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Development and Characterization of Microsatellite Markers for Three Pollination Morphs of *Cimicifuga simplex* (Ranunculaceae)

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Abstract

Cimicifuga simplex Wormsk. (Ranunculaceae) is a perennial herb distributed in eastern and northeastern Asia for which at least three different pollination morphs have been reported. It is classified as endangered or near threatened in some Japanese regions, and its rhizome is commercially used as a crude drug. To examine genetic differentiation and gene flow among the three morphs, we developed eight microsatellite markers by using next-generation sequencing and estimated the genetic structure of *C. simplex*. We tested eight primer pairs on 93 individuals from six populations of *C. simplex* in Nagano, central Japan, and found that heterozygosity in morphs I and III was low compared to expected heterozygosity. Bayesian clustering performed with the STRUCTURE program clearly distinguished the three morphs of *C. simplex*, and only a little gene flow was detected among the morphs. These eight microsatellite markers are expected to be useful in conservation genetic studies of this species and for future conservation planning.

Keywords

Cimicifuga simplex, Genetic Structure, Microsatellite Marker, Next-Generation Sequencing, Pollination Morph

1. Introduction

Cimicifuga simplex Wormsk. (Ranunculaceae) is a perennial herb distributed in eastern and northeastern Asia [1] [2] that is classified as endangered or near threatened in three of the 47 Prefecture Red Data Book lists of Japan [3]. Its rhi-

zome is commercially used as a crude drug [4]. Accordingly, for the conservation and sustainable pharmacological use of this species, it is important to evaluate its genetic diversity and genetic structure.

C. simplex comprises at least three different pollination morphs [5] (henceforth morphs I, II, and III). Morphs I and III are pollinated mainly by bumblebees, and morph II is pollinated mainly by butterflies [5]. The three morphs differ in their altitudinal distribution, habitats, flowering season, and nuclear internal transcribed spacer gene sequences [6], although the actual state of gene flow among the different morphs is unknown.

Therefore, we used next-generation sequencing to develop eight nuclear microsatellite markers for *C. simplex* and estimated its genetic structure for a preliminary assessment of genetic differentiation and gene flow among the three morphs.

2. Materials and Methods

2.1. DNA Extraction and Library Construction

Each sample was a fresh leaf collected from an individual *C. simplex* plant growing in Nagano Prefecture, Japan. Genomic DNA was extracted from the leaf with the DNeasy Plant Mini Kit (QIAGEN, Germantown, Maryland, USA), and the extracted DNA was used for library preparation. The library was constructed from morph II individual. A 5' and 3' adapter was ligated for random fragmentation DNA. The adapter-ligated fragments were then amplified by polymerase chain reaction (PCR) and gel purified. Each fragment was amplified into distinct clonal clusters by bridge amplification.

2.2. DNA Sequencing and Microsatellite Isolation

We performed DNA sequencing on a Miseq sequencer (Illumina, San Diego, CA) using approximately 10% of a plate. The filtered reads were assembled de novo into contigs by using Newbler software (Roche, Basel, Switzerland). The QDD version 3.1 [7] bioinformatics pipeline was used to identify contigs possessing microsatellite motifs as well as to design primer pairs. We searched for primers with the following specifications: melting temperature (T_m) of 57˚C - 63˚C, PCR product between 100 and 300 bp long, and primer length between 18 and 27 nucleotides. We identified 96 candidate primer pairs meeting these specifications and tested them using DNA samples from eight *C. simplex* individuals. For all tested microsatellite loci, the forward primers were synthesized with one of four different universal fluorescent sequences added,
5'-GCCTCCCTCGCGCCA-3', 5'-GCCTTGCCAGCCCGC-3',
5'-CAGGACCAGGCTACCGTG-3', or 5'-CGGAGAGCCGAGAGGTG-3' [8], and a 5'-GTTTCTT-3' PIG-tail [9] was added to all reverse primers.

2.3. Microsatellite Genotyping

The PCR amplification was performed in a 6 μL reaction volume containing 10

ng template DNA, 3.0 μL 2 × Type-it Multiplex PCR Master Mix (QIAGEN, Valencia, California, USA), 0.1 μM forward primer, 0.2 μM reverse primer, and 0.1 μM fluorescent labeled universal primer. In the multiplex PCR, the final concentration of each forward primer and fluorescent-labeled universal primer was 0.1 μM, and the final concentration of each reverse primer was 0.2 μM. The PCR thermal profile was as follows: initial denaturation at 95˚C for 5 min; followed by 35 cycles of 95˚C for 30 s, 60˚C for 1.5 min, and 72˚C for 30 s; and a final extension at 60˚C for 30 min. The PCR products were detected by using a ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA). Fragment lengths were calculated by using the GeneMapper version 4.0 program (Applied Biosystems).

2.4. Genetic Analysis

We used the eight primer pairs identified by the screening to analyze 93 individuals from six populations of *C. simplex* in Nagano Prefecture, central Japan (morph I, Norikura_1 36˚12'29"N 137˚58'48"E, Norikura_2 36˚12'19"N 137˚57'98"E; morph II, Sanjiro 36˚21'24"N 138˚08'78"E, Hinokizawa 36˚20'35"N 138˚09'18"E; morph III, Hora 36˚28'03"N 137˚98'47"E, Misuzu 36˚26'32"N 138˚01'26"E). Population genetic analysis and tests for deviation from Hardy-Weinberg equilibrium (HWE) were performed with FSTAT version 2.9.3 software [10]. Genetic structure was analyzed after Bayesian clustering with STRUCTURE 2.1.3 software by assuming an admixture model and correlated allele frequencies [11] [12]. The simulated K value ranged from 1 to 10. Ten runs were conducted for each K value, and ΔK was calculated from the mean log likelihood LnP (D) value of each K. The simulations conducted with 1,000,000 burn-in iterations and 100,000 Markov chain Monte Carlo repetitions after burn-in.

3. Results and Discussion

A total of 3,922,364 reads identified as microsatellite loci were generated in a run of the Miseq genetic sequencer. The QDD pipeline analysis detected 4492 putative microsatellite loci. Among those, 96 primer pairs were tested. A total of seven primer pairs amplified microsatellite loci in all 93 individuals. In some populations, marker of cisi 8 was not amplified (**Table 1**).

The number of alleles per locus in morph I ranged from 1 to 7 (population means, 2.6 and 3.0), in morph II from 1 to 9 (population mean, 3.4 and 4.3), and in morph III from 1 to 10 (population means, 3.7 and 4.9) (**Table 2**). In morph I populations, the average observed heterozygosity (H_o) was 0.05 and 0.15 and the average expected heterozygosity (H_e) was 0.28 and 0.33. In morph II populations, average H_o was 0.34 and 0.36 and average H_e was 0.43. In morph III populations, average H_o was 0.11 and 0.19 and average He was 0.36 and 0.49 (**Table 2**). The average H_e and H_o values thus indicated significant deviation from HWE ($p < 0.01$) in the morph I and III populations.

Table 1. Characteristics of eight microsatellite loci in *Cimicifuga simplex*[†].

Locus		Primer sequences (5'-3')	Repeat motif	Fluorescent label[‡]	Size range (bp)	GenBank accession no.
Multiplex 1						
Cisi1	F:	TTCCTATGAATCCAACGAGCA	AAT	FAM	316 - 339	LC279064
	R:	GCGTTCGATTCAATATCCATATGGG				
Cisi2	F:	CGCGTTCGTTGTCTTGACAT	AG	VIC	147 - 209	LC279065
	R:	GCGCACCCAATGATACAACG				
Cisi3	F:	GTTCCTAGGACAGGGCATCA	AT	NED	100 - 201	LC279066
	R:	GGGACAAGCTCTAGGTATACCC				
Cisi4	F:	ATCACAACCCAATCCCGTCC	AG	PET	103 - 153	LC279067
	R:	AGGTTTCTTCGACAGTTGCT				
Multiplex 2						
Cisi5	F:	TCCAATGAATCATACATGAAATGAGT	AAT	FAM	248 - 300	LC279068
	R:	GTGACAGGTGTGGTCCGAAA				
Cisi6	F:	AGCAATTAGTAGTCAGCCGGG	AAG	VIC	202 - 211	LC279069
	R:	GACCTCCAGACCATTCAGCA				
Cisi7	F:	TTCGATGCCAACCTGCTACA	AC	NED	106 - 233	LC279070
	R:	ATCCATTATCCAAGCCTCAGG				
Cisi8	F:	CCTGTAACATGGCTCCCATT	AT	PET	246 - 377	LC279071
	R:	GCAGGTACCACGGAGGATTTA				

[†]Annealing temperature was 60˚C for all loci. [‡]Sequence of fluorescent labels: FAM = 5'-GCCTCCCTCGCGCCA-3', VIC = 5'-GCCTTGCCAGCCCGC-3', NED = 5'-CAGGACCAGGCTACCGTG-3', PET = 5'-CGGAGAGCCGAGAGGTG-3'.

Bayesian clustering with STRUCTURE identified three *C. simplex* populations (**Figure 1**) consistent with the three recognized pollination morphs. This result indicates that the eight microsatellite markers are valid for identifying the three morphs of *C. simplex*. Further, a little gene flow from morph I to III was detected.

The eight microsatellite markers described in this study were successfully used to detect genetic variation within populations and genetically distinguish the three pollination morphs. Thus, they will be useful in conservation genetic studies of *C. simplex*. In addition, the finding that pollination morphs I and III have relatively low genetic diversity is useful information for future conservation planning for *C. simplex*.

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Table 2. Results of primer screening for eight microsatellite loci in six population of *Cimicifuga simplex*.

	morph I					
	Norikura_1 (n = 12)			Norikura_1 (n = 12)		
Locus	*A*	*He*	*Ho*	*A*	*He*	*Ho*
Cisi 1	1	0.000	0.000	1	0.000	0.000
Cisi 2	2	0.083	0.083	2	0.030	0.030
Cisi 3	7	0.879	0.286*	4	0.857	0.000*
Cisi 4	7	0.824	0.667	3	0.190	0.000*
Cisi 5	1	0.000	0.000	1	0.000	0.000
Cisi 6	1	0.000	0.000	1	0.000	0.000
Cisi 7	2	0.533	0.000*	6	0.879	0.286*
Cisi 8	-	-	-	-	-	-
Average	3.0	0.33	0.15*	4.3	0.43	0.34*
	morph II					
	Norikura_1 (n = 12)			Norikura_1 (n = 12)		
Locus	*A*	*He*	*Ho*	*A*	*He*	*Ho*
Cisi 1	1	0.000	0.000	1	0.000	0.000
Cisi 2	2	0.091	0.091	3	0.342	0.375
Cisi 3	5	0.407	0.455	6	0.683	0.875
Cisi 4	3	0.621	0.333	2	0.527	0.000*
Cisi 5	4	0.333	0.273	1	0.000	0.000
Cisi 6	2	0.337	0.200	2	0.458	0.375
Cisi 7	9	0.856	0.778	7	0.835	0.857
Cisi 8	8	0.825	0.625	5	0.593	0.429
Average	4.3	0.43	0.34	3.4	0.43	0.36
	morph III					
	Norikura_1 (n = 12)			Norikura_1 (n = 12)		
Locus	*A*	*He*	*Ho*	*A*	*He*	*Ho*
Cisi 1	1	0.000	0.000	1	0.000	0.000
Cisi 2	1	0.000	0.000	2	0.067	0.067
Cisi 3	6	0.711	0.800	5	0.660	0.222*
Cisi 4	8	0.895	0.111*	8	0.909	0.111*
Cisi 5	3	0.329	0.000*	3	0.543	0.000*
Cisi 6	1	0.000	0.000	1	0.000	0.000
Cisi 7	6	0.579	0.400	10	0.844	0.250*
Cisi 8	-	-	-	9	0.855	0.250*
Average	3.7	0.36	0.19*	4.9	0.48	0.11*

Note: *A* = number of alleles; *He* = expected heterozygosity; *Ho* = observed heterozygosity; n = number of samples screened. *Significant deviation from Hardy-Weinberg equilibrium ($p < 0.01$).

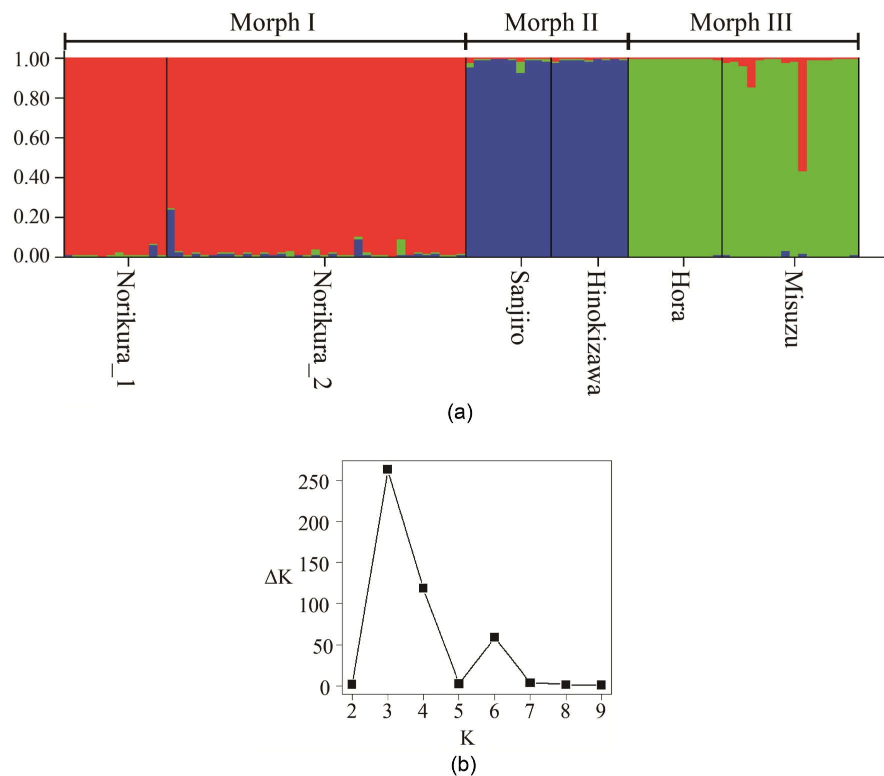

Figure 1. (a) STRUCTURE analysis results for eight microsatellite loci in six *C. simplex* populations. Each individual is represented by a vertical bar consisting of three colored segments representing the estimated fraction of the genome belonging to each cluster (b) Delta *K* plot showing a peak at $K = 3$ (best *K*).

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