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PRIMER NOTE

DEVELOPMENT OF MICROSATELLITE MARKERS FOR *ISODON LONGITUBUS* (LAMIACEAE)[1]

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- *Premise of the study:* Microsatellite markers were developed for *Isodon longitubus* to study the natural hybridization of the species and its congeners.
- *Methods and Results:* A total of 10 primer sets were developed for *I. longitubus*. From the initial screening, all of 10 loci were polymorphic with five to 19 alleles per locus in the Mt. Ishizuchi population, whereas nine loci were polymorphic with two to 12 alleles per loci in the Toon population. Although one locus was monomorphic at one population, the observed and expected heterozygosity values estimated from 34 *I. longitubus* samples ranged from 0.273 to 1.000 and from 0.483 to 0.918, respectively. Six primer sets could amplify all three species examined in this study (*I. inflexus*, *I. japonicus*, and *I. shikokianus*).
- *Conclusions:* The 10 microsatellite markers developed here will be useful in analyzing the population genetic structure of *I. longitubus* and in studying the natural hybridization between *Isodon* species.

Key words: *Isodon longitubus*; Lamiaceae; microsatellite; polymorphism.

Isodon (Schrad. ex Benth.) Spach (Lamiaceae) is composed of approximately 100 species (Li, 1988). The genus is distributed widely from the Far East to Africa. In Japan, seven species and six varieties are currently recognized (Murata and Yamazaki, 1993).

Suzuki et al. (2007) reported that most of the species belonging to section *Isodon* (four species and six varieties) are pollinated by bumblebees in Japan. The corolla lengths of these *Isodon* taxa generally correspond to the proboscis lengths of the bumblebee species visiting the flower of the taxa (Suzuki et al., 2007). Consequently, differences in the flower visitors could cause reproductive isolation between *Isodon* taxa in Japan.

A previous phylogenetic study on *Isodon* in Japan and Korea based on chloroplast DNA revealed that individuals from different populations in the same species were rarely monophyletic in the tree (Maki et al., 2010), suggesting that interspecific reproductive isolation might be incomplete and that extensive hybridization may occur in *Isodon* in Japan and Korea (Maki et al., 2010). To resolve whether natural hybridization is responsible for speciation and genetic diversity in these species, population genetic analyses using a combination of rapidly evolving genetic markers and extensive taxon sampling will be necessary (Buerkle and Lexer, 2008). In this respect, highly polymorphic microsatellite markers will provide valuable insights into natural hybridization among *Isodon* species. In this study, we isolated and characterized 10 microsatellite markers for *I. longitubus* (Miq.) Kudô and explored their applicability to congeneric species.

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METHODS AND RESULTS

For developing microsatellite markers, an enriched genomic DNA library was constructed using the streptavidin-coated magnetic bead method described by Bloor et al. (2001). The brief procedure from library construction to primer design was presented in Yamashiro et al. (2013). Genomic DNA was extracted from leaf samples of *I. longitubus* collected at Mt. Ishizuchi, Ehime Prefecture, Japan (*T. Yamashiro 11843*; see Appendix 1 for voucher information), using the procedure of Doyle and Doyle (1987). We designed a total of 25 primer sets for cloned sequences, of which 10 successfully amplified the target regions. PCR was performed in 10 µL of reaction mixture containing 3 ng of template DNA, 0.5 µM each primer, 0.25 mM each dNTP, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, and 0.25 U of Ex Taq polymerase (TaKaRa Biotechnology Co., Otsu, Shiga, Japan). The PCR amplification conditions were as follows: initial denaturation at 94°C for 2 min; followed by 30 cycles of 20 s at 94°C, 15 s at the annealing temperature (see Table 1), and 45 s at 72°C; and final extension at 72°C for 10 min. Two *I. longitubus* populations (*T. Yamashiro 11843* and *T. Yamashiro 11845*; Appendix 1) were used for initial polymorphism screening. We tested cross amplification in *I. inflexus* (Thunb.) Kudô ($N = 10$), *I. japonicus* (Burm. f.) H. Hara ($N = 10$), and *I. shikokianus* (Makino) H. Hara ($N = 9$). Voucher specimens representing the populations examined have been deposited in the herbarium at Tohoku University (TUS; Appendix 1). For nine of the polymorphic loci (Isodon 2, 6, 7, 9, 36, 47, 64, 69, and 70), PCRs were conducted using the above-mentioned reaction mixture volume and cycling conditions, with primers labeled with one of three Beckman WellRED dyes (D2–4: Sigma-Aldrich, St. Louis, Missouri, USA). To amplify locus Isodon3, we employed the single-tube nested PCR methods described by Schuelke (2000). All PCRs were conducted using a PC-818S program temperature control system (Astec, Fukuoka, Japan). The PCR product sizes were measured using CEQ 8000 Genetic Analysis System (Beckman Coulter, Pasadena, California, USA) and CEQ 8000 fragment analysis software (Beckman Coulter). We used the GenomeLab DNA Size Standard Kit 400 (Beckman Coulter) to determine allele size.

TABLE 1. Characteristics of the 10 microsatellites developed in *Isodon longitubus*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)[a]	T_a (°C)	WellRED dye[b]	GenBank accession no.
Isodon2	F: ACTGCTAACTACCTGCAAAACTGG	$(GA)_{18}$	201–231	60	D4	AB809448
	R: CAAAACTCTCTCTCTCTCTCT					
Isodon3	F: GTCAGTCAGTCATGTTTCAGAAAA	$(CT)_{18}$	102–142	55	D3	AB809449
	R: TCAAAAAGTAGAGGGACATCAGCC					
Isodon6	F: ATGCCCACATTTCGCTCTAT	$(CT)_{13}$	182–224	55	D2	AB809450
	R: GTGGCCATGGCTAACTATTT					
Isodon7	F: GTTATGATCCAGAGCAAGCG	$(GA)_{15}$	140–156	55	D3	AB809451
	R: CTCCTCCGACCTTTTTGGGCAT					
Isodon9	F: CGCACACTGACGTCGTAAAA	$(AG)_8$	172–212	55	D2	AB809452
	R: AGTTTTTCTGCCCCCCTGACCT					
Isodon36	F: GACTACATGGAGGTGGTGAT	$(TG)_{10}$	307–325	55	D2	AB809453
	R: GAAACATTCTTCCAAACACAG					
Isodon47	F: TTCTTGGGGTCGCCAAGAAA	$(TG)_{28}$	162–192	55	D3	AB809454
	R: AGCCTTTCCCAGGTTGGGTT					
Isodon64	F: CTTCGCAGTCTGTTGCTTCT	$(CA)_{15}$	174–190	55	D4	AB809455
	R: TGTGCGGATCTCGTGAGTGA					
Isodon69	F: GTTTGGAACCTACCCGCCGA	$(CT)_{10}(CA)_{10}$	312–394	55	D3	AB809456
	R: CAGGTCCGAACTTGACTCGT					
Isodon70	F: GTGTTAGGAGAAGCCTTGCA	$(TG)_{11}$	134–140	55	D4	AB809457
	R: TGGTGCCAATACGGCAATGT					

Note: T_a = annealing temperature.
[a] Observed size range from 34 samples of *I. longitubus*.
[b] Forward primer label.

The basic genetic diversity parameters obtained from two *I. longitubus* populations are presented in Table 2. In the Mt. Ishizuchi population, all 10 loci were polymorphic, with five to 19 alleles per locus. The observed and expected heterozygosity values ranged from 0.350 to 0.950 and 0.525 to 0.918, respectively. In contrast, in the Toon population, all loci except Isodon70 were polymorphic, with two to 12 alleles per locus. The observed and expected heterozygosity values ranged from 0.273 to 1.000 and 0.483 to 0.872, respectively. Hardy–Weinberg equilibrium and linkage disequilibrium were analyzed using GENEPOP version 3.3 (Raymond and Rousset, 1995). Although significant linkage disequilibrium was not found for all 45 possible pairwise comparisons, significant heterozygote deficiency ($P < 0.05$) was detected only at Isodon36 in the Mt. Ishizuchi population. Results of the cross amplification of the three congeneric species examined are summarized in Table 3. For *I. inflexus*, all 10 loci were amplified, although two loci (Isodon6 and Isodon70) were monomorphic. For *I. japonicus*, eight loci were amplified and three were monomorphic. For *I. shikokianus*, seven loci were polymorphic, one locus was monomorphic, and amplification failed for two loci.

TABLE 2. Results of initial primer screening in two *Isodon longitubus* populations.

	Mt. Ishizuchi ($N = 22$)				Toon ($N = 12$)			
Locus	A	H_o	H_e	HWE	A	H_o	H_e	HWE
Isodon2	11	0.950	0.876	0.683	12	1.000	0.872	1.000
Isodon3	12	0.950	0.884	0.869	7	1.000	0.816	1.000
Isodon6	11	0.700	0.839	0.104	9	0.833	0.851	0.375
Isodon7	8	0.800	0.818	0.580	6	1.000	0.719	1.000
Isodon9	8	0.700	0.739	0.133	6	0.750	0.726	0.665
Isodon36	5	0.350	0.558	0.050*	2	0.273	0.483	0.158
Isodon47	13	0.947	0.875	0.625	10	1.000	0.865	1.000
Isodon64	6	0.500	0.530	0.668	7	1.000	0.795	1.000
Isodon69	19	0.900	0.918	0.230	7	0.750	0.764	0.452
Isodon70	7	0.650	0.525	1.000	1	0.000	0.000	—

Note: — = monomorphic; A = number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium; N = number of individuals.
* Deviations from HWE ($P < 0.05$).

CONCLUSIONS

The primers described here may provide useful markers to investigate the natural hybridization among the studied *Isodon* species. These microsatellite markers will also be useful to examine intra- and interpopulation genetic diversity in *I. longitubus* and its congeners.

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TABLE 3. Cross-species amplification of 10 microsatellite primers in three congeneric *Isodon* species.

	I. inflexus ($N = 10$)				*I. japonicus* ($N = 10$)				*I. shikokianus* ($N = 9$)			
Locus	A	H_o	H_e	Size range (bp)	A	H_o	H_e	Size range (bp)	A	H_o	H_e	Size range (bp)
Isodon2	7	0.889	0.821	201–231	4	0.600	0.510	201–217	—	—	—	—
Isodon3	5	0.900	0.690	110–130	—	—			8	1.000	0.802	110–128
Isodon6	1	0.000	0.000	184	7	0.900	0.765	190–216	6	0.667	0.586	180–196
Isodon7	4	0.600	0.545	142–148	2	0.500	0.455	144–146	5	0.444	0.580	138–152
Isodon9	5	0.600	0.630	206–224	2	0.100	0.095	196–202	6	0.556	0.691	186–204
Isodon36	5	0.500	0.750	308–318	1	0.000	0.000	308	1	0.000	0.000	252
Isodon47	9	0.900	0.830	166–186	1	0.000	0.000	164	8	1.000	0.895	164–174
Isodon64	9	0.800	0.840	166–188	—	—	—	—	4	0.556	0.758	176–190
Isodon69	11	0.900	0.880	322–358	6	0.600	0.695	314–342	—	—	—	—
Isodon70	1	0.000	0.000	280	1	0.000	0.000	280	2	0.222	0.209	274–276

Note: — = unsuccessful amplification; A = number of alleles; H_e = expected heterozygosity; H_o = observed herterozygosity; N = number of individuals.

APPENDIX 1. List of vouchers of *Isodon* species used in this study. All vouchers are deposited at Tohoku University (TUS), Aoba, Sendai, Japan.

Species	Voucher specimen	Collection locality	Geographic coordinates
I. longitubus	*T. Yamashiro 11843*[a]	Mt. Ishizuchi, Ehime	33°42′49″N, 133°05′53″E
I. longitubus	*T. Yamashiro 11845*	Toon, Ehime	33°45′57″N, 132°58′46″E
I. inflexus	*T. Yamashiro 11887*	Toon, Ehime	33°45′57″N, 132°58′46″E
I. japonicus	*T. Yamashiro 11875*	Sadamitsu, Tokushima	33°42′49″N, 133°05′53″E
I. shikokianus	*T. Yamashiro 11844*	Mt. Ishizuchi, Ehime	33°59′20″N, 134°04′46″E

[a] Used in library construction.