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Short Note

Development of 16 Microsatellite Markers for Prince's Pine, *Chimaphila japonica* (Pyroleae, Monotropoideae, Ericaceae)

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Abstract: The perennial evergreen herb, *Chimaphila japonica* is found exclusively in East Asian temperate coniferous or sometimes in deciduous forests. By using the Fast Isolation by Amplified Fragment Length Polymorphism (AFLP) of Sequences Containing repeats (FIASCO) protocol, 20 microsatellite primer sets were identified in two wild populations. Of these primers, 16 displayed polymorphisms and 4 were monomorphic. The number of alleles per locus ranged from one to six among populations, values for expected and observed heterozygosities ranged from 0.000 to 0.848 and from 0.000 to 1.000, respectively. The new SSR markers will be useful in obtaining estimates of population-level genetic diversity and in phylogeographic studies of *C. japonica*.

Keywords: Chimaphila japonica; microsatellite marker; polymorphism; population genetics

1. Introduction

Members of the small genus *Chimaphila* are evergreen herbs, found in patches on the floor of coniferous or sometimes deciduous forests across the Northern Hemisphere. *Chimaphila japonica*, the focal species of the present study, is exclusively distributed in East Asia [1]. Floristic studies suggested that the forest area in the East Asiatic Kingdom could be divided into two subkingdoms, namely the Sino-Japanese Forest subkingdom and the Sino-Himalayan Forest subkingdom [2,3]. It has long been thought that the formation of these spatiotemporal patterns of biodiversity correlates with physical environmental changes resulting from the Himalaya-Tibet Plateau uplifting [4,5]. However, the nature and extent of this correlation remains unexplained. Intensive biological observation of widespread species at the population level may provide insights into the biogeography of this region. As part of our broader research on the systematic and evolutionary history of *Chimaphila*, in this study we isolate and characterize fast-evolving codominant microsatellite markers to gain a better understanding of the relationships among populations of *C. japonica*, and the amount and geographic distribution of its genetic diversity.

2. Results and Discussion

A total of 260 positive clones were captured: among these 158 clones (61%) were found to contain simple sequence repeats (SSR). Of the 125 primer sets, 20 microsatellite loci successfully amplified in *C. japonica*; 16 of them were found to be polymorphic and the remaining four were monomorphic (Table 1).

Locus	Primer sequence (5'–3')	Repeat motif	Size range	T _a	GenBank
			(bp)	(U)	Accession No.
Chim004 *	F: CGTCACTTCTAACTCAAATCC	(AC) ₃₁	249–297	50	JQ392547
	R: TCATTGGGTGTTGTTGTGT				
Chim009 *	F: CGCTTCTCTTCACAGCTATT	(AC) ₂₄	240-272	52	JQ392548
	R: ACATCCCTTAGTTTACCTCAG				
Chim011 *	F: CCATCCATTTCCTCAATCAT	$(AG)_6CT (AG)_6$	157–191	50	JQ392549
	R: TTAGGCTTACCACCAAGAAC				
Chim012 *	F: TTTACTTTCTCTTCTTCCCC	(AG) ₁₉	176–192	40	JQ392550
	R: TTTTTGCTTGCTTTACATCT				
Chim025 *	F: TATTTCACATAGACGAGGGT	(AG) ₁₈	133–151	52	JQ392551
	R: GACGAACACAGTAACGGAC				
Chim029 *	F: GTCAGGAAACAAGCGAGGAA	(AC) ₂₃	291-357	52	JQ392553
	R: CATCAGTTGAGAAATGGGTA				
Chim035 *	F: TCCAGGAGTGTGAGACATAG	(AC) ₂₄	148–160	50	JQ392554
	R: TACCGAAGGCACGATAAACA				
Chim037 *	F: TTCTCAAAAGCCAATCACAC	(AC) ₉	125–141	46	JQ392555
	R: CCTCATTCCATCCGAAGTCC				

Table 1. Characteristics of 20 microsatellite loci developed in Chimaphila japonica.

Chim054 *	F: TGAGGAGACGAACACAACAA	(AC) ₈	195–213	52	JQ392556
	R: CATCCATCCAAAGGCTCTAC				
Chim064 *	F: CGCACGCATTTGTATTTA	$(AG)_{10}(TG)_{12}$	127–155	48	JQ392557
	R: GGTGTATGGAACATCACGCA				
Chim069 *	F: TGCTCTAACAGATATGCGTG	(AC) ₁₀	76–86	48	JQ392558
	R: CGTGTATGTAGAATGGGAG				
Chim092 *	F: ATGAGAAGAAGTCTGGGAGAG	(TG) ₉ (AG) ₁₀	87–101	50	JQ392560
	R: CATAGTGAACCACAAAGCC				
Chim107 *	F: ACCATCTCAAACTTCACTAAG	(AC) ₈	237-249	52	JQ432377
	R: CCATTCTCTCCCTCATTACC				
Chim110 *	F: GCTTTATTTTGTCTTAGGGT	(AG) ₁₅	197–205	48	JQ432378
	R: AAACTTGAACTTGAATGGAA				
Chim119 *	F: CAGGATGCTCTTTGAATTAG	$(AG)_{23}(AC)_{11}$	143–171	50	JQ432379
	R: AAGGTATGAGAAGCGAAGGG				
Chim122 *	F: GACCAAGGGTGGACCAAGAG	(AG) ₁₅	107-125	48	JQ432380
	R: AAACAACTTACGCATAGAAA				
Chim27	F: TTTCTCATCTCTCCCTACTG	(AG) ₄ TT(AG)-	100	48	JQ392552
	R: GAGTTTATTCCTTATGCCTT	(AC) ₅			
Chim80	F: GGCATACAACATCTAACGAG	(AG) ₁₇	254	50	JQ392559
	R: TCTCTCAAGGTTTTGCTTC				
Chim97	F: TGAGATGCCCATCCTCTCG	$(AC)_8$	262	52	JQ392561
	R: CGAACAAATCACACAAATTCC				
Chim99	F: ATCCCAGATATACGGCACAC	(AG) ₁₂	221	52	JQ392562
	R: GAACACCAAAAGGCTATGTA				

 Table 1. Cont.

* Displayed polymorphisms in *Chimaphila japonica*; *T*a, PCR annealing temperature.

In the Kangding population, the number of alleles per locus ranged from one to six, with an average of 2.9, and the expected and observed heterozygosities ranged from 0.000 to 0.848 and from 0.000 to 1.000, respectively. In the Kunming population, the number of alleles per locus varied between one and six, with an average of 2.6, and the expected and observed heterozygosities ranged from 0.000 to 0.775 and from 0.000 to 1.000, respectively (Table 2).

Table 2. Locus-specific measures of genetic diversity of two populations of Chimaphila japonica.

	Population KD $(N = 12)$			Population KM $(N = 12)$		
Locus	$N_{\scriptscriptstyle m A}$	$H_{\scriptscriptstyle m E}$	$H_{ m o}$	$N_{\scriptscriptstyle m A}$	$H_{\scriptscriptstyle m E}$	$H_{ m o}$
Chim004	5	0.688	1.000	4	0.775	1.000
Chim009	3	0.565	0.000	4	0.670	0.083
Chim011	3	0.649	0.083	2	0.083	0.083
Chim012	4	0.576	0.333	6	0.775	0.250
Chim025	4	0.703	0.333	4	0.544	0.333
Chim029	3	0.594	0.000	3	0.652	0.000
Chim035	2	0.228	0.250	1	0.000	0.000
Chim037	2	0.228	0.083	1	0.000	0.000
Chim054	3	0.638	1.000	1	0.000	0.000

Chim064	3	0.565	0.167	3	0.489	0.583
Chim069	2	0.159	0.000	2	0.507	0.000
Chim092	2	0.083	0.083	2	0.464	0.667
Chim107	2	0.228	0.250	1	0.000	0.000
Chim110	1	0.000	0.000	2	0.159	0.167
Chim119	6	0.848	0.250	4	0.736	0.417
Chim122	2	0.083	0.083	2	0.083	0.083

Table 2. Cont.

N = population sample size; $N_A =$ number of alleles revealed; H_E : expected heterozygosity; H_0 : observed heterozygosity; Population KD (Kangding, Sichuan: 30 09'N, 102 09'E, 2857 m a.s.l.); Population KM (Kunming, Yunnan: 25 °11'N, 102 °44'E, 2345 m a.s.l.).

An excess of homozygotes was simultaneously observed for seven loci (Chim009, Chim011, Chim029, Chim037, Chim069, Chim110 and Chim122) in both populations (Table 2), which were possibly caused by the limited sample size. And the possibility of the occurrence of some null alleles at these loci could not be completely ruled out. Compared with the one monomorphic locus (Chim110) found in the Kangding population, four loci (Chim035, Chim037, Chim054 and Chim107) were observed in the Kunming population (Table 2). This difference is possibly caused by the areas' different population histories. The Qinghai-Tibetan Plateau distributed Kangding population may have been extensively influenced by the uplift of the Himalayas and the Tibetan Plateau. However, the Yungui Plateau-distributed Kunming population may have experienced simpler geological events, and notably, it is the most southern distribution as far as we know, having a relatively short evolutionary history.

3. Experimental Section

Genomic DNA samples of C. japonica were extracted from silica-gel-dried leaves of two different individuals using a modified hexadecyltrimethylammonium bromide (CTAB) method [6]. The extracted DNA was dissolved in ddH₂O. The fast isolation by AFLP of Sequences Containing repeats (FIASCO) was performed in this study [7]. Total genomic DNA (approximate 250 ng) was completely digested with 2.5 U of MseI restriction enzyme (New England Biolabs, Beverly, MA, USA), and then ligated to an MseI AFLP adaptor (5'-TAC TCA GGA CTC AT-3'/5'-GAC GAT GAG TCC TGA G-3') using T4 DNA ligase (Fermentas, Burlington, ON, Canada). The digested-ligated fragments were diluted in a ratio of 1:10, and 5 µL were used amplification reaction with MseI-N primer (5'-GAT GAG TCC TGA GTA AN-3'). The amplified DNA fragments (200-800 bp) were enriched by magnetic bead selection with 5-biotinylated (AG)₁₅ and (AC)₁₅ probes, respectively. The recovered DNA fragments were reamplified with MseI-N primers. The PCR products were purified using the EZNA Gel Extraction Kit (Omega Bio-Tek, Guangzhou, China), and were ligated into a pGM-T vector (Tiangen, Beijing, China), and then transformed into E. coli strain DH5a competent cells (TaKaRa, Dalian, Liaoning, China). The positive clones were picked out and tested using vector primers T7/SP6 and primer $(AC)_{10}/(AG)_{10}$ respectively to select appropriate fragments which contained SSRs. In other words, a set of tested PCRs included three reactions was performed using T7 and SP6, T7 and (AC)₁₀, (AC)₁₀ and SP6 as primers, respectively. The second set of tested PCRs was tested using T7 and SP6,

T7 and $(AG)_{10}$, $(AG)_{10}$ and SP6 as primers, respectively. All of these PCR reactions had the same conditions: 94 °C for 4 min followed by 35 cycles at 94 °C for 45 s, 52 °C for 45 s, 72 °C for 1 min, and a final extension step at 72 °C for 3 min. The selected positive clones were sequenced using an ABI PRISM 3730XL DNA sequencer (Applied Biosystems, Foster City, CA, USA). Sequences containing simple sequence repeats and enough flanking regions were selected for primer design using Primer Premier 5.0 [8].

The designed Primer pairs were assessed in 24 individuals of *C. japonica* pooled from two natural populations (KD, Kangding, Sichuan: 30 09'N, 102 09'E, 2857 m a.s.l. and KM, Kunming, Yunnan: 25 °11'N, 102 °44'E, 2345 m a.s.l.). Herbarium vouchers were deposited in the Kunming Institute of Botany, Chinese Academy of Science (code LZW 0128-0151). The PCR reactions were performed in 20 μ L of reaction volume containing 30–50 ng genomic DNA, 0.35 μ M of each primer, 10 μ L 2× Taq PCR MasterMix (Tiangen; 0.1 U Taq Polymerase/ μ L, 0.5 mM dNTP each, 20 mM Tris-HCl (pH 8.3), 100 mM KCl and 3 mM MgCl₂). PCR amplifications were conducted under the following conditions: 94 °C for 3 min followed by 32 cycles at 94 °C for 30 s, the annealing temperature for each specific primer (optimized for each locus, Table 1) for 30 s and 72 °C for 45 s with a final extension step at 72 °C for 3 min. The amplification products were separated and visualized using a QIAxcel capillary gel electrophoresis system (QIAGEN, Irvine, CA, USA).

The number of alleles per locus, observed heterozygosity (H_0), and expected heterozygosity (H_E) were estimated in POPGENE version 1.31 [9].

4. Conclusions

All 16 microsatellite loci described here showed a useful degree of polymorphism at the population level and will be useful to reveal the history of *C. japonica* in East Asia, population dynamics, and genetic structure.

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