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PRIMER NOTE

DEVELOPMENT AND CHARACTERIZATION OF MICROSATELLITE MARKERS FOR *LILIUM LONGIFLORUM* (LILIACEAE)[1]

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- *Premise of the study:* Ten microsatellite primers were developed to obtain information on genetic variation in *Lilium longiflorum*, a bulbous species showing high intraspecific genetic differentiation.
- *Methods and Results:* Of 61 microsatellite loci isolated using the dual suppression PCR technique, 10 loci were effective to characterize and estimate genetic variation in two populations of *L. longiflorum*. The number of alleles at each locus was different between the populations (averages = 3.2 and 10.3 alleles per locus), and the mean observed heterozygosity values were 0.245 and 0.732.
- *Conclusions:* Our results demonstrate that there is significant genetic variation between the populations and that the microsatellite markers developed in this study will be useful tools for the investigation of the genetic structure and mating system of natural *L. longiflorum* populations.

Key words: genetic variation; Liliaceae; *Lilium longiflorum*; natural population; self-compatibility; self-incompatibility.

Lilium longiflorum Thunb., which is native to the Ryukyu Archipelago in Japan and the mainland seacoast and satellite islands in the eastern part of Taiwan, is a bulbous species in the Liliaceae and shows high intraspecific genetic differentiation (Hiramatsu et al., 2001; Sakazono et al., 2012). It is an important horticultural species throughout the world and is the most studied of the *Lilium* L. species, both morphologically and physiologically (Miller, 1993). Cultivars of *L. longiflorum* are self-incompatible and, therefore, it has long been believed that it is a self-incompatible species. However, Sakazono et al. (2012) identified completely self-compatible individuals from natural populations of *L. longiflorum*, and some populations located in the northern distribution area consisted predominantly of self-compatible individuals. The factors leading to establishment of the predominantly self-compatible populations, however, are unclear. Development of codominant molecular markers, such as microsatellite (simple sequence repeat [SSR]) markers, could lead to clarification of genetic variation, mating system, and life-history evolution. Microsatellite markers have been developed for *L. philadelphicum* L. (Horning et al., 2003), which is distantly related to *L. longiflorum*, but these markers are unsuitable for use with *L. longiflorum* (S. Sakazono, personal communication). Here, we report 10 microsatellite markers designed from microsatellite loci of *L. longiflorum* and they are used to genetically characterize two natural populations, from Yakushima Island (predominantly self-compatible population) and Ishigakijima Island (predominantly self-incompatible population).

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METHODS AND RESULTS

Total DNA was extracted from approximately 100 mg of *L. longiflorum* 'Hinomoto' fresh leaf by the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987), with some modifications (Nakazawa et al., 1997).

Microsatellite loci of *L. longiflorum* were isolated according to the dual-suppression PCR technique (Lian et al., 2006). DNA was digested with *Eco*RV, and the restricted fragments were ligated with a specific adapter (48-mer: 5′-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3′ and 8-mer with the 3′-end capped with an amino residue: 5′-ACCAGCCC-NH_2-3′) using the DNA Ligation Kit version 1 (TaKaRa Bio Inc., Otsu, Shiga, Japan). The ligated fragments were treated with ddGTP using AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, California, USA) to block polymerase-catalyzed extension of the 8-mer adapter strand. Then, specific fragments were amplified using an SSR compound primer ($(AC)_6(AG)_5$ or $(TC)_6(AC)_5$) and an adapter primer (5′-CTATAGGGCACGCGTGGT-3′) from the *Eco*RV DNA library of *L. longiflorum*. The fragments (400–800 bp) were purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and cloned using the Perfectly Blunt Cloning Kit (Novagen, Madison, Wisconsin, USA) in accordance with the manufacturer's instructions. Recombinant clones were identified using blue/white screening on Luria–Bertani agar plates containing carbenicillin, tetracycline, X-gal, and IPTG. Insert-positive clones were amplified using M13 forward or reverse primer. Plasmid DNA was extracted from each of 89 insert-positive clones using the Plasmid Mini Purification Kit (LaboPass, COSMO Genetech, Seoul, South Korea), and sequenced using the BigDye Terminator Cycle Sequencing Kit version 1.0 (Applied Biosystems). For 32 of 61 fragments containing the $(AC)_6(AG)_5$ or $(TC)_6(AC)_5$ sequence at one end, specific primers were designed using Primer3 software (Rozen and Skaletsky, 2000).

To demonstrate the effectiveness of 10 primer pairs (Table 1) in amplifying clear and polymorphic bands, we investigated *L. longiflorum* individuals from

TABLE 1. Characteristics of 10 microsatellite primers developed for analysis of *Lilium longiflorum*.

Locus	Primer sequences (5′–3′)[a]	Repeat motif	Size range (bp)	T_a (°C)	Fluorescent label	GenBank accession no.
Llon001	F: ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_7$	115–121	55	6-FAM	KC631411
	R: **GTTTCTT**TGGTAACCCACCTCTGACTT					
Llon002	F: ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_{18}$	173–225	55	6-FAM	KC631412
	R: **GTTTCTT**ATTTGACCTCCAACTCCTCA					
Llon003	F: ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_6$	186–242	58	6-FAM	KC631413
	R: **GTTTCTT**ATCCTCCTTCAATGGACTCA					
Llon004	F: ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_8$	200–224	55	6-FAM	KC631414
	R: **GTTTCTT**GGCTGGTATCATTGATGATGTG					
Llon005	F: ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_8$	128–138	55	6-FAM	KC631415
	R: **GTTTCTT**GGTACATGCAGCAGGTTATG					
Llon006	F: ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_5$	171–197	58	6-FAM	KC631416
	R: **GTTTCTT**CGGATCATATTGGTGATGAGG					
Llon007	F: ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_{20}$	240–284	55	6-FAM	KC631417
	R: **GTTTCTT**CTAGTGAGTGAACCCAATCTAGG					
Llon008	F: ACACACACACACAGAGAGAGAG	$(AC)_6(AG)_5$	150–154	63	6-FAM	KC631418
	R: **GTTTCTT**CAAGTTTGGGGGTAGTGTATG					
Llon009	F: TCTCTCTCTCTCACACACACAC	$(TC)_6(AC)_{10}$	192–228	55	VIC	KC631419
	R: **GTTTCTT**CCCAAATGGAGCAAAAATAC					
Llon010	F: TCTCTCTCTCTCACACACACAC	$(TC)_6(AC)_{14}$	154–176	63	VIC	KC631420
	R: **GTTTCTT**TCACATCATGGGTACCTCTAGTT					

Note: T_a = annealing temperature.
[a]The additional PIG-tail sequence of the reverse primer is boldfaced.

two populations in Japan. Leaf samples were collected from 22 plants from the Yakushima Island population (Anbo, Yakushima Island, Kagoshima, Japan, 30°30′N, 130°65′E) and the Ishigakijima Island population (Sakieda, Ishigakijima Island, Okinawa, Japan, 24°43′N, 124°07′E) for microsatellite analysis. Voucher specimens (Yakushima Island population: SS001; Ishigakijima Island population: SS002) were deposited in the Herbarium of Tohoku University (TUS). Template DNA was extracted from each silica gel–dried leaf sample, and PCR amplification was performed using $(AC)_6(AG)_5$ or $(TC)_6(AC)_5$ primers labeled with fluorochromes 6-FAM or VIC (Applied Biosystems) and a specific reverse primer with a PIG-tail sequence at the 5′ end (GTTTCTT) (Brownstein et al., 1996). The PCR mixture (10 µL) contained 0.2 µM of each primer, 0.05 U AmpliTaq Gold DNA Polymerase (Applied Biosystems), 1× PCR Buffer II, 0.2 mM each dNTP, 2.5 mM $MgCl_2$, and 20 ng template DNA. The PCR cycle parameters were 95°C for 12 min; followed by 35 cycles of 94°C for 30 s, 30 s at the annealing temperature for each primer, and 72°C for 1 min; and final extension at 72°C for 7 min. The reaction products were electrophoresed using the Applied Biosystems 3500 Genetic Analyzer with GeneScan 600 LIZ size standard (Applied Biosystems) and analyzed by GeneMapper software (Applied Biosystems). Observed heterozygosity (H_o) and expected heterozygosity (H_e) were calculated using IDENTITY version 1.0 software (Wagner and Sefc, 1999). Deviation from Hardy–Weinberg equilibrium (HWE) at each locus and linkage disequilibrium between loci were calculated using GENEPOP version 4.0.10 (http://genepop.curtin.edu.au/; Rousset, 2008) and sequential Bonferroni correction was applied for multiple tests.

There was a marked difference in genetic variation between the populations. In the Yakushima Island population, mean H_o and H_e values were 0.245 (0.000–0.591) and 0.346 (0.000–0.834), respectively, and the number of alleles per locus was 1 to 10, with an average of 3.2 (Table 2). Three loci (Llon004, Llon005, and Llon007) were monomorphic, and a significant deviation from HWE was observed at four loci (Llon002, Llon003, Llon008, and Llon009). The fixation index of the Yakushima Island population was 0.303 ± 0.349. In contrast, in the Ishigakijima Island population, mean H_o and H_e values were 0.732 (0.333–1.000) and 0.745 (0.512–0.924), respectively, and the number of alleles per locus was 3 to 18, with an average of 10.3 (Table 2). Significant deviation from HWE was observed only at Llon005, and the fixation index was 0.026 ± 0.173. Linkage disequilibrium was estimated for the data set from the Ishigakijima Island population and was found to be not significant between any loci ($P < 0.01$).

CONCLUSIONS

Sakazono et al. (2012) determined that the Ishigakijima Island population predominantly contains self-incompatible individuals and the Yakushima Island population predominantly contains

TABLE 2. Results of primer screening in two populations of *Lilium longiflorum*.

	Yakushima Island population ($N = 22$)				Ishigakijima Island population ($N = 22$)			
Locus	A	H_o	H_e	F_{IS}[a]	A	H_o	H_e	F_{IS}[a]
Llon001	3	0.591	0.509	–0.138	4	0.591	0.524	–0.105
Llon002	3	0.091	0.368	0.763***	17	0.909	0.896	0.008
Llon003	10	0.455	0.834	0.473***	18	1.000	0.924	–0.060
Llon004	1	0.000	0.000	—	10	0.944	0.856	–0.039
Llon005	1	0.000	0.000	—	6	0.333	0.742	0.416*
Llon006	3	0.318	0.274	–0.140	7	0.545	0.512	–0.041
Llon007	1	0.000	0.000	—	12	0.545	0.715	0.259
Llon008	3	0.182	0.377	0.535**	3	0.636	0.561	–0.112
Llon009	3	0.227	0.408	0.462*	14	0.909	0.895	0.007
Llon010	4	0.591	0.687	0.163	12	0.909	0.830	–0.073
Average	3.2	0.245	0.346	0.303	10.3	0.732	0.745	0.026

Note: A = number of alleles; F_{IS} = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity.
[a]Significant deviation from Hardy–Weinberg equilibrium: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

self-compatible individuals. The microsatellite analysis reported here has clarified that genetic diversity is very low in the Yakushima Island population, while the Ishigakijima Island population has retained high genetic variation. These results indicate that *L. longiflorum* shows intraspecific variation in mating systems among populations; that is, inbreeding occurs in the Yakushima Island population and random outcrossing occurs in the Ishigakijima Island population. The microsatellite markers developed in this study have the potential to be useful tools for estimating the genetic diversity and mating system of natural *L. longiflorum* populations.

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