Development of 13 microsatellite markers in *Cadiocrinum cordatum* var. *glehnii*

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Abstract

Cadiocrinum cordatum var. *glehnii* is a monocarpic perennial herb distributed in northern Japan. We developed 13 polymorphic microsatellite loci from genomic DNA of *Cadiocrinum cordatum* var. *glehnii* using a dual-suppression polymerase chain reaction technique and an improved method. The number of allele per locus ranged from two to four, and the expected heterozygosities ranged from 0.052 to 0.670. These markers will be available to identify genets and evaluate genetic diversity of *C. cordatum* var. *glehnii*.

Cadiocrinum cordatum (Thunb.) Makino (Liliaceae) is a monocarpic perennial herb growing in temperate broad-leaved deciduous forests of the Japanese Islands and adjacent Far East regions. The northern populations of *C. cordatum* especially in Sakhalin, Kuriles, Hokkaido, and particularly on the Japan sea side of Honshu, Japan are characterized by taller and more robust morphologies of the flowering individuals, with more numerous and larger flowers than other populations, and thus are referred to as a local variety, var. *glehnii* (Kawano *et al.* 2004). *Cardiocrinum cordatum s. lat.* (including var. *glehnii*) is a monocarpic and shows unique life-history characteristics (Ohara *et al.* 2006). This plant reproduces by two different manners; sexual reproduction via seeds and vegetative reproduction via formation of daughter bulbs (bulbils) (Kawano 1975; Ohara *et al.* 2006). Thus, in order to assess the extent and the contribution of sexual and vegetative reproduction to the maintenance of populations, we isolated microsatellite markers from *C. cordatum* var. *glehnii.*

Total DNA was extracted from fresh leaves of *C. cordatum* var. *glehnii* using a cetyl trimethyl ammonium bromide (CTAB) miniprep procedure (Stewart & Via 1993). Microsatellite regions were isolated using a dual-suppression polymerase chain reaction (PCR) method (Lian & Hogetsu 2002) and an improved technique (Lian *et al.* 2006). To construct adaptor-ligated, restricted DNA libraries, DNA was separately digested with six restriction enzymes *Acc*II, *Afa*I, *Alu*I, *Eco*RV, *Hae*III and *Ssp*I. The fragments produced by each restriction enzyme were then ligated to an unequal-length adaptor, consisting of a 48-mer and an 8-mer (see, Lian & Hogetsu 2002), using DNA Ligation Kit version 2.1 (TaKaRa). The DNA fragments with a microsatellite at one end were amplified from the *Afa*I and *Ssp*I libraries using compound SSR primers $(AC)_6(AG)_5$, $(AG)_6(AC)_5$, $(AC)_6(TC)_5$ or $(TC)_6(AC)_5$ and an adaptor primer AP2 (5'-CTATAGGGCACGCGTGGT-3') which was designed from the longer strand of the adaptor (Lian *et al.* 2006). The fragments amplified by

46 $(AC)_6(TC)_5$ or $(TC)_6(AC)_5$ were purified by QIAquick PCR Purification Kit (QIAGEN). The
47 purified DNA fragments were cloned into pDrive Cloning Vector, and the plasmids were
48 transformed into QIAGEN EZ Competent Cells, using QIAGEN PCR Cloning plus Kit. The
49 cloned fragments were amplified using the M4 forward and M13 reverse primers from the
50 plasmid DNA of positive clones. These products were purified as described above. The PCR
51 products were sequenced directly using the BigDye Terminator Cycle Sequencing Kit (Applied
52 Biosystems) with the ABI PRISM 3100 automated sequencer (Applied Biosystems). For each
53 fragment containing $(AC)_6(TC)_n$ or $(TC)_6(AC)_n$ compound SSR sequences at one end, a specific
54 primer (IP1) was designed from the sequenced region flanking the SSR. The primer pairs of
55 IP1 primer and SSR primer were used as a SSR marker (Lian *et al.* 2006).
56 To investigate microsatellite polymorphism, leaves of 38 flowering individuals were
57 collected from a *C. cordatum* var. *glehnii* population in Hokkaido, Japan. PCR amplification
58 was performed with the GeneAmp PCR system 9700 thermal cycler (Applied Biosystems) in a
59 reaction mixture (10 μL) containing 0.5 μL of template DNA, 0.2 mM of each dNTP (Applied
60 Biosystems), 10× PCR buffer with 1.5 mM $MgCl_2$ (Applied Biosystems), 0.25 U of Ampli*Taq*
61 Gold DNA polymerase (Applied Biosystems), and two primers: IP1 (0.5μM) and the SSR
62 primer (fluorescent dye-labelled, 0.5μM). The PCR conditions were 9 min at 94 °C, 35 cycles of
63 30 s at 94 °C, 30 s at the annealing temperature, and 1 min at 72 °C, followed by 72 °C for
64 7 min. The PCR products were detected with the ABI PRISM 3100 automated sequencer and
65 GeneScan analysis software (Applied Biosystems).
66 We successfully isolated 13 SSR loci with polymorphism and codominance from *C.*
67 *cordatum* var. *glehnii* (Table 1). To conduct the following tests, all 38 individuals were assayed
68 for every locus. The number of alleles per locus ranged from two to four per locus, with an
69 average of 3.08 (Table 1). The observed and expected heterozygosities calculated using

CERVUS version 3.0 (Kalinowski *et al.* 2007) ranged from 0.053 to 0.579 and from 0.052 to 0.670, respectively (Table 1). Statistical tests for Hardy-Weinberg equilibrium and linkage disequilibrium were conducted with GENEPOP (Raymond & Rousset 1995) using default values for the Markov chain parameters. One locus, cc6 significantly deviated from the Hardy-Weinberg equilibrium after Bonferroni correction. This deviation was due to deficiency of heterozygosities, and a significant level of null allele frequency (0.176; Brookfield 1 estimator) was detected by MICRO−CHECKER version 2.2.3 (Van Oosterhout *et al.* 2004). There were no significant linkage disequilibria between the different pairwise combinations of the 13 loci ($P > 0.01$). These markers are currently being used to analyze the genetic structure and reproductive success of *C. cordatum* var. *glehnii* populations in Hokkaido, Japan.

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Table 1 Characteristics of 13 microsatellite loci from *Cardiocrinum cordatum* var. *glehnii*

Locus	Repeat	Primer sequences (5'-3')	Fluorescent label	T_a (°C)	Size range (bp)	No. of alleles	H_O	H_E	Frequency of null allele	GeneBank Accession no.
cc1	$(AC)_6(TC)_7TTTC$	ACCTTTTGATTGTGATAGAGTTTG ACACACACACACTCTCTCTCTC	VIC	55	105-110	3	0.579	0.513	-0.049	AB512096
cc6	$(AC)_6(TC)_7$	CCTCCCGACGTCTTTCAGTTAGTT ACACACACACACTCTCTCTCTC	PET	60	115-121	4	0.316	0.604*	**0.176**	AB512097
cc15	$(AC)_6(TC)_5(TA)_6$	TTCTCCAGCGGATCTTTATCACATT ACACACACACACTCTCTCTCTC	FAM	57	205-213	3	0.162	0.153	-0.010	AB512098
cc19	$(AC)_6(TC)_6CCCC(TC)_2$	CTCGTAGCAACGATGACCTAAGAAG ACACACACACACTCTCTCTCTC	FAM	63	179-182	3	0.474	0.670	0.113	AB512099
cc22	$(AC)_6(TC)_5CCCCC(TC)_5$	TTCAATGATGATGATGTCAAATACC ACACACACACACTCTCTCTCTC	FAM	55	104-120	4	0.108	0.106	-0.004	AB512100
cc24	$(TC)_6(AC)_{12}$	GTTATGACTAACCTCCAACGCTCTC TCTCTCTCTCTCACACACACAC	VIC	63	120-126	4	0.553	0.643	0.050	AB512101
cc30	$(TC)_6(AC)_5(AT)_5$	TGGTCATACGTGATATGGGGGACAA TCTCTCTCTCTCACACACACAC	VIC	60	186-198	4	0.474	0.529	0.032	AB512102
cc37	$(AC)_6(TC)_5\cdots(TC)_3T(TC)_4\cdots(TC)_4$	AATGGATAAGGGTTAAGGTTGAGTC ACACACACACACTCTCTCTCTC	PET	56	200-203	2	0.333	0.493	0.103	AB512103
cc41	$(AC)_6(TC)_7$	CACAATCAAAACTTGCAATCTCTTAC ACACACACACACTCTCTCTCTC	VIC	56	161-173	3	0.081	0.176	0.079	AB512104
cc44	$(TC)_6(AC)_5(CA)_2\cdots(CT)_3\cdots(CT)_7$	TGACGTGGATACTAAGACATCTCAA TCTCTCTCTCTCACACACACAC	VIC	56	112-114	2	0.053	0.052	-0.001	AB512105
cc45	$(TC)_6(AC)_{11}\cdots(AC)_4$	ATATAAATCTAGGGTAAGGGTTTCA TCTCTCTCTCTCACACACACAC	FAM	54	127-133	4	0.368	0.499	0.083	AB512106
cc50	$(TC)_6(AC)_9AT(AC)_3$	AACTTATGATAGAATGGGGAAGAGG TCTCTCTCTCTCACACACACAC	FAM	56	108-110	2	0.324	0.506	0.117	AB512107
cc52	$(TC)_6(AC)_7$	TTCATACCTGATCATAGTTGCACTC TCTCTCTCTCTCACACACACAC	NED	56	103-110	2	0.237	0.405	0.117	AB512108

T_a, annealing temperature of the primer pair; H_O, observed heterozygosity; H_E, expected heterozygosity. *Indicates significant deviation from Hardy-Weinberg equilibrium. The value in bold indicates significant evidence for the presence of a null allele ($P<0.01$).