

Development of microsatellite markers for the endangered orchid *Calanthe izu-insularis* (Orchidaceae)

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(Received 1 June 2017, accepted 3 October 2017; J-STAGE Advance published date: 17 January 2018)

Microsatellite markers were developed for the endangered orchid *Calanthe izu-insularis* (Orchidaceae). This species is unique to the Izu Islands in Japan. Unfortunately, its population size has decreased because of excessive collection for horticultural purposes. In addition, although natural hybridization between *C. izu-insularis* and *C. discolor* var. *discolor* has been reported, morphological differences between *C. izu-insularis* and the hybridized individuals remain unclear. Using next-generation sequencing, 11 polymorphic microsatellite markers were developed. All developed markers could amplify *C. aristulifera* and nine markers could amplify *C. d.* var. *discolor*, two other orchid species that are also endangered in Japan. The number of alleles and expected heterozygosity at each locus were 1–6 (mean, 2.35) and 0.00–0.79 (mean, 0.30), respectively. These microsatellite markers will help conservation geneticists in their investigation of the proportion of pure *C. izu-insularis* individuals in the Izu Islands.

Key words: *Calanthe*, conservation, endangered orchid, natural hybridization, next-generation sequencing

INTRODUCTION

Calanthe izu-insularis (Satomi) Ohwi et Satomi (Orchidaceae) is an endemic perennial plant species in the Izu Islands, Japan (Cribb and Bailes, 2001). This species is now classified as endangered according to the Red List of Japan (<http://www.env.go.jp/press/files/jp/105449.pdf>). The main factor for the decline of *C. izu-insularis* is excessive collection for horticulture because of the rare, beautiful and fragrant nature of this orchid (Ministry of the Environment, Government of Japan, 2015). Recently, the collection of wild *C. izu-insularis* has been prohibited in Mikura and Kodzu Islands; however, the risks of illegal collection persist. Thus, ubiquitous genotyping is essential for the conservation of this plant (Shiga et al., 2013;

Isagi and Kaneko, 2014). Ubiquitous genotyping data would be based on all remaining individuals and would be useful for assisting in the prevention of their illegal collection (Shiga et al., 2013).

Based on morphological data, *C. izu-insularis* is closely related to two other species, *C. aristulifera* and *C. discolor* var. *discolor*, which are widely distributed throughout the islands of Japan (Cribb and Bailes, 2001). Natural hybridization has also been reported between *C. izu-insularis* and *C. d.* var. *discolor* (Takahashi, 1972; Cribb and Bailes, 2001). These two species sympatrically grow in the Izu Islands (Cribb and Bailes, 2001; Suetsugu et al., 2016). In addition, they are pollinated by the same bee species, *Lasioglossum occidens* (Suetsugu et al., 2016, 2017). Although the morphological difference between *C. izu-insularis* and the hybridized individuals is unclear, the application of molecular techniques should make it possible to distinguish them.

In this study, we developed 11 microsatellite mark-

Edited by Yoshihiko Tsumura

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DOI: <http://doi.org/10.1266/ggs.17-00021>

ers for *C. izu-insularis* using next-generation sequencing and examined the availability of these markers for two related species of *Calanthe*, namely *C. aristulifera* and *C. d. var. discolor* (Table 1).

MATERIALS AND METHODS

Isolation of microsatellite loci To develop microsatellite markers for *C. izu-insularis* and related species, we constructed DNA libraries from *C. izu-insularis*, *C. d. var. discolor* and *C. d. var. kanashiroi*. Fresh leaf samples of one individual of *C. izu-insularis* (collected from Mikura Island, Tokyo prefecture, Japan), two individuals of *C. d. var. discolor* (collected from Mikura Island, Tokyo prefecture and Tokunoshima Island, Kagoshima Prefecture) and one individual of *C. d. var. kanashiroi* (collected from Okinawa Island, Okinawa Prefecture) were used for DNA extraction. Genomic DNA of all species was extracted using the DNeasy Plant Mini Kit (Qiagen), nebulized at 0.24 MPa for 1 min and purified using the MinElute PCR

Purification Kit (Qiagen).

The purified DNA fragments of *C. izu-insularis* underwent end repair and A-tailing using the Rapid Library Prep Kit (Roche) and were ligated to the Rapid Library Adapter (Roche) with RL Ligase (Roche). Suitably sized DNA fragments were selected by removing short fragments using AMPure XP beads (Agilent). Fragments were then mixed with capture beads and amplified using emulsion polymerase chain reaction (emPCR) with the GS Junior Titanium emPCR kit (Roche). After emPCR, beads were collected and those that captured the DNA library were enriched before annealing with sequencing primers. Amplified fragments were sequenced using the GS Junior Bench Top system (Roche). Consequently, a total yield of 35,982 reads was achieved.

Genomic DNA from two individuals of *C. d. var. discolor* and one individual of *C. d. var. kanashiroi* was digested with MspI and PstI (New England Biolabs) at 37 °C for 2 h. Adapters were ligated at 22 °C overnight in a 40-μl solution containing 20 μl of digested DNA, 2 μl of 10×

Table 1. Characteristics of microsatellite loci for *Calanthe izu-insularis*, *C. aristulifera* and *C. discolor*. All values are based on 112 samples from one, one, and two populations in Japan, respectively

Locus	Primer sequences (5'-3')	Repeat motif	Fluorescent label ^a	T _a (°C)	Size range (bp)	Total number of alleles in all species	Species used for primer design	GenBank Accession No.
<i>Caiz_005</i>	F: GTGCACAACACATGAAGTCTG R: TCTTTAGGTTATGTAACACCAGGG	(CT)9	FAM	57	422–428	3	<i>C. izu-insularis</i>	LC271144
<i>Caiz_010</i>	F: CCATCCGTCAAACCAACGG R: AAATTCGCGGCGGACAAAG	(TC)10	VIC	57	332–345	8	<i>C. izu-insularis</i>	LC271145
<i>Caiz_020</i>	F: AGCCTAAGAGTAAGGTTGAAATTGG R: TGGCATTGTACAAGGGCTATTC	(TG)10	NED	57	226–250	11	<i>C. izu-insularis</i>	LC271146
<i>Caiz_024</i>	F: AACCCGACGCCAGAATAG R: TGATAACCACCAAGCAAGGAC	(AC)8	FAM	57	255–257	2	<i>C. izu-insularis</i>	LC271147
<i>Caiz_036</i>	F: CATCTGGTGTATCACGCC R: TGCATAAGGTCATCAATCCTTTAC	(GA)9	FAM	57	299–339	3	<i>C. izu-insularis</i>	LC271148
<i>Caiz_041</i>	F: TCAGGCTCTAACTTGTTGGG R: TCTGCCTCAAATTCATGGACTG	(AAC)8	NED	57	282–296	4	<i>C. izu-insularis</i>	LC271149
<i>Caiz_045</i>	F: CTCAACAGCATTTGGCCTCG R: AGAAGCAACCCGAAACATCC	(TGA)6	NED	57	352–373	6	<i>C. izu-insularis</i>	LC271150
<i>Caiz_047</i>	F: GAGCCTGGTCTTGTGCATC R: TGCCTAGGATTTCTTTGGCTG	(AAG)7	VIC	57	266–270	3	<i>C. izu-insularis</i>	LC271151
<i>Caiz_068</i>	F: TGCTAAGTTAAACCCGTGTGC R: ATTCAAACCTCGAGGGCTTGG	(AAT)5	NED	57	167–170	2	<i>C. izu-insularis</i>	LC271152
<i>Caiz_071</i>	F: GGGACTCGCACATCCCTAG R: GTAAGGCGTCGCTGCCCTCGC	(CCG)9	VIC	57	127–145	7	<i>C. discolor</i> <i>var. discolor</i>	LC271153
<i>Caiz_083</i>	F: CGGCTGAGGACCCCGTCTT R: TCCGTTTCGACATTATGGCCG	(CGG)6	FAM	57	214–223	4	<i>C. discolor</i> <i>var. discolor</i>	LC271154

Note: T_a annealing temperature.

^aSequence of the fluorescent labels: FAM = 5'-CACGACGTTGTAAAACGAC-3', NED = 5'-CTATAGGGCACGCGTGGT-3', VIC = 5'-TGTGGAATTGTGAGCGG-3'.

NEB buffer 4, 0.02 μ M adapter 1, 3 μ M adapter 2, 1 mM ATP and 0.5 μ l of T4 DNA ligase (Enzymatics). The DNA was then purified with the QIAquick PCR purification kit (Qiagen), and 7.5 μ l of purified DNA was used for PCR amplification in a 25- μ l solution containing 12.5 μ l of 2 $\times$ Multiplex PCR Master Mix (Qiagen), 0.2 μ M Illumina forward primer and 0.2 μ M Illumina reverse primer. Thermal cycling was initiated with a 95 °C step for 15 min, followed by 27 cycles of 94 °C for 30 s, 62 °C for 1.5 min and 72 °C for 1.5 min and a final extension at 72 °C for 10 min. PCR products were purified again with the QIAquick PCR purification kit. DNA fragments of 500–600 bp were extracted using Pippin Prep (Sage Science). The amplification and extraction was checked using MultiNA (Shimadzu), and the fragments were sequenced using the Illumina Miseq v2 (Illumina). The total numbers of reads obtained from *C. d.* var. *discolor* and *C. d.* var. *kanashiroi* were 4,003 and 1,228, respectively.

The reads were screened using PRIMER3 software (Rozen and Skaletsky, 2000) embedded in msatcommander version 0.8.2 (Faircloth, 2008) to identify dinucleotide and trinucleotide loci of 5–18 repeats. A total of 90 primer pairs were designed, comprising 68, 18 and 4 primer pairs from *C. izu-insularis*, *C. d.* var. *discolor* and *C. d.* var. *kanashiroi* reads, respectively.

Primer check Amplification by and specificity of the primer pairs were tested using four *C. izu-insularis* and four *C. d.* var. *discolor* individuals. For all confirmed microsatellite loci in *C. izu-insularis*, locus-specific forward primers were synthesized with one of the following M13 sequence tails, 5'-CACGACGTTGTAAAACGAC-3', 5'-CTATAGGGCACGCGTGGT-3', 5'-TGTGGAATTGTGAGCGG-3' (Boutin-Ganache et al., 2001), added to their 5' ends (Table 1). Each PCR amplification was performed in a final volume of 5 μ l containing approximately 16 ng of template DNA, 2.5 μ l of 2 $\times$ Multiplex PCR Master Mix (Qiagen), 0.01 μ M forward primer, 0.2 μ M reverse primer and 0.1 μ M M13 (fluorescent-labeled) primer. The PCR thermal profile was as follows: initial denaturation at 95 °C for 15 min, followed by 30–35 cycles of 94 °C for 30 s, 57 °C for 1.5 min and 72 °C for 1 min and a final extension at 60 °C for 30 min. PCR products were detected on the ABI Prism 3130 Genetic Analyzer (Applied Biosystems). Fragment lengths were calculated using Gene Mapper (Applied Biosystems).

Polymorphism of microsatellite markers For population genetic analysis, DNA was extracted from 78, 3 and 31 individuals of *C. izu-insularis*, *C. aristulifera* and *C. d.* var. *discolor* using the CTAB method (Milligan, 1992). Leaf samples of *C. izu-insularis* and *C. aristulifera* were collected from Mikura Island, and those of *C. d.* var. *discolor* were collected from two populations located

in Ibaraki and Hiroshima Prefectures. PCR conditions were the same as used for the above primer check. The following three summary statistics were calculated using GenAlEx version 6.4 (Peakall and Smouse, 2006) and FSTAT version 2.9.3 (Goudet, 1995): number of alleles (A), observed heterozygosity (H_O) and expected heterozygosity (H_E). Tests for deviation from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium (LD) between loci were performed using FSTAT version 2.9.3. The combined first-parent non-exclusion probabilities for an identity were calculated using Cervus 3.0 for each species (Marshall et al., 1998; Kalinowski et al., 2007). These probabilities were calculated using 11 markers in *C. izu-insularis* and *C. aristulifera* and nine markers in *C. d.* var. *discolor*.

To evaluate genetic differentiation among *C. izu-insularis*, *C. aristulifera* and *C. d.* var. *discolor*, we calculated pairwise codominant genotypic distances (Smouse and Peakall, 1999) among each species. We also performed principal coordinates analysis (PCoA) using GenAlEx version 6.41 (Peakall and Smouse, 2006).

RESULTS AND DISCUSSION

Of the 90 primer pairs tested, 11 amplified all four *C. izu-insularis* and four *C. d.* var. *discolor* individuals. The lengths of the amplified fragments were unambiguously scored. Their size range for each primer pair is shown in Table 1. Nine and two primer pairs were designed from the library of *C. izu-insularis* and *C. d.* var. *discolor*, respectively. Four primer pairs designed from the library of *C. d.* var. *kanashiroi* were unusable for amplification.

All primers successfully amplified all of the samples of *C. izu-insularis* and *C. aristulifera*; however, two primer pairs, *Caiz_005* and *Caiz_024*, could not amplify 27 and 23 samples, respectively, of a total of 31 *C. d.* var. *discolor* samples (Table 2).

The number of alleles per locus ranged from 1 to 6 (mean, 2.35) (Table 2). The observed (H_O) and expected (H_E) heterozygosities per locus ranged from 0.00 to 1.00 (mean, 0.32) and from 0.00 to 0.79 (mean, 0.30), respectively (Table 2). The total number of alleles in all three species ranged from 2 to 11 (mean, 4.82) (Table 1). All 11 loci showed no evidence of significant deviation from HWE ($P > 0.05$). Significant LD was observed in the Ibaraki and Hiroshima populations of *C. d.* var. *discolor* for two pairs of loci (*Caiz_010* and *Caiz_020*, *Caiz_020* and *Caiz_071*; $P < 0.05$). In the Hiroshima populations of *C. d.* var. *discolor*, there was also significant LD for an additional pair of loci (*Caiz_010* and *Caiz_071*; $P < 0.05$). The combined non-exclusion probabilities for an identity in *C. izu-insularis*, *C. aristulifera* and *C. d.* var. *discolor* were 0.0069, 0.0004 and 0.0001, respectively. According to PCoA based on codominant genotypic distances, 64.49% and 5.17% of the variation was

Table 2. Results for primer screening of all samples for 11 microsatellite loci in a total of four populations of *Calanthe izu-insularis*, *C. aristulifera* and *C. discolor* var. *discolor*

Locus	<i>Calanthe izu-insularis</i>			<i>Calanthe aristulifera</i>			<i>Calanthe discolor</i> var. <i>discolor</i>					
	Mikura Island (N = 78)			Mikura Island (N = 3)			Ibaraki (N = 14)			Hiroshima (N = 17)		
	A	H _O	H _E	A	H _O	H _E	A	H _O	H _E	A	H _O	H _E
Caiz_005	2	0.35	0.39	1	0.00	0.00	—	—	—	—	—	—
Caiz_010	2	0.27	0.37	3	1.00	0.61	4	0.64	0.59	6	1.00	0.79
Caiz_020	4	0.45	0.42	3	1.00	0.61	6	0.50	0.79	5	0.76	0.70
Caiz_024	2	0.13	0.14	1	0.00	0.00	—	—	—	—	—	—
Caiz_036	2	0.03	0.03	1	0.00	0.00	2	0.07	0.07	1	0.00	0.00
Caiz_041	2	0.01	0.01	2	0.33	0.28	1	0.00	0.00	1	0.00	0.00
Caiz_045	2	0.06	0.06	3	1.00	0.61	2	0.43	0.35	2	0.12	0.11
Caiz_047	1	0.00	0.00	1	0.00	0.00	2	0.14	0.53	2	0.24	0.21
Caiz_068	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00	1	0.00	0.00
Caiz_071	2	0.40	0.45	3	0.33	0.61	4	0.36	0.48	4	0.82	0.70
Caiz_083	3	0.46	0.49	3	0.67	0.50	3	0.86	0.55	2	0.18	0.41
Average	2.09	0.20	0.22	2.00	0.39	0.29	2.78	0.33	0.37	2.67	0.35	0.32

Note: N = sample size; A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity.

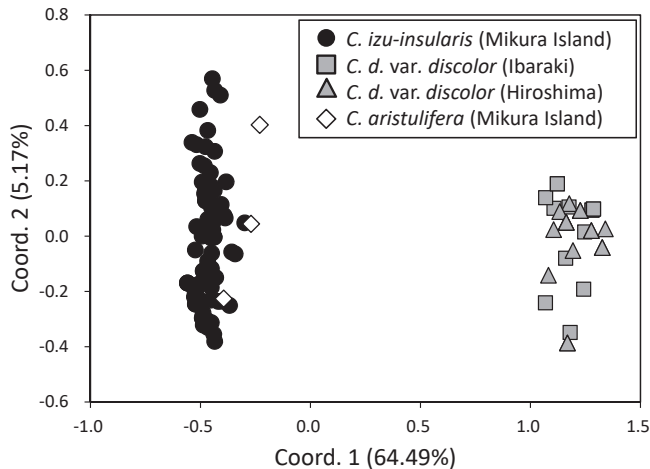


Fig. 1. Principal coordinates analysis plot of individuals based on codominant genotypic distances (Smouse and Peakall, 1999). Axis 1 explains 64.49% of the variance and axis 2 explains 5.17% of the variance.

described with the first and second axes, respectively (Fig. 1). Although individuals between *C. izu-insularis* and *C. aristulifera* were not clearly divided, *C. izu-insularis* and *C. d. var. discolor* individuals were plotted as independent groups. This may be attributed to the small number of samples of *C. aristulifera*.

Performing population genetic analysis using these microsatellite markers may contribute to the conservation of *C. izu-insularis*. Considering that the main factor for the decline of *C. izu-insularis* is excessive collection for horticulture, the use of these markers to identify genotypes of *C. izu-insularis* could prevent their illegal collection (Shiga et al., 2013). Furthermore, the assessment

of hybridization between *C. izu-insularis* and *C. d. var. discolor* should enable us to determine the proportion of pure *C. izu-insularis* individuals.

These markers may be capable of identifying other species of *Calanthe*, such as *C. aristulifera*. As described in the Red List of Japan, several species of *Calanthe* are now classified as *near threatened*, *endangered* or *critically endangered* (<http://www.env.go.jp/press/files/jp/105449.pdf>). Therefore, population genetic studies of the species of *Calanthe*, such as those evaluating genetic diversity and structure within and between populations, are essential for constructing successful conservation programmes.

We are grateful to Mr. Kuniaki Ohtake, Dr. Tomohisa Yukawa and the Hiroshima Botanical Garden for providing *C. d. var. discolor* samples. We are also grateful to Mr. Masayuki Hino and Mr. Toshiaki Kurimoto for helping us to collect samples of *C. aristulifera* and *C. izu-insularis* from Mikura Island. This work was supported by JSPS KAKENHI Grant Number 15H04414 and the Environment Research and Technology Development Fund (4-1605) of the Ministry of the Environment, Japan.

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