Biochemical Systematics and Ecology 61 (2015) 462–464

Contents lists available at ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

# Development of 12 microsatellite markers for *Aconitum brachypodum* (Ranunculaceae), a critically endangered and endemic medicinal plant

Ya-Qiong Li [a, *], Yi Meng [b], Jie Zhang [a]

[a] College of Pharmacology, Yunnan University of TCM, Kunming 650500, China
[b] Key Laboratory of Biodiversity and Biogeography, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, China

ARTICLE INFO

*Article history:*
Received 22 April 2015
Received in revised form 19 June 2015
Accepted 27 June 2015
Available online 23 July 2015

*Keywords:*
*Aconitum brachypodum*
Microsatellite markers
Genetics
Critically endangered
Medicinal plant

ABSTRACT

In this study, 12 microsatellite markers were developed from two microsatellite-enriched libraries (AG, AC) of *Aconitum brachypodum*, which were constructed using a FIASCO method. Polymorphism of each locus was assessed in 24 individuals of *A. brachypodum*. Number of alleles per locus ($N_A$) ranged from 2 to 4. The average allele number of the microsatellites was 2.58 per locus. The observed ($H_O$) and expected ($H_E$) heterozygosities ranged from 0.125 to 0.875 and 0.117 to 0.612, respectively. Polymorphic information content (PIC) ranged from 0.110 to 0.531. Among the 12 microsatellite markers, three deviated from Hardy–Weinberg equilibrium significantly. These markers will facilitate further studies on the population genetics of *A. brachypodum*.



## 1. Introduction

*Aconitum* L.(Ranunculaceae) includes at least 350 species in temperate regions of the Northern Hemisphere. About 76 *Aconitum* species have been used as medicinal plants in China, and mainly for treatment of rheumatoid arthritis and various types of pains (Xiao et al., 2006). *Aconitum brachypodum* Diels is a critically endangered (following IUCN Red List Categories and Criteria) and endemic medicinal plant, and is restricted to the alpine meadow area at an altitude range of 2800–4500 m in southwest China. It is a perennial herb with various kinds of chemical components including diterpenoid alkaloids mainly. Our recent field surveys have further confirmed the threatened status of this species and only six isolated natural populations were discovered. The habitats of all the investigated populations have been greatly degraded and the populations are fragmented. As an endangered and endemic medicinal herb, *A. brachypodum* is facing a very high risk of extinction, and its effective and long-term conservation is urgently needed. Assessment of genetic background by molecular markers is essential for the efficient conservation and rational utilization of plant genetic resources.

Although there are many kinds of molecular markers, microsatellite or simple sequence repeat (SSR) markers are becoming the markers of primary choice for the molecular characterization of different plant species because of their abundance, high polymorphism content, codominance, easy detection and transferability across studies (Tautz, 1989). Nonetheless, there are no microsatellite markers available for the molecular characterization of *A. brachypodum* so far. In

* Corresponding author.
*E-mail address:* aconitum@163.com (Y.-Q. Li).

http://dx.doi.org/10.1016/j.bse.2015.06.036
0305-1978/

order to provide a set of powerful tools for the characterization of genetic resources present in wild populations, we developed 12 microsatellite markers for *A. brachypodum.*

## 2. Materials and methods

### 2.1. SSR development and genotyping

Total genomic DNA samples of *A. brachypodum* were extracted from silica-gel-dried leaves using a CTAB methodology (Doyle and Doyle, 1987). The fast isolation by AFLP of sequences containing repeats (FIASCO) (Zane et al., 2002) was performed in this study. Total genomic DNA (ca. 500 ng) was completely digested with 2.5 U of *Mse* I restriction enzyme and then ligated to an *Mse* I amplified fragment length polymorphism (AFLP) adaptor (5′ -TACTCAGGACTCAT-3′/5′ -GACG AT GAGTCCTGAG-3′) using T4 DNA ligase (New England Biolabs, Beverly, Massachusetts, USA). The digested-ligated fragments were diluted in a ratio of 1:10, and 5 μL of them were used for amplification reactions with adaptor-specific primers (5′ -GATGAGTCCTGAGTAAN-3′/5′ -TTACT C AGGA CT CATCN-3′). The amplified DNA fragments (200–800 bp) were enriched for SSRs by magnetic bead selection with a 5-biotinylated probe [$(AG)_{10}$ or $(AC)_{10}$, respectively]. Enriched DNA fragments were reamplified with *Mse* I–N primers. The PCR products were purified using the E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Guangzhou, China). Purified DNA fragments were ligated into pBS-T II vector (Tiangen, Beijing, China) and then transformed into DH5 α competent cells (Tiangen). The positive clones were tested by PCR using vector primers T3/T7 and primers $(AC)_{10}$/$(AG)_{10}$.

Positive clones were sequenced with an ABI PRISM 3730XL DNA sequencer. The microsatellite loci were identified with SSRHunter (Li and Wan, 2005) and primer design for the loci with sufficient flanking sequences were carried out using Oligo 7.0 (Rychlik, 2007). Polymorphism of all loci with newly-designed primer pairs was assessed with 24 individuals. The PCR reactions were performed in 20 μl of reaction volume containing 50–100 ng genomic DNA, 0.5 μM of each primer, 10 μl 2 × Taq PCR MasterMix [Tiangen, 0.1 U *Taq* Polymerase/μl, 0.5 mM dNTP each, 20 mM Tris–HCl (pH 8.3), 100 mM KCl, 3 mM $MgCl_2$]. PCR amplifications were conducted under the following conditions: 95 °C for 3 min followed by 32–35 cycles at 94 °C for 45 s, at the annealing temperature for each specific primer or 45 s, 72 °C for 45 s, and a final extension step at 72 °C for 5 min. PCR products were separated by 8% non-denaturing PAGE gel and stained with a silver-staining method. A portion of PCR products were checked with QIAxcel of capillary gel electrophoresis (QIAGEN, Irvine, USA).

### 2.2. Data analysis

The variability at each locus was evaluated in terms of the numbers of alleles ($N_A$) and observed heterozygosities ($H_O$), expected heterozygosity ($H_E$) using the package GENEPOP version 4.0 (Raymond and Rousset, 1995). The same program was

**Table 1**
Descriptive statistics over all loci for the two natural populations of *Aconitum brachypodum.*

| Locus name | NCBI GenBank Accession no. | Primer sequence (5′–3′) | Repeat motif | Size (bp) | Ta (°C) | $N_a$ | $H_o$ | $H_e$ | *PIC* |
|---|---|---|---|---|---|---|---|---|---|
| AcoB01 | KP774836 | F:AATAACGCCGTCCAATCTCA<br>R:TTGTGTGCTTTCTCTCTCTTCA | (GCA)7 | 196 | 55 | 3 | 0.25 | 0.226 | 0.212 |
| AcoB02 | KP774837 | F:GTATAGCCAGATTGTCAACC<br>R:CCCATCTTTTCACAGTGTAG | (TA)8(TG)12AG(TG)11 | 252 | 51 | 3 | 0.875 | 0.612 | 0.531 |
| AcoB03* | KP774838 | F:TTTGAGGTTTCGCATTGAGG<br>R:CGTCGTTTATGATTTTCTC | (TG)8 | 246 | 50 | 4 | 0.292 | 0.4 | 0.358 |
| AcoB04 | KP774839 | F:TCGGTTCCAATGATTCTTCC<br>R:GCTCTCACTTCCATTCTCGG | (AG)8 | 127 | 55 | 2 | 0.125 | 0.117 | 0.110 |
| AcoB05* | KP774840 | F:TACAAGAAATGAGAACCAGTT<br>R:GTAAGCCAAAAGATGAAGAC | (TC)9 | 268 | 51 | 3 | 0.375 | 0.603 | 0.525 |
| AcoB06 | KP774841 | F:TCCTCCTCTCGACCCATTCT<br>R:ATCCATAACACCTACAAACG | (TC)16 | 143 | 50 | 2 | 0.125 | 0.117 | 0.110 |
| AcoB07 | KP774842 | F:TAAAATCCGCACGAGTCCAA<br>R:CTTGATTAGGTGAGTTTGAA | (AG)6 | 287 | 49 | 2 | 0.208 | 0.187 | 0.169 |
| AcoB08 | KP774843 | F:TGAGAAAACACACTGCCTGA<br>R:TCATAGTCGTAATAGGGGTC | (TG)6 | 236 | 49 | 2 | 0.583 | 0.444 | 0.346 |
| AcoB09 | KP774844 | F:CTCTGCCTACCCTCCATTCA<br>R:AAACCCACCAAAGATTCCGA | (TC)8 | 191 | 54 | 2 | 0.208 | 0.187 | 0.169 |
| AcoB10 | KP774845 | F:TTATTGACAGATGAACGAGC<br>R:GGGTTTAGGGTTTGGGAGTA | (TC)9 | 145 | 52 | 2 | 0.458 | 0.353 | 0.291 |
| AcoB11 | KP774846 | F:ATGCGATTAGAGATGGATGC<br>R:CCAAGCGAACACCGAATGAT | (TC)12 | 262 | 55 | 3 | 0.375 | 0.573 | 0.479 |
| AcoB12* | KP774847 | F:GGAATCACTGAGAAACCTAT<br>R:GCTTTCCATTATTTCCCCTA | (TG)10 | 202 | 50 | 3 | 0.833 | 0.503 | 0.396 |

Note: Ta, PCR annealing temperature;$N_a$, number of alleles revealed; $H_o$, observed heterozygosity; $H_e$, expected heterozygosity; * Indicate significant departures from Hardy–Weinberg equilibrium ($P < 0.01$).

used to test the Hardy–Weinberg equilibrium (HWE). Polymorphic information content (PIC) analyses was performed by using PowerMarker version 3.25 (Liu and Muse, 2005).

## 3. Results and discussion

### 3.1. SSR development

Positive clones from the SSR enriched library were sequenced extensively. Of 273 positive clones sequenced, 114 clones contained microsatellites. Within the 114 microsatellite-bearing clones, 86 were suitable for primer designing using Oligo 7.0, others had too short flanking sequence of SSRs. Newly-designed SSR markers were tested for polymorphism in a panel of 24 accessions from Yunnan. As a result, among the 86 primer pairs, 25 successfully amplified in all samples and 12 primer pairs displayed polymorphism.

### 3.2. SSR polymorphism

The variability at each SSR locus was measured in terms of the numbers of alleles ($N_A$) and observed heterozygosities ($H_O$), expected heterozygosity ($H_E$). The number of alleles per lous ($N_A$) for the 12 loci was 2–4 with an average of 2.58, values for observed heterozygosities ($H_O$) and expected heterozygosities ($H_E$) ranged from 0.125 to 0.875 and from 0.117 to 0.612 respectively. Polymorphic information content (PIC) ranged from 0.110 to 0.531 (Table 1).

Three (AcoB03, AcoB05 and AcoB12) were deviated from Hardy–Weinberg equilibrium significantly ($P < 0.01$). Although microsatellites have classically been considered to be evolutionary neutral, these HWE deviated loci might be under selective pressure to some extent because accumulating evidence suggests that SSRs may play a pivotal role in evolutionary processes and response to environment, particularly in the context of a genic region (Eustice et al., 2008). Therefore, the three loci should be used with cautions in the future. However, the deviation from HWE for the three loci might be simply due to stochasticity because sample size is very limited and pooled data from different populations were used in this study. Ten loci pairwise showed significant genotypic linkage disequilibrium (LD) between pairs of loci ($P < 0.001$).

## Acknowledgements

This study is funded by National Natural Science Foundation of China (No. 31460277).

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bse.2015.06.036.

## References

Doyle, J.J., Doyle, J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytoch. Bull. 19, 11–15.
Eustice, M., Yu, Q., Lai, C.W., Hou, S., Thimmapuram, J., Liu, L., Alam, M., Morre, P.H., Presting, G.G., Ming, R., 2008. Development and application of microsatellite markers for genomic analysis of papaya. Tree Genet. Genome 4, 333–341.
Li, Q., Wan, J.M., 2005. SSRHunter: development of a local searching software for SSR sites. Hereditas 27, 808–810.
Liu, K., Muse, S.V., 2005. PowerMarker: an integrated analysis environment for genetic marker analysis. Bioinformatics 21, 2128–2129.
Raymond, M., Rousset, F., 1995. GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J. Hered. 86, 248–249.
Rychlik, W., 2007. OLIGO 7 primer analysis software. Methods in Mol. Biol. 402, 35–59. Clifton, N.J.
Tautz, D., 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucleic. Acids Res. 17, 6463–6471.
Xiao, P.G., Wang, F.P., Gao, F., Yan, L.P., Chen, D.L., Liu, Y., 2006. A pharmacophylogenetic study of *Aconitum* L. (Ranunculaceae) from China. Acta Phytotaxon. Sin. 44 (1), 1–46.
Zane, L., Bargelloni, L., Patarnello, T., 2002. Strategies for microsatellite isolation: a review. Mol. Ecol. 11, 1–16.