Isolation and Characterization of Novel Microsatellite Loci For The Endangered Orchid Cypripedium japonicum (Orchidaceae)

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Primer Note

Isolation and characterization of novel microsatellite loci for the endangered orchid *Cypripedium japonicum* (Orchidaceae)[1]

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- *Premise of the study:* Twenty-six microsatellite markers were developed for the endangered orchid *Cypripedium japonicum* (Orchidaceae) to estimate the clonal diversity and genetic structure of the remaining populations in Japan.
- *Methods and Results:* Microsatellite loci of *C. japonicum* were isolated using Ion Personal Genome Machine (PGM) sequencing. The primer sets were tested on 55 ramets sampled from two populations in Japan. Sixteen loci showed polymorphism in at least one population, with two to five alleles per locus. Observed and expected heterozygosities for the two populations ranged from 0.00 to 0.92 and 0.00 to 0.71, respectively.
- *Conclusions:* The microsatellite markers developed here provide a useful tool to analyze clonal structure and sexual regeneration status and will help to manage the remaining genetic variation within *C. japonicum*.

Key words: clonal analysis; conservation genetics; *Cypripedium japonicum*; Ion PGM sequencing; microsatellites; Orchidaceae.

Cypripedium L. (Orchidaceae) is a genus rich in horticulturally important species, including many endangered taxa. *Cypripedium japonicum* Thunb. is an attractive terrestrial orchid distributed widely throughout temperate forests in Japan, Korea, and China (Cribb, 1997). However, because of recent habitat destruction and extraction for horticultural purposes, remaining populations of this species have declined and become fragmented, and *C. japonicum* is now classified as Critically Endangered in Korea (Lee, 2009) and Vulnerable in Japan (Ministry of the Environment, 2015). In this critical situation, the necessity for the in situ and ex situ conservation of genetic resources has been highlighted in Korea (Lee, 2009), and in situ recovery programs are underway for several populations in Japan.

Although immediate establishment of appropriate conservation programs is needed for *C. japonicum*, ecological studies and knowledge of sexual regeneration for this species are limited. Field observations reported a low fruiting rate in Japan (Hasegawa et al., 1987; Yamashita, personal observation), and observations of connections of underground organs by careful excavation suggested a high reliability on asexual reproduction by stoloniferous rhizomes (Chiba Prefecture Board of Education, 1980). The bias toward asexual reproduction may have made it difficult to investigate sexual regeneration of this species, and suitable vegetation for safe sites and the fungal symbionts for its germination are not yet known. Therefore, clarifying the current status of sexual reproduction in remaining populations can provide basic information about in situ conservation programs of this endangered species.

In this study, we developed microsatellite markers for *C. japonicum* using the Ion Personal Genome Machine (PGM; Life Technologies, Waltham, Massachusetts, USA) sequencing to investigate the current status of sexual and asexual regeneration. This genetic analysis aims to reveal clonal structures accumulated as a result of asexual reproduction as well as sexual regeneration status. The data will also provide information about genetic variation and differences among remaining populations, which have not been possible to elucidate by either allozyme analysis (Chung et al., 2009) or intersimple sequence repeat (ISSR) analysis (Qian et al., 2014).

METHODS AND RESULTS

A fresh leaf sample was taken from a ramet growing in a native population in Soma, Fukushima Prefecture, Japan (Appendix 1). Genomic DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Germantown, Maryland, USA) following the manufacturer's instructions. The DNA fragment library was constructed using an Ion Xpress Plus Fragment Library Kit (Life Technologies). Emulsion PCR was performed for the fragment library with capture beads using an Ion PGM Template OT2 400 Kit (Life Technologies). After amplification, the desired beads were enriched and the amplified DNA fragments were sequenced using an Ion PGM Sequencing 400 Kit (Life Technologies) and an Ion 318 Chip v2 (Life Technologies). A total of 326,901 sequences (mean read length 220 bp) were obtained. After filtering for identical reads, the resulting 325,984 sequences were screened for potential microsatellite loci

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using MSATCOMMANDER (Faircloth, 2008) using default settings. Primers were designed for all sequences containing more than six di- or trinucleotide repeats using Primer3 software (Rozen and Skaletsky, 1999) with the default settings, resulting in a total of 238 primer pairs for screening. Twenty-six primer pairs showing clear peak patterns were selected after an amplification trial using eight ramets from populations in the Chiba and Fukushima prefectures, Japan (Appendix 1).

To test the genetic variation of the 26 selected microsatellite loci, 24 ramets from a population in the Chiba Prefecture and 31 ramets from a population in the Hokkaido Prefecture were used (Appendix 1). A tag sequence for fluorescent labeling was added to each of the forward primers (Boutin-Ganache et al., 2001). PCR amplification was done in 5-μL reactions using the QIAGEN Multiplex PCR Kit (QIAGEN). Each reaction contained the following components: 10 ng of genomic DNA, 2.5 μL of Multiplex PCR Master Mix, 0.01 μM of forward primer, 0.2 μM of reverse primer, and 0.1 μM of fluorescently labeled tag primer. Amplifications used the following program: 95°C for 15 min; 33 cycles at 94°C for 30 s, 57°C for 1.5 min, and 72°C for 1 min; and an extension at 60°C for 30 min. Product sizes were determined using an ABI PRISM 3130 Genetic Analyzer and GeneMapper software (Applied Biosystems, Foster City, California, USA).

Of the 26 loci tested, 16 were polymorphic and 10 were monomorphic (Table 1). In the Chiba population, all 24 ramets showed distinct multilocus genotypes, whereas in the Hokkaido population, 19 multilocus genotypes were detected across 31 ramets. The combined nonexclusion probability of each population calculated by CERVUS 3.0 (Kalinowski et al., 2007) was 0.000018 in Chiba and 0.00012 in Hokkaido, respectively. Thus, these

TABLE 1. Characteristics of 26 microsatellite primers developed for *Cypripedium japonicum*.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Fluorescent label[b]	Allele size range (bp)	GenBank accession no.
Cypj025	F: TTCGAGATGCTTCCGACCC	$(TTG)_9$	VIC	236–239	LC73788
	R: TTGGCCGAGTTCGTTCGAG				
Cypj047	F: TGTCAGTGTCGCTGCCTTC	$(GCG)_{10}$	FAM	191–199	LC73789
	R: AGTTCACGACCCGATTGTC				
Cypj060	F: TCACTGAGAGGTGTGATTCC	$(AC)_{12}$	FAM	160	LC73790
	R: CATTGCATGCTTGTGTTGT				
Cypj061	F: TTTTGGATCAAATCATCACCT	$(AC)_{10}$	FAM	154–156	LC73791
	R: CTTCTTTAGAGGAAGATCCAAGA				
Cypj062	F: TGAGGCTACCAGTTAATGTCTG	$(AG)_{12}$	FAM	131–135	LC73792
	R: ATCTTCCTCTCCACCAATCA				
Cypj065	F: ACAAGAACCTGCCAGAAAAC	$(AG)_{10}$	FAM	122–124	LC73793
	R: GACAAGATTTTCAATTCATCACTC				
Cypj069	F: GCATCATTCAAGGTGTCAAA	$(GA)_{10}$	VIC	104	LC73794
	R: CTTCCTCCCTCTCTCTTTCC				
Cypj082	F: ATTCATAAAACCAGGGCTGA	$(GA)_{11}$	FAM	158–162	LC73795
	R: TCAAAGGATGGTGGAGAAGT				
Cypj091	F: TCGATGACATTGATATGGAAG	$(GA)_{23}$	FAM	125–131	LC73796
	R: AGGGATGATCTTTTCCTTCA				
Cypj094	F: CCTCAATAGGGACACACACA	$(AG)_{11}$	VIC	128–157	LC73797
	R: AGTTCAATGGAACCCTCAAA				
Cypj100	F: GGTGAATTATATGATGGAAGCA	$(AC)_{11}$	VIC	173–177	LC73798
	R: TTGCTGTTATTACTCCCACCT				
Cypj114	F: TTAAGGGACTTTCTCTGATTCAAC	$(CT)_9$	FAM	240	LC73799
	R: CCAATCACTTCCTAGCTGGC				
Cypj122	F: CCATCAGGCCACCATTCTG	$(GA)_7$	FAM	221–223	LC73800
	R: TGGTGTCTCCTTATTGTGATTGC				
Cypj140	F: AGTTGGGTATCGAGGTGGC	$(GA)_{13}$	FAM	174–176	LC73801
	R: AGACTAAGCTATGGTAACTACATTCC				
Cypj147	F: CCAGGACCTTAGCCCTGAC	$(GA)_6$	VIC	375	LC73802
	R: CCCTCTCAGATCTCCTACAAAGG				
Cypj179	F: AGTTGGCAAGGATCTTATTGGC	$(TA)_6$	VIC	247–249	LC73803
	R: GCCCAGGCCCTTATTCAAAG				
Cypj180	F: ACACCCATATTTGAGGATGGC	$(TG)_9$	FAM	311	LC73804
	R: AGCAGTTCCTAATGGCAAGG				
Cypj196	F: AGCTCTCATACTGAGGGTTG	$(CT)_{10}$	VIC	217–219	LC73805
	R: TATGCACTTGGCACATTCG				
Cypj197	F: ACCGATGAAATTTGGCAGAGG	$(CT)_8$	FAM	258	LC73806
	R: CACTCCCGCCATTAGAACC				
Cypj202	F: TGCTAACATTTGCAACAAAGC	$(AG)_{10}$	FAM	174–176	LC73807
	R: TGCTTGGTGATGGAGGAAAC				
Cypj204	F: TCCTCCAGCACTTTGTCGG	$(AG)_{10}$	VIC	180	LC73808
	R: TCCTACAAGCCTCCACTGC				
Cypj205	F: ACTAGCATCGCTGAAAGTGC	$(GA)_{10}$	VIC	277	LC73809
	R: TGAGGAGAGACTCCATGAACG				
Cypj216	F: AATCAATTCCCATTTAAAACTCTC	$(CT)_{10}$	VIC	234	LC73810
	R: ATTTAGGCCAAAACAGAGGA				
Cypj218	F: ACCGGTGATGAAGGAAAATA	$(TA)_{10}$	VIC	220–226	LC73811
	R: TGATTTGAAGCCTAATATATAT				
Cypj224	F: AAGAGGTTGGCTTTTGGATT	$(TC)_{11}$	VIC	168–170	LC73812
	R: CAACGATGAGTTCGTAAAGG				
Cypj233	F: AAGCCAAAAGAGAAGCTTGA	$(CT)_{10}$	FAM	214	LC73813
	R: GAACTTGAACCCGAGAGAGA				

[a]Annealing temperature for all reactions was 57°C.
[b]Sequence of the fluorescent labels: FAM = 5′-CACGACGTTGTAAAACGAC-3′, VIC = 5′-TGTGGAATTGTGAGCGG-3′.

TABLE 2. Genetic variation of the 16 polymorphic microsatellite loci for two populations of *Cypripedium japonicum* in Japan.

	Chiba (N = 24, G = 24)				Hokkaido (N = 31, G = 19)			
Locus	A	H_o	H_e	P_{ID}	A	H_o	H_e	P_{ID}
Cypj025	2	0.04	0.04	0.92	1	0.00	0.00	1.00
Cypj047	4	0.17	0.16	0.71	2	0.05	0.15	0.74
Cypj061	2	0.38	0.40	0.44	2	0.63	0.51	0.38
Cypj062	2	0.08	0.08	0.85	1	0.00	0.00	1.00
Cypj065	2	0.46	0.51	0.38	1	0.00	0.00	1.00
Cypj082	3	0.17	0.16	0.72	1	0.00	0.00	1.00
Cypj091	4	0.92	0.71	0.14	3	0.42	0.35	0.48
Cypj094	5	0.54	0.64	0.21	3	0.58	0.65	0.21
Cypj100	3	0.58	0.58	0.26	2	0.32	0.40	0.45
Cypj122	2	0.38	0.36	0.48	1	0.00	0.00	1.00
Cypj140	2	0.38	0.31	0.53	1	0.00	0.00	1.00
Cypj179	2	0.29	0.25	0.59	2	0.26	0.23	0.62
Cypj196	2	0.04	0.04	0.92	2	0.42	0.40	0.45
Cypj202	2	0.29	0.25	0.59	1	0.00	0.00	1.00
Cypj218	4	0.46	0.38	0.43	2	0.16	0.15	0.74
Cypj224	1	0.00	0.00	1.00	2	0.26	0.23	0.62

Note: A = number of alleles; G = number of genets; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of analyzed ramets; P_{ID} = probability of identity.

microsatellite markers have sufficient resolution in clonal analysis, and ramets that showed identical genotypes in the Hokkaido population were from the same genets, probably produced by asexual propagation via rhizome elongation.

Genetic variation was evaluated for 24 genets from the Chiba population and 19 genets from the Hokkaido population using GenAlEx version 6.2 (Peakall and Smouse, 2006) and CERVUS 3.0 (Kalinowski et al., 2007). The observed and expected heterozygosities (H_o and H_e) were 0.00–0.92 (mean 0.20) and 0.00–0.71 (mean 0.19), respectively (Table 2). Deviations from Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium (LD) between loci were tested using FSTAT version 2.9.3 (Goudet, 1995). Significance levels were adjusted using the Bonferroni correction for multiple testing. None of the loci exhibited a significant deviation from HWE ($P < 0.05$) in either of the populations, and there was no evidence of LD for any locus pairs.

CONCLUSIONS

We have developed 26 microsatellite markers for *C. japonicum* that will be useful for assessing the clonal structure and sexual regeneration status of remaining populations of *C. japonicum*. The results presented here indicate that sexual regeneration may be contributing more to maintaining the number of ramets than previously expected. These markers also have enough resolution to investigate genetic variation and differences among remaining populations, which are essential for handling the priority of genets and populations for the in situ and ex situ conservation of this species.

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APPENDIX 1. Voucher and location information for the *Cypripedium japonicum* populations used in this study. One voucher was collected from each population sampled.

Collector	Collection locality	GPS coordinates[a]	Voucher specimen accession no.[b]	No. of ramets
Kazuko Iga and Yumi Yamashita	Soma, Fukushima Prefecture, Japan	37°46′N, 140°42′E	FKSE 22462 (*Kazuko Iga 977*)	1
Yumi Yamashita	Yotsukaido, Chiba Prefecture, Japan	35°39′N, 140°12′E	FKSE 87328 (*Yumi Yamashita 735*)	24
Yumi Yamashita	Nikappu, Hokkaido Prefecture, Japan	42°21′N, 142°18′E	FKSE 86822 (*Yumi Yamashita 636*)	31

[a] Precise GPS coordinates were not included for conservation purposes.
[b] All vouchers were deposited in the Herbarium of the Faculty of Symbiotic Systems Science (FKSE), Fukushima University, Fukushima, Japan.