Microsatellites for Phytolacca acinosa (Phytolaccaceae), a Traditional Medicinal Herb

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PRIMER NOTE

MICROSATELLITES FOR Phytolacca acinosa (Phytolaccaceae), a traditional medicinal herb

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Methods and Results:

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.

Microsatellites for Phytolacca acinosa (Phytolaccaceae), a traditional medicinal herb

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. americana (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.

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 Rsal and XmnI enzymes. Then, two adapters were ligated to the digested DNA (forward: 5′-GGTTTAA-AGGGCTACGTACGAAATG-3′; reverse: 5′-GATTCTGCTAGCTAGGCC-TTAACAAAAA-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) n, (AC) n, and (ATG) n. 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′-TGTAAAGACGACCGCCTGTT-3′) and M13R (5′-CAGGAAACGCTATGACC-3′) primers.

In total, 110 positive clones were 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 (TaKaRa Biotechnology Co.). 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)

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Table 1. Characterization of 15 microsatellite loci from Phytolacca acinosa.

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | Tm (°C) | GenBank accession no. |
|-------|--------------------------|--------------|-----------------------|---------|----------------------|
| SL-34 | F: TGTCCACCATAAAAACACTT  | (ATC)₃       | 165–182               | 49.1    | KP133119             |
|       | R: CCCCCTTTCTGTACTTGC    |              |                       |         |                      |
| SL-58 | F: CTCTCTGATCTGAAAGAGGA  | (ATC)₆       | 162–167               | 51.1    | KP133120             |
|       | R: ATTTGTGCCCTTGAGAGAG   |              |                       |         |                      |
| SL-116| F: AGCCCCATCTCCTACAC     | (ATC)₃       | 305–315               | 53.3    | KP133121             |
|       | R: CCTCCCTTCTCTCTCTG     |              |                       |         |                      |
| SL-160| F: CATAACCAAGAGCCAGA     | (ATC)₆       | 376–419               | 53.7    | KP133122             |
|       | R: ACAAGAGAAAGAGGTTAAC   |              |                       |         |                      |
| SL-164| F: ATGTGCACACAAAGGG      | (AC)₆        | 234–244               | 49.6    | KP133123             |
|       | R: AGGCTTAAACAGCGATA     |              |                       |         |                      |
| SL-200| F: TGCCACCCCATCCTCAAG    | (GAT)₅       | 157–166               | 55.9    | KP133124             |
|       | R: CAAGATGCACCACAAATGA   |              |                       |         |                      |
| SL-287| F: CAAGAGAGAGACAAGG      | (CAT)₂       | 130–135               | 49.0    | KP133125             |
|       | R: TAGGTGAGAGGAGGT       |              |                       |         |                      |
| SL-307| F: GCCCCATTTCTTTATTC     | (TCA)₄       | 229–235               | 49.6    | KP133126             |
|       | R: AAAGGCTCCGTGGTTGT     |              |                       |         |                      |
| SL-324| F: TGGAAGGCTCCATAC      | (TCA)₄       | 125–131               | 52.2    | KP133127             |
|       | R: AAACACAAAGCTCTGAG     |              |                       |         |                      |
| SL-377| F: TGACCCCCCTCTGACT      | (TGA)₄       | 253–307               | 53.2    | KP133128             |
|       | R: GGACACCTCATCAGTAAA    |              |                       |         |                      |
| SL-379| F: ATTTGGGTATTGGGAC      | (GTT)₄       | 443–447               | 52.3    | KP133129             |
|       | R: TTTGAATTTGAGGAGACT    |              |                       |         |                      |
| SL-385| F: GAATGATGGGGAGAGG      | (ATG)₃       | 383–394               | 53.3    | KP133130             |
|       | R: CAACGCGAAACGTGATATA   |              |                       |         |                      |
| SL-546| F: CATCCATTTCTCTCTTTC    | (ATC)₄       | 299–306               | 52.2    | KP133131             |
|       | R: ATTCATATTTCTCTGGTCTC  |              |                       |         |                      |
| SL-269| F: GCCCAATAGCCCAACAAT    | (AC)₂        | 251                   | 46.9    | KY810486             |
|       | R: CTATGAGGTGGATGAGGT    |              |                       |         |                      |
| SL-362| F: ATATAGACACCTCTCCAC    | (GAT)₃       | 215                   | 45.5    | KY810487             |
|       | R: ATCCATATACCAACACAAAT  |              |                       |         |                      |

Note: Tm = 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 (Hₒ), expected heterozygosity (Hₑ), 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 Hₒ and Hₑ 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 Hₒ and Hₑ 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

| Locus | ZMD (N = 21) | XM (N = 23) | BJ (N = 18) | ZZ (N = 33) |
|-------|-------------|-------------|-------------|-------------|
|       | A  Hₒ  Hₑ  Hₑ | A  Hₒ  Hₑ  Hₑ | A  Hₒ  Hₑ  Hₑ | A  Hₒ  Hₑ  Hₑ |
| SL-34 | 6  0.438  0.470 | 3  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  0.474  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  0.813  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| 4  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; Hₒ = expected heterozygosity; Hₑ = observed heterozygosity; N = number of individuals.

aLocality and voucher information are provided in Appendix 1.

aAsterisks indicate significant deviation from Hardy–Weinberg equilibrium: *P < 0.05, **P < 0.01.
*Phytolacca acinosa*. 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 | 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.

* Specimens are deposited at Henan Agricultural University (HEAC), Zhengzhou, Henan, China.