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Isolation and characterization of microsatellite markers for *Hydrangea luteovenosa* (Hydrangeaceae), an endangered species in Korea

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한국 제주도에 자생하는 멸종위기종 성널수국(수국과)의 microsatellite 분자마커 개발

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ABSTRACT: *Hydrangea luteovenosa* is a critically endangered plant species of Jeju Island in Korea, though it is widely distributed in western Japan. We isolated and characterized five microsatellite loci in this species. The number of alleles ranged from 3 to 27, observed heterozygosity from 0.27 to 0.86, and expected heterozygosity from 0.34 to 0.91. The markers described here will be useful for investigating the genetic diversity, genetic structure, and gene flow of *H. luteovenosa*, and the genetic findings would contribute to the establishment of effective conservation measures for this species in Korea.

Keywords: Conservation, Endangered species, *Hydrangea luteovenosa*, Korea, Microsatellite

적 요: 성널수국은 일본 관서지방에 널리 분포하고 있지만, 한반도에서는 제주도에서만 발견되고 있는 멸종위기 식물이다. 본 연구에서는 성널수국에서 총 5개의 microsatellite 유전자좌를 형질화하여 마커를 개발하였다. 유전자좌당 대립유전자수의 범위는 3-27개, 이형접합률의 관측치와 기대치는 각각 0.27-0.86과 0.34-0.91로 나타났다. 본 연구에서 개발된 microsatellite 마커는 성널수국의 유전적 다양성 및 구조, 그리고 유전자 이동을 밝히는데 사용될 수 있을 것이며, 그 결과들은 한국산 성널수국에 대한 효과적인 보전 전략을 제시하는데 일조할 것으로 기대된다.

주요어: 보전, 멸종위기종, 성널수국, 한국, Microsatellite 마커

Hydrangea luteovenosa Koidz. (Hydrangeaceae Dumort.) is a deciduous shrub that grows on the forest floor of temperate forest in Jeju Island of Korea and western Japan (Kim, 2009). Although *H. luteovenosa* is common in Japan, it should be listed as a critically endangered plant species in Korea at regional level based on the criteria of IUCN (Kim, 2009) with a noticeably small size population. Only one population has been known in Seongneol-oreum region of Mt. Hallasan in Jeju

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Island (Moon et al., 2004; C. S. Kim, pers. comm.). In conservation of endangered species with limited distribution like *H. luteovenosa* in Korea, it is important to understand the ecological status such as its breeding system and clonal distribution (Izuno et al., 2012). At the same time, genetic information is also crucial for designing its management strategy because small sized population are likely to be vulnerable to the impact of genetic factors in extinction such as inbreeding depression and loss of genetic diversity (Frankham, 1995; Schwartz et al., 2007; Izuno et al., 2012). Furthermore, microsatellite markers will provide effective genetic information with its high variability (Guichoux et al., 2011; Yun et al., 2011). Therefore, we developed five microsatellite markers in order to understand current genetic status of critically endangered Korean *H. luteovenosa* comparing with Japanese populations.

Materials and Methods

We obtained samples from four populations of *H. luteovenosa* in Korea (Jeju Island) and Japan (Hyogo, Kagoshima, and Yamaguchi prefectures). Microsatellite markers were developed using the technique for isolating codominant compound microsatellite markers of Lian and Hogetsu (2002) and Lian et al. (2006). An adaptor-ligated, restricted DNA library for *H. luteovenosa* was generated according to the following procedure: genomic DNA was extracted from silica-dried leaves using a modified CTAB method (Milligan, 1992) and digested with the blunt-end restriction enzyme *Eco*RV. The restriction fragments were then ligated with a specific blunt adaptor (consisting of the 48-mer: 5'-GTAATACGACTCACT ATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3' and an 8-mer with the 3'-end capped with an amino residue: 5'-ACCAGCCC-NH_2-3') using the Takara DNA ligation kit (Takara). Fragments were amplified by polymerase chain reaction (PCR) from the *Eco*RV DNA library using compound SSR primer $(AC)_6(TC)_7$ and an adaptor primer (5'-CTATAGG GCACGCGTGGT-3'). The amplified fragments, ranging in size from 400 to 800 bp, were then separated on a 1.5% LO3 agarose gel (Takara) and purified using a QIAquick Gel Extraction Kit (Qiagen). The purified DNA fragments were cloned using the QIAGEN PCR Cloning plus Kit (Qiagen) following the manufacturer's instructions. The cloned fragments were amplified using the M13 forward and reverse primers from the plasmid DNA. Amplified fragments were sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). For each fragment containing a compound SSR sequence at one end, a specific primer was designed from the sequence flanking the compound SSR using Primer3 (v. 0.4.0, Rozen and Skaletsky, 2000) (National Human Genome Research Institute). PCR amplifications were performed following the standard protocol of the Qiagen Multiplex PCR Kit (Qiagen) in a final volume of 5 μL, which contained 5 ng of extracted DNA, 5 μL of 2 × Multiplex PCR Master Mix, and 0.2 μM of each primer. Compound SSR primers $(AC)_6(TC)_7$ were labeled with fluorochromes FAM or PET (Applied Biosystems), respectively. PCR amplifications were performed with the GeneAmp PCR System 2700 thermal cycler (Applied Biosystems) using the following conditions: initial denaturation at 95°C for 15 min, followed by 30 cycle of denaturation at 94°C for 30 s, annealing at 57°C for 1 min 30 s, extension at 72°C for 1 min, and extension at 60°C for 30 min. The size of the PCR products was measured using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and GENOTYPER analysis software (Applied Biosystems).

Results and Discussion

In total, 170 cloned DNA fragments were screened in a *H.*

Table 1. Characteristics of the five compound microsatellite loci for *Hydrangea luteovenosa*.

Locus	Repeat motif	Primer sequences (5'-3')	T_a (°C)	Size range (bp)	*A*	Accession no.
Hlut157	$(AC)_6(TC)_7$	ACACACACACACTCTCTCTCTCTCTC ATTGACTACGGCGAAGGTGT	57	195-243	25	AB775654
Hlut170	$(AC)_6(TC)_7$	ACACACACACACTCTCTCTCTCTCTC CAGAGTAATTATTGGGGCACAAC	57	135-173	15	AB775655
Hlut214	$(AC)_6(TC)_7$	ACACACACACACTCTCTCTCTCTCTC ATGATCTGTTTTGTGGTGCTTG	57	137-163	14	AB775656
Hlut226	$(AC)_6(TC)_7$	ACACACACACACTCTCTCTCTCTCTC TGATTCGATGATCTGTGTTTGA	57	164-174	3	AB775657
Hlut234	$(AC)_6(TC)_{15}$	ACACACACACACTCTCTCTCTCTCTC ACCCATCATCATCGCTAATC	57	197-254	27	AB775658
Average					17.3	

T_a = annealing temperature of primer pair, *A* = total number of allele for each locus.

Table 2. Variability of five microsatellite loci in four population of *Hydrangea luteovenosa* in Korea and Japan. X indicates that the locus was not amplified, a significant deviation from Hardy-Weinberg equilibrium expectations is indicated by * ($P < 0.05$), and † indicates that deviation from HWE cannot be checked because only two genotypes was observed.

	Jeju (Korea) (n = 30)			Hyogo (Japan) (n = 31)			Kagoshima (Japan) (n = 45)			Yamaguchi (Japan) (n = 25)		
	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E	A	H_O	H_E
Hlut157	2	1.00†	0.50	13	0.81	0.86	18	0.84	0.92	15	0.81	0.83
Hlut170	1	0.00†	0.00	X	-	-	X	-	-	X	-	-
Hlut214	2	1.00†	0.50	9	0.45*	0.8	6	0.47*	0.52	2	0.08*	0.18
Hlut226	1	0.00†	0.00	3	0.45	0.43	2	0.44	0.47	2	0.04	0.04
Hlut234	2	0.93†	0.50	15	0.48*	0.9	21	0.71	0.76	11	0.73	0.83

A = total number of allele for each locus, H_O = observed heterozygosity, H_E = expected heterozygosity, n = number of individuals.

luteovenosa genomic library. A total of 63 positive clones were sequenced, and primers were designed for 23 repeats. From the 23 primer pairs tested five microsatellite loci were identified that showed a clear, strong single band for each allele (Table 1). The polymorphism was evaluated for 143 individuals from four populations of *H. luteovenosa* in Korea (Jeju Island) and Japan (Hyogo, Kagoshima, and Yamaguchi prefectures) (Table 2). The number of alleles per locus ranged from 3 to 27 with an average of 17.3 (Table 1). The observed and expected heterozygosities (H_O and H_E) ranged from 0.27 to 0.86 and from 0.34 to 0.91 with an average of 0.49 and 0.71, respectively. Linkage disequilibrium between loci and deviation from Hardy–Weinberg equilibrium (HWE) were tested with FSTAT (version 2.9.3; Goudet, 1995). Significance levels were adjusted using Bonferroni correction for multiple testing. There was no evidence of significant linkage disequilibrium ($P < 0.05$) for all pair of loci. However, we cannot check the deviation from HWE in Jeju population, because only two multilocus genotypes were observed through the whole data set (Table 2). Though locus *Hlut170* was amplified in Jeju population, it was not amplified in most of the tested Japanese individuals, indicating the high frequency of null allele in Japanese populations (Table 2). In *Hlut214* and *Hlut234*, significant deviation ($P < 0.05$) from HWE was observed in all the Japanese populations and in the Hygo population only, respectively, indicating the existence of null allele (Table 2).

In conclusion, all the microsatellite markers described here are expected to be useful in understanding the current genetic status of the only known Korean *H. luteovenosa* population in Jeju Island. The genetic results from the developed markers will therefore be helpful to develop effective conservation strategies for this species in Korea. In addition, three microsatellite markers of *Hlut157*, *Hlut226*, and *Hlut234* are also practicable for investigating the genetic diversity, genetic structure, and gene flow among populations of *H. luteovenosa* in Korea and Japan. Although the other two markers (*Hlut170* and *Hlut214*) are not useful in population genetic studies because of the existence of null alleles, it is expected to be useful in individual identification and in understanding the clonal structure of this species in Korea.

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