PRIMER NOTE

ISOLATION AND CHARACTERIZATION OF NOVEL MICROSATellite
LOCI FOR THE ENDANGERED ORCHID CYPRIPEDIUM JAPONICUM
(Orchidaceae)

YUMI YAMASHITA², AYAKO IZUNO³, YUI ISAGI³, TAKAHIDE KUROSAWA², AND SHINGO KANEKO²,⁴

²Graduate School of Symbiotic Systems Science and Technology, Fukushima University, Kanayagawa 1, Fukushima, Fukushima 960-1296, Japan; and ³Laboratory of Forest Biology, Division of Forest and Biomaterials Science, Graduate School of Agriculture, Kyoto University, Kitashirakawa, Oiwake-cho, Sakyo-ku, Kyoto 606-8502, Japan

• 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 DNAeasy 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

1 Manuscript received 21 August 2015; revision accepted 2 October 2015. The authors thank Kazuko Iga, Shoue Tohmi, Noriko Hasegawa, and Katsuroh Hasegawa for their help in sampling. We also thank Daisuke Sugimori for the support in genetic analysis. This study was partly supported by the Fukushima University research project for the Regeneration of Harmonies between Human Activity and Nature in Bandai-Asahi National Park.

²²Author for correspondence: kaneko.shingo@gmail.com

doi:10.3732/apps.1500097

Applications in Plant Sciences 2016 4(2): 1500097; http://www.bioone.org/loi/apps © 2016 Yamashita et al. Published by the Botanical Society of America.

This work is licensed under a Creative Commons Attribution License (CC-BY-NC-SA).
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 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:

- 0.000018 in Chiba and 0.00012 in Hokkaido, respectively. Thus, these repeats using Primer3 software (Rozen and Skaletsky, 1999) with the default settings, resulting in a total of 238 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 95°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 0.00018 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 labelb | Allele size range (bp) | GenBank accession no. |
|-------|--------------------------|--------------|-------------------|-----------------------|-----------------------|
| Cyp105 | F: TTCGAGATGGCTTCGCAGCC | (TTG)6       | VIC               | 236–239               | LC73788               |
|       | R: TGGCCGGATGCTTGCAG     |              |                   |                       |                       |
| Cyp107 | F: TGTCAGTGTCGTGCTTCTC  | (GCC)10      | FAM               | 191–199               | LC73789               |
|       | R: AGTTCAGCAGCGATACTTC   |              |                   |                       |                       |
| Cyp106 | F: TACCTGAGAGGTGAGTTCC   | (AC)12       | FAM               | 160                   | LC73790               |
|       | R: CAGTGGATCTGGGTGTTG    |              |                   |                       |                       |
| Cyp1061 | F: TTTCGATCAACTACATACAC  | (AG)10       | FAM               | 154–156               | LC73791               |
|       | R: CTTGCTTACATGAGTACAA   |              |                   |                       |                       |
| Cyp1062 | F: TGAGCCTACAGTATTATGCTG | (AG)12       | FAM               | 131–135               | LC73792               |
|       | R: ATCTCCTCTCCACCACCTCA  |              |                   |                       |                       |
| Cyp1065 | F: ACAAAAGATCGCAGAAAAC  | (AG)10       | FAM               | 122–124               | LC73793               |
|       | R: GACAAGATTTCATCATTACCT |              |                   |                       |                       |
| Cyp1069 | F: GCATCATTCAAGTGTCAAA  | (GA)10       | VIC               | 104                   | LC73794               |
|       | R: CTCTCCTCCTCTCCTCC     |              |                   |                       |                       |
| Cyp1082 | F: ATCTAATAACACAGGCCCTGA | (GA)11       | FAM               | 158–162               | LC73795               |
|       | R: TCAAGAGGTGTTGGAGAAGT  |              |                   |                       |                       |
| Cyp1091 | F: TCGATGACATTGATAGGAAG  | (GA)13       | FAM               | 125–131               | LC73796               |
|       | R: AGGGAATCTTCCTTCTTCCTCA|              |                   |                       |                       |
| Cyp1094 | F: CCTCAATTAGGAGCAACACA  | (AG)11       | VIC               | 128–157               | LC73797               |
|       | R: ATGTGCAATGGAGACCTCGAA |              |                   |                       |                       |
| Cyp1100 | F: GGTGAATTATATGATGAGCCA | (AC)11       | VIC               | 173–177               | LC73798               |
|       | R: TTGCTGTATAATTACTCCACCT|              |                   |                       |                       |
| Cyp114 | F: TTAAGGGCTTCTCTGTATCAA | (CT)9        | FAM               | 240                   | LC73799               |
|       | R: CCAATCTTCCTCTGAGGTC   |              |                   |                       |                       |
| Cyp122 | F: CCATACGGCAGACACTTC    | (GA)7        | FAM               | 221–223               | LC73800               |
|       | R: TGAGTCGCTCTATTTGAGTTCG|              |                   |                       |                       |
| Cyp140 | F: AGAGAGTTACCTGAGGGGCCG | (GA)13       | FAM               | 174–176               | LC73801               |
|       | R: AGACTAAGCTGTAGTAAACTACATTCC | (TG)9       | FAM               | 311                   | LC73804               |
| Cyp147 | F: CCAAGGACCTTAGCCTGAG   | (GA)6        | VIC               | 375                   | LC73802               |
|       | R: CTCCTCGATCTCTACATACAGG|              |                   |                       |                       |
| Cyp179 | F: AGTTTGGCAAGTTTATTTGGGC| (TA)6        | FAM               | 247–249               | LC73803               |
|       | R: GCCAGGCCCCTATTTCCAAGG|              |                   |                       |                       |
| Cyp180 | F: ACACCAATTTGAGAGGGGCCG | (TG)9        | FAM               | 311                   | LC73804               |
|       | R: AGACGTCTCTTAATTGGCAGG  |              |                   |                       |                       |
| Cyp196 | F: AGCTCTCATTACAGAGGTTG  | (CT)10       | VIC               | 217–219               | LC73805               |
|       | R: TATGCGCTTGGCAACATTCC |              |                   |                       |                       |
| Cyp197 | F: ACCGAGAAATTTGGCAGAGG  | (CT)9        | FAM               | 258                   | LC73806               |
|       | R: CACTCCGCCATTAGAACCC   |              |                   |                       |                       |
| Cyp202 | F: TGGCCATGGTGCAGAACCCAG| (AG)10       | FAM               | 174–176               | LC73807               |
|       | R: TGTTGAGGTAGGAGGAAAC   |              |                   |                       |                       |
| Cyp204 | F: TCTCAGACCTTGGTCCGG   | (AG)10       | VIC               | 180                   | LC73808               |
|       | R: TCTCTACAGCCTTGGGAGG   |              |                   |                       |                       |
| Cyp205 | F: ACTAGCATGCTGAAGATGC  | (GA)10       | VIC               | 277                   | LC73809               |
|       | R: TGGAGAAGACTCTCATGGAAG |              |                   |                       |                       |
| Cyp216 | F: ATCTCAATCCCCATTATGACTCTC| (CT)10       | VIC               | 234                   | LC73810               |
|       | R: ATTTAGGCAAAACAGAGGA  |              |                   |                       |                       |
| Cyp218 | F: ACCGGTGATGGAGGAAATAA  | (TA)10       | VIC               | 220–226               | LC73811               |
|       | R: TGATTTGAAATGCATATATATAT|              |                   |                       |                       |
| Cyp224 | F: AAAGGTTGCGCTTTGGAAGT | (TC)11       | VIC               | 168–170               | LC73812               |
|       | R: CAAAGTAGTGGTTGTAAGAG  |              |                   |                       |                       |
| Cyp233 | F: AAAGCAAAAGAGAGCTGGA  | (CT)10       | FAM               | 214                   | LC73813               |
|       | R: GAACCTGACGGCAGGAGA    |              |                   |                       |                       |

*a Annealing temperature for all reactions was 57°C.

*b Sequence of the fluorescent labels: FAM = 5′-CAGCAGCTTGTAAACGAC-3′, VIC = 5′-TGTGAATTTGTGACCGG-3′.

http://www.bioone.org/loi/apps
TABLE 2. Genetic variation of the 16 polymorphic microsatellite loci for two populations of *Cypripedium japonicum* in Japan.

| Locus    | A | *H*<sub>o</sub> | *H*<sub>e</sub> | *P*<sub>id</sub> | A | *H*<sub>o</sub> | *H*<sub>e</sub> | *P*<sub>id</sub> |
|----------|---|----------------|----------------|----------------|---|----------------|----------------|----------------|
| Cypj025  | 2 | 0.04           | 0.04           | 0.92           | 1 | 0.00           | 0.00           | 1.00           |
| Cypj047  | 3 | 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  | 1 | 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*<sub>e</sub> = expected heterozygosity; *H*<sub>o</sub> = observed heterozygosity; N = number of analyzed ramets; *P*<sub>id</sub> = probability of identity.

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.

LITERATURE CITED

Boutin-Ganache, I., M. Raposo, M. Raymond, and C. F. Deschepper. 2001. M13-tailed primers improve the readability and usability of microsatellite analyses performed with two different allele-sizing methods. *BioTechniques* 31: 24–26.

CHIBA PREFECTURE BOARD OF EDUCATION. 1980. Report on *Cypripedium japonicum* at Noruto-machi, the natural monument of Chiba Prefecture. Chiba Prefecture Board of Education, Chiba, Japan [in Japanese].

Chung, J. M., K. W. Park, C.-S. Park, S.-H. Lee, M. G. Chung, and M. Y. Chung. 2009. Contrasting levels of genetic diversity between the historically rare orchid *Cypripedium japonicum* and the historically common orchid *Cypripedium macranthos* in South Korea. *Botanical Journal of the Linnean Society* 160: 119–129.

Chubb, P. 1997. The genus *Cypripedium*. Timber Press, Portland, Oregon, USA.

Faircloth, B. C. 2008. MSATCOMMANDER: Detection of microsatellite repeat arrays and automated, locus-specific primer design. *Molecular Ecology Resources* 8: 92–94.

Goudet, J. 1995. FSTAT: A computer program to calculate F-statistics, version 1.2. *Journal of Heredity* 86: 485–486.

Hasegawa, A., M. Nakasugi, and M. Gol. 1987. A seed harvesting method of *Cypripedium japonicum* Thunberg. *Technical Bulletin of Faculty of Agriculture, Kagawa University* 38: 63–70 [in Japanese with English abstract].

Kalinowski, S. T., M. L. Taper, and T. C. Marshall. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Molecular Ecology* 16: 1099–1106.

Lee, B. C. 2009. Rare plants: Data book of Korea. Korea National Arboretum, Pocheon, Korea.

MINISTRY OF THE ENVIRONMENT. 2015. Red Data Book 2014—Threatened wildlife of Japan, Vol. 8. Vascular Plants. Gyosei, Tokyo, Japan [in Japanese].

Peakall, R., and P. E. Smouse. 2006. *GenAlEx*6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6: 288–295.

Qian, X., Q.-J. Li, F. Liu, M.-J. Gong, C.-X. Wang, and M. Tian. 2014. Conservation genetics of an endangered Lady’s Slipper Orchid: *Cypripedium japonicum* in China. *International Journal of Molecular Sciences* 15: 11578–11596.

Rozen, S., and H. Skalesky. 1999. Primer3 on the WWW for general users and for biologist programmers. In S. Misener and S. A. Krawetz [eds.], Methods in molecular biology, vol. 132: Bioinformatics methods and protocols, 365–386. Humana Press, Totowa, New Jersey, USA.
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 coordinatesa | 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 626) | 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.