellite-rich library using the following method: genomic DNA was double-digested with a mixture of *Rsa*I and *XMn*I enzymes. Then, two adapters were ligated to the digested DNA (forward: 5′-GTTTAAGGCCTAGCTAGCAGAATC-3′; reverse: 5′-GATTCTGCTAGCTAGGCCTTAAACAAAA-3′). The fragments with adapters at both ends were separated using 1.5% agarose gel electrophoresis, and DNA fragments with lengths of between 400 and 1200 bp were recovered using the QIAquick Gel Extraction Kit (QIAGEN, Shanghai, China). The collected fragments were hybridized with three different biotin-labeled probes (New England Biolabs, Beijing, China): $(AG)_8$, $(AC)_8$, and $(ATG)_{12}$. The hybridization products were then captured using streptavidin-coated magnetic beads. Finally, the obtained fragments were inserted into the pMD18-T Simple Vector (TaKaRa Biotechnology Co., Dalian, China) and then transformed and cloned into DH5α cells (TaKaRa Biotechnology Co.). The positive clones were tested by PCR amplification using the M13F (5′-TGTAAAACGACGGCCAGT-3′) and M13R (5′-CAGGAAACAGCTATGACC-3′) primers.

In total, 110 positive clones were selected and sequenced using an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, California, USA), 46 of which contained SSRs. Twenty-three pairs of microsatellite primers were designed using Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA) and tested in six randomly selected individuals of *P. acinosa* that came from different populations. PCRs were performed in a total volume of 10 μL containing approximately 5–20 ng of DNA template, 0.25 μM forward primers, 0.25 μM reverse primers, and 1× PCR Mix (Tiangen Biotech, Beijing, China). Microsatellite loci were tested for amplification under the following conditions: 94°C for 5 min; 35 cycles of 94°C for 35 s, the annealing temperature optimized for each primer for 30 s (Table 1), and 72°C for 1 min; and a final extension at 72°C for 10 min. The amplified products were separated on a 6% polyacrylamide gel and visualized using silver staining. A total of 15 primer pairs were successfully amplified, generating legible products of the expected fragment size. Among them, 13 primer pairs each able to amplify two or more alleles were selected to determine the genotypes of all of the samples from the three *P. acinosa* populations from Zhumadian (ZMD), Xinmi (XM), and Beijing (BJ)

TABLE 1. Characterization of 15 microsatellite loci from *Phytolacca acinosa*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
SL-34	F: TGTCCACCATAAAACACTT R: CCTCTTTCGCTACTTGC	$(ATC)_3$	165–182	49.1	KP133119
SL-58	F: CTCCTGAATCTGATGGTGAA R: AGTTGTGCGTGTTGAAGAAG	$(ATC)_6$	162–167	51.1	KP133120
SL-116	F: AGCCCCATACTCTACATC R: CTCTTCTTTCTTTTCTGTG	$(ATC)_5$	305–315	53.3	KP133121
SL-160	F: CATAACCAAGGAGGCAGA R: ACAAGAAGAAAGAGGGTGAC	$(ATC)_6$	376–419	53.7	KP133122
SL-164	F: AGTTCACACACAAAGGG R: AGGCTAATACCACGGATA	$(AC)_9$	234–244	49.6	KP133123
SL-200	F: TCCAACCCCCATCTCAAG R: CAAGATGCCCACCAATGA	$(GAT)_5$	157–166	55.9	KP133124
SL-287	F: CAAGGAAGAACAAGAGG R: TAGGTGAGAGAAGGAGT	$(CAT)_7$	130–135	49.0	KP133125
SL-307	F: GCCCCATTTCTTTTATTC R: AAGGGTCTTGGTGTT GAT	$(TCA)_4$	229–235	49.6	KP133126
SL-324	F: TGGAAAGGTCGCTAATAC R: AAACACAAGGCTTCTGAG	$(TCA)_4$	125–131	52.2	KP133127
SL-377	F: TGGACCCCTCTGCTACT R: GGACACCTCATCAGTAAA	$(TGA)_8$	253–307	53.2	KP133128
SL-379	F: ATTTGGGTACTTGGGGAC R: TTTGATTTGGGAGGGACT	$(GTT)_4$	443–447	52.3	KP133129
SL-385	F: GAATGATGGGGACAAGGA R: CAAACGCAAACGCTAGTATA	$(ATG)_3$	383–394	53.3	KP133130
SL-546	F: CCATCCATTCATCCTTTG R: ATTCACTATTCTTTGGCTTC	$(ATC)_4$	299–306	52.2	KP133131
SL-269	F: GCCCACTAAGCCCAAACT R: CTATGGAGGTTGAGGATGGT	$(AC)_{12}$	251	46.9	KY810486
SL-362	F: ATATAGACAGCCTTCCAC R: ATCCATACACAACACAAAT	$(GAT)_3$	215	45.5	KY810487

Note: T_a = annealing temperature.

(Table 2). Genotyping reactions were performed using three primers: the forward SSR-specific primer with the M13 tail at the 5′ end, the reverse SSR-specific primer, and a fluorescent dye–labeled (FAM or HEX) M13 universal primer according to the method of Schuelke (2000). Alleles were analyzed with GeneMapper version 4.0 (Applied Biosystems). The number of alleles per locus (A), observed heterozygosity (H_o), expected heterozygosity (H_e), and Hardy–Weinberg equilibrium (HWE) were calculated using Arlequin suite version 3.5 (Excoffier and Lischer, 2010). In addition, cross-amplification was conducted in one population ($N = 33$) of *P. americana* (Table 2).

Thirteen primers were successfully used to amplify SSR loci for all samples from the populations of *P. acinosa* and *P. americana*. For *P. acinosa*, A varied from two to seven per locus, and H_o and H_e ranged from 0.267 to 1.000 and 0.331 to 0.743, respectively (Table 2). A few loci were found to significantly deviate from HWE: two in the ZMD population, four in the XM population, and five in the BJ population. For *P. americana*, A ranged from two to four, and H_e and H_o varied from 0.280 to 0.760 and 0.313 to 1.000, respectively (Table 2).

CONCLUSIONS

In this work, we developed 13 polymorphic microsatellite markers for *P. acinosa* that were also successfully applied in

TABLE 2. Genetic diversity in four *Phytolacca* populations based on the 13 developed polymorphic microsatellite markers.[a]

	Phytolacca acinosa									*Phytolacca americana*		
	ZMD ($N = 21$)			XM ($N = 23$)			BJ ($N = 18$)			ZZ ($N = 33$)		
Locus	A	H_o	H_e[b]	A	H_o	H_e[b]	A	H_o	H_e[b]	A	H_o	H_e[b]
SL-34	6	0.438	0.470	2	0.267	0.331	3	0.412	0.348	3	0.313	0.280
SL-58	3	0.813	0.571	3	0.667	0.497	4	0.824	0.586	3	0.813	0.571
SL-116	4	0.417	0.525	3	0.667	0.480	3	0.470	0.383	2	0.625	0.444
SL-160	2	0.625	0.444	2	0.571	0.476	3	0.765	0.508*	2	0.625	0.444
SL-164	4	0.867	0.582	4	0.733	0.545*	2	0.688	0.466	4	0.813	0.760*
SL-200	6	0.813	0.669	3	0.933	0.591*	5	0.941	0.722*	3	0.938	0.615*
SL-287	6	0.733	0.605*	2	0.667	0.460	2	0.941	0.513**	3	0.813	0.534*
SL-307	3	0.692	0.495	2	0.533	0.405	3	0.529	0.415	3	0.500	0.401
SL-324	6	0.688	0.641	3	0.533	0.497*	3	0.563	0.462	2	0.625	0.444
SL-377	3	0.727	0.589*	3	1.000	0.618*	7	0.882	0.743**	4	1.000	0.724*
SL-379	5	0.667	0.656	3	0.429	0.540	3	0.412	0.426*	2	0.688	0.466
SL-385	4	0.750	0.558	2	0.467	0.370	2	0.529	0.401	2	0.625	0.444
SL-546	5	0.358	0.429	3	0.400	0.432	3	0.529	0.415	2	0.688	0.466

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]Asterisks indicate significant deviation from Hardy–Weinberg equilibrium: *$P < 0.05$, **$P < 0.01$.

P. americana. These loci would be useful for probing the genetic diversity, genetic structure, and mating systems of *P. acinosa*, which could provide information about protecting and harvesting this species.

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Appendix 1. Voucher information for *Phytolacca* species used in this study.

Species	Population	*N*	Voucher information[a]	Locality	Geographic coordinates
Phytolacca acinosa Roxb.	ZMD	21	ZMD201301	Zhumadian, Henan, China	35°18′15″N, 110°46′35″E
P. acinosa	XM	23	XM201303	Xinmi, Henan, China	35°18′15″N, 110°46′35″E
P. acinosa	BJ	18	BJ201301	Beijing, China	41°01′47″N, 115°15′00″E
P. americana L.	ZZ	33	ZZ201401	Zhengzhou, Henan, China	34°26′24″N, 113°25′12″E

Note: *N* = number of individuals.

[a] Specimens are deposited at Henan Agricultural University (HEAC), Zhengzhou, Henan, China.