Isolation and Characterization of Microsatellite Loci for the Endangered Wetland Plant Adenophorapalustris (Campanulaceae)

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PRIMER NOTE

ISOLATION AND CHARACTERIZATION OF MICROSATELLITE LOCI FOR THE ENDANGERED WETLAND PLANT *ADENOPHORA PALUSTRIS* (CAMPANULACEAE)[1]

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- *Premise of the study: Adenophora palustris* (Campanulaceae) is an endangered wetland plant species in Japan. Although it is widely distributed in East Asia, only six extant populations are known in Japan, with fewer than 1000 individuals in total. We developed 15 microsatellite markers for this species and confirmed their utility for the closely related species *A. triphylla* var. *japonica*.
- *Methods and Results:* Ten polymorphic loci were characterized for genetic variation within three populations of *A. palustris*. The number of alleles per locus ranged from four to 15, with an average of 9.3; the expected heterozygosity ranged from 0.48 to 0.89, with an average of 0.74. Nine loci were successfully amplified in *A. triphylla* var. *japonica*, and three of these loci showed polymorphism.
- *Conclusions:* These markers are useful for investigating genetic diversity and gene flow within and among remnant populations of *A. palustris* in Japan, and the results will provide crucial information for conservation.

Key words: *Adenophora palustris*; Campanulaceae; endangered plant species; microsatellite; molecular marker.

Adenophora palustris Kom. (Campanulaceae) is a perennial herb that inhabits small wetland areas in hilly regions. *Adenophora palustris* is widely distributed in East Asia, including eastern China, Korea, and Japan (Miyajima Natural Botanical Garden of Hiroshima University and Hiba Society of Natural History, 1997; Hong et al., 2011). However, only six extant populations are known in Japan—five in western Japan and one in central Japan. Although approximately 800 individuals grow in the largest population, which is located in the Sera region of western Japan, only a few to a few dozen individuals grow in the other populations. Consequently, fewer than 1000 individuals are estimated to remain in Japan, and *A. palustris* is currently classified as "critically endangered" in the Japanese Red Data Book (Ministry of the Environment Japan, 2015). Therefore, understanding its genetic diversity will play a key role in the future management of the species.

Masumoto et al. (2011) developed eight microsatellite markers for *A. palustris*. Although four of the eight loci showed moderate polymorphism (5–8 alleles among 31 individuals), the other loci had limited polymorphism (2–4 alleles). Therefore, we have newly developed highly polymorphic genomic microsatellite markers to investigate the genetic diversity and gene flow within remnant populations of *A. palustris*. This paper reports 15 markers developed using next-generation sequencing technology.

METHODS AND RESULTS

A fresh leaf sample was taken from a ramet growing in a native population in the Sera region, Hiroshima Prefecture, Japan (Appendix 1). Genomic DNA was extracted using the hexadecyltrimethylammonium bromide mini-prep procedure (Stewart and Via, 1993) and used for library preparation with a TruSeq Nano DNA Sample Prep Kit (Illumina, San Diego, California, USA). The amplified DNA fragments were sequenced using a MiSeq Benchtop Sequencer (Illumina) in 2 × 300-bp read mode, and the data were assembled into contigs in fastq-join software (Aronesty, 2011), resulting in a total of 1,738,214 contigs. Contigs were screened using Primer3 version 2.2.3 software (Rozen and Skaletsky, 1999) embedded in MSATCOMMANDER version 1.0.8 software (Faircloth, 2008) to identify dinucleotide motifs with a minimum of 15 repeats. Consequently, a total of 5049 primer pairs were designed. In the amplification trial using four ramets of *A. palustris*, 15 of 48 randomly selected primer pairs showed a clear, strong, single band for each allele.

To test the genetic variation of the 15 selected microsatellite markers, we sampled three *A. palustris* populations in the Sera and Toyosaka regions, Hiroshima Prefecture (western Japan), and the Ena region, Gifu Prefecture (central Japan; Appendix 1). To prevent illegal collection, we have withheld the exact locations of the sites. The sample size ranged from eight to 12 individuals per population, with a total sample size of 32. To confirm the utility of the 15 selected microsatellite markers for a close relative, one population of *A. triphylla* (Thunb.) A. DC. var. *japonica* H. Hara (n = 8) was also sampled (34°30′31″N, 132°44′06″E; Appendix 1).

PCR amplifications were performed in a final volume of 10 µL, which contained 5 ng of extracted DNA, 0.2 µL of KOD FX Neo polymerase (Toyobo,

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TABLE 1. Characteristics of the 15 microsatellite markers of *Adenophora palustris*.

Locus	Primer sequences (5′–3′)	Repeat motif	Fluorescent label	Allele size range (bp)	GenBank accession no.
AP01585	F: [F9AGG] TGCAAGTACGAGTCGGTCC R: GTCACCAACGGGCATTCTAG	$(AT)_{16}$	VIC	386	LC127427
AP02665	F: [F9CCG] GGGTGTCAACTTCATGTCAGG R: CGGTCTGCTAACTGGAGAGA	$(AG)_{25}$	FAM	305–353	LC122486
AP04413	F: [F9GCC] ACGGGAGGGTAGATAGGTCC R: GCCAAACATTCTTCCCAAGGT	$(AC)_{18}$	PET	280	LC127428
AP04430	F: [F9GTC] GGAACACTGCTCCTTGACTC R: GTAACACCATTGCTGCCTCC	$(GA)_{17}$	VIC	158–206	LC122487
AP07350	F: [F9CCG] TTTGAGATCACCCGAAACGC R: AAGGGCCGCAAATTGGATTG	$(GT)_{19}$	FAM	335–355	LC122488
AP07489	F: [F9AGG] GCTCAAACACCAAATTCAAC R: ACCTTCCAACCCACTAATCCC	$(AG)_{20}$	VIC	355–385	LC122489
AP08053	F: [F9TAC] AAACGGACGGAGGGAGTAG R: TGCGTTATTGGAGGAGGGTG	$(AG)_{21}$	NED	255–275	LC122490
AP08837	F: [F9CCG] CGCGGATCATGAGCTAAACC R: GGCGTCTCCTTCAGAAATGG	$(CT)_{24}$	FAM	232	LC127429
AP12435	F: [F9GAC] ACAAGACTGGGACCACTCTC R: CTCCAAGGTAGGCAGTGTATTG	$(AC)_{18}$	FAM	135–175	LC127430
AP14144	F: [F9GCC] AGTTTCTTTGAGCCGCGTTG R: AAATCTGAGGACTTGTGCGC	$(AG)_{19}$	PET	298–316	LC122491
AP14331	F: [F9GAC] GCCATTCCTCCATCTTC R: TCCGCCATGACAAGCAATTC	$(AG)_{16}$	FAM	138	LC122492
AP14371	F: [F9AGG] ACAGATGCAGATAGGTGGCC R: ACAGATGCAGATAGGTGGCC	$(AG)_{22}$	VIC	403–409	LC122493
AP15792	F: [F9GCC] GTGATTATTCCTGCTGGCCG R: CCTCCGTGCCATTGTGAAAG	$(AG)_{19}$	PET	288–314	LC122494
AP15867	F: [F9GAC] AGTTCATAGCCCGTCGAAATTC R: GGGACCTCTTCAACAACCAAC	$(AG)_{16}$	FAM	150	LC127431
AP20216	F: [F9CCG] GCCGAGATGATATAAAGAGCC R: AGTGGGTATTCGGGAGCAAG	$(AT)_{25}$	FAM	289–321	LC122495

Osaka, Japan), 5 μL of 2× PCR buffer, 0.4 mM dNTPs, and 0.2 μM primers (forward primer with a bar-coded split tag, reverse primer, and fluorescent bar-coded split tag primer). A bar-coded split tag (BStag), comprising a common basal region among six BStags, a three-nucleotide "bar-code" sequence, and a mismatched nucleotide at the middle position, was added at the 5′ end of the forward primer of each locus to enable postlabeling (Shimizu and Yano, 2011). We labeled the BStag primers with fluorescent dyes to create F9GAC-FAM (5′-CTAGTATCAGGACGAC-3′), F9GTC-VIC (5′-CTAGTATGAGGACGTC-3′), F9TAC-NED (5′-CTAGTATCAGGACTAC-3′), F9GCC-PET (5′-CTAGTATTAGGACGCC-3′), F9CCG-FAM (5′-CTAGTATTAGGACCCG-3′), and F9AGG-VIC (5′-CTAGTATTAGGACAGG-3′) (Shimizu and Yano, 2011). The sequences of forward and reverse primers are listed in Table 1. Amplification was performed with a Veriti Thermal Cycler (Life Technologies, Carlsbad, California, USA) under the following conditions: initial denaturation at 94°C for 2 min; 30 cycles of denaturation at 98°C for 10 s, annealing at 58°C for 30 s, and extension at 68°C for 30 s; 12 cycles of 98°C for 10 s, 49°C for 30 s, and 68°C for 10 s; and a final extension at 68°C for 7 min. The size of the PCR products was measured using an ABI PRISM 3130XL Genetic Analyzer (Life Technologies) by GeneMapper software (Life Technologies). We conducted PCR amplifications twice in four randomly selected individuals because microsatellite markers with a high number of repeat units may cause unstable results due to PCR error (Shinde et al., 2003).

The genetic polymorphism at each locus was assessed by calculating the observed number of alleles (A), observed heterozygosity (H_o), and expected

TABLE 2. Genetic variation of the 10 polymorphic microsatellite loci for three populations of *Adenophora palustris* and one population of *A. triphylla* var. *japonica*.[a]

	A. palustris									*A. triphylla* var. *japonica*		
		Sera (n = 12)			Toyosaka (n = 12)			Ena (n = 8)			Hiroshima (n = 5)		
Locus	A_T	A	H_o	H_e	A	H_o	H_e	A	H_o	H_e	A[b]	H_o	H_e
AP02665	15	13	0.667*	0.886	9	0.750	0.809	6	1.000	0.781	4 (1)	0.400	0.640
AP04430	11	8	0.667	0.583	7	0.750	0.694	5	0.500	0.624	—	—	—
AP07350	9	8	0.750	0.756	6	0.917*	0.750	3	0.750	0.594	—	—	—
AP07489	8	4	0.250*	0.642	6	0.333	0.659	2	0.875	0.491	1 (0)	0.000	0.000
AP08053	10	5	0.250	0.517	6	0.750	0.743	5	0.750	0.702	3 (2)	0.600	0.480
AP12435	8	4	0.000*	0.513	4	0.917*	0.642	3	0.250	0.593	—	—	—
AP14144	10	9	0.833	0.816	7	0.917	0.827	4	0.750	0.608	—	—	—
AP14371	4	4	0.500	0.538	3	0.583	0.434	2	0.500	0.375	4 (2)	1.000	0.640
AP15792	8	6	0.583	0.611	4	0.500	0.632	3	0.750	0.507	1 (1)	0.000	0.000
AP20216	10	6	0.750	0.781	8	0.750	0.792	1	0.000	0.000	—	—	—

Note: — = not amplified; A = number of alleles per population; A_T = total number of alleles observed in *A. palustris*; H_e = expected heterozygosity; H_o = observed heterozygosity.

[a] Voucher and locality information are provided in Appendix 1.

[b] Numbers in parentheses are the numbers of alleles only observed in the *A. triphylla* var. *japonica* population.

* Significant deviation from Hardy–Weinberg equilibrium expectations ($P < 0.01$).

heterozygosity (H_e). Deviations from Hardy–Weinberg equilibrium in each population and linkage disequilibrium were tested using FSTAT version 2.9.3 software (Goudet, 1995) and the web version 4.2 of GENEPOP software (Raymond and Rousset, 1995), respectively. Significance levels were adjusted using the Bonferroni correction for multiple comparisons.

Of the 15 loci tested, 10 were polymorphic and five were monomorphic. PCR slippage was not observed. The evaluation of polymorphism in all 32 individuals showed that the 10 polymorphic loci had moderate to high levels of polymorphism (Table 2). *A* ranged from four (AP14371) to 15 (AP02665), with an average of 9.3 alleles per locus. H_e for each locus was generally high, ranging from 0.48 (AP14371) to 0.89 (AP02665). At the population level, *A* ranged from one to 13 (average: 5.4), H_o from 0.00 to 1.00 (average: 0.62), and H_e from 0.00 to 0.89 (average: 0.63). Among all loci in the three populations, five combinations of locus and population deviated significantly from Hardy–Weinberg equilibrium ($P < 0.01$; Table 2). There was no evidence of significant linkage disequilibrium ($P < 0.05$) in any pair of loci. Nine loci were successfully amplified in *A. triphylla* var. *japonica*, and of these, three loci showed polymorphism (Table 2).

CONCLUSIONS

The microsatellite markers described here will be useful for investigating genetic diversity and gene flow within and among remnant native populations of the critically endangered *A. palustris* in Japan. Assessment of their genetic variation will also contribute to elucidating how *A. palustris* populations in Japan have been declining, and the results will play a key role in the future management of the species. Furthermore, these markers have enough resolution to detect localities of individuals that were illegally collected and marketed, and the results will provide crucial information for revitalization of populations by complementary planting of illegally collected individuals.

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Appendix 1. Voucher information for *Adenophora* populations used in this study. One voucher was collected from each population sampled.

Species	Collection locality	Voucher collection no.[a]
A. palustris Kom.	Sera, Hiroshima Prefecture, Japan	*K. Otake0001*
A. palustris	Toyosaka, Hiroshima Prefecture, Japan	*K. Otake0003*
A. palustris	Ena, Gifu Prefecture, Japan	*K. Otake0006*
A. triphylla (Thunb.) A. DC. var. *japonica* H. Hara	Higashi-Hiroshima, Hiroshima Prefecture, Japan	*K. Otake0007*

[a]All vouchers were deposited in the Herbarium of the Graduate School for International Development and Cooperation, Hiroshima University, Hiroshima, Japan. To prevent illegal collection, we have withheld the exact locations of the sites.