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Full Length Research Paper

Development and characterization of nine polymorphic microsatellite markers in the seven-spotted lady beetle, *Coccinella septempunctata* (Coleoptera: Coccinellidae)

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In this study, nine microsatellite loci were isolated and characterized from the seven-spotted lady beetle, *Coccinella septempunctata* (Coleoptera: Coccinellidae). The loci were validated and characterized using 20 samples collected from five Korean localities. These results indicate that some loci were highly variable in terms of number of alleles (2 to 13), heterozygosity (0.10 to 0.40), and polymorphic information content (0.31 to 0.85). These microsatellite markers will be very valuable for population genetic studies of *C. septempunctata.*

Key words: Seven-spotted lady beetle, *Coccinella septempunctata*, microsatellite Deoxyribonucleic acid (DNA).

INTRODUCTION

The seven-spotted lady beetle, *Coccinella septempunctata* (Coleoptera: Coccinellidae), is one of the best known insects capable of predation. In North America, the species has repeatedly been introduced starting in the 1950s and has become a more effective predator than some native lady beetle species in some areas (Angalet et al., 1979; Schaefer et al., 1987). The species is widely-distributed across Eurasia to the Palearctic (Iablokoff-Khnzorian, 1982), including Korea. Due partly to an extensive and discontinuous distribution of the species, taxonomic status of the seven-spotted lady beetle has long been investigated (Rao, 1962) and some populations in fact, have been designated as a distinct species (Dobzhansky, 1933). Recently, Marin et al. (2010) assessed species status of the geographic populations collected throughout the Paleartic region using mitochondrial *COI* gene, inter-simple sequence repeat (ISSR) marker, and a few morphological characters, along with cross breeding experiment. They found a high sequence divergence of COI gene in a population, but other experimental results consistently confirmed that all populations of the species belong to the same species (Marin et al., 2010). Nevertheless, microsatellite or other polymorphic markers were necessitated to further illustrate population genetic perspective of the species.

Previously, a large set of microsatellite markers were developed from a within-tribe species, *Harmoniaaxyridis*, but cross-species amplification was only successful from the within-generic species, but not from *Coccinella* species (Loiseauat al., 2009). Therefore, in this study, we isolated and characterized nine microsatellite markers that will be useful to address various questions raised previously regarding this species, such as dispersal (Van der Werf et al., 2000), high individual variation (Hodek and Michaud, 2008), and species complexity (Marin et al., 2010).

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MATERIALS AND METHODS

Insects

Adult *C. septempunctata* were caught from five Korean localities named Gangneung, Anmyeon-Island, Chungju, Jeju-Island and Yeongam from May to June 2009. Each adult was collected at least 3 m from the neighboring one, in order to prevent over-representation of siblings from each locality. The samples were frozen at -70°C until use in molecular analysis.

Enrichment for microsatellite-containing DNA fragments

A microsatellite library was developed using methods adapted from Glenn and Schable (2005). A single individual of *C. septempunctata* was crushed in a glass grinder in liquid nitrogen. After removing the midgut, genomic DNA was extracted using a DNA Extraction Kit, in accordance with the manufacturer's instruction (Qiagen, Valencia, CA).The Genomic DNA was then digested with *RsaI* (Promega, Madison, WI), ligated to SuperSNX linkers, hybridized independently to three mixtures of biotinylated oligonucleotides, and captured on streptavidin beads. Microsatellite enriched fragments were amplified using the SuperSNX24-F primer and cloned with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA). A total of 195 clones were sequenced using M13 forward and reverse primers with an ABI 3730xl genetics analyzer (Applied Biosystems, Foster City, CA). Sequences were assembled using Sequncher 4.2.2 (Gene Codes, Ann Arbor, MI). Primers for 96 candidate loci that contained the microsatellite motif were designed using the web-based program Primer 3 (Rozen and Skaletsky, 2000) to detect amplification and redundancy.

Polymerase chain reaction amplification

PCR was conducted in a 10 µL reaction volume in an ABI 2720 Thermo cycler (Applied Biosystems, Foster City, CA) under the following condition: an initial denaturation step at 95°C for 3 min, followed by 30 cycles at 94°C for 30 s, annealing at 48 to 56°C for 30 s and 72°C for 1 min, and a final extension for 5 min at 72°C. Each reaction contained 30 ng of DNA, 1× PCR buffer, 200 µM of dNTPs, 5 pmol of each primer, and 0.5 unit of FR-Taq DNA polymerase (Biomedic, Bucheon, Gyeonggi-do). The 34 pairs of primers that were designed using the OLIGO 4.0 software (National Bioscience (National Biosciences, Plymouth, MN) were labeled with the 6-carboxyfluorescein fluorescent dye.

Genotyping

To validate the applicability of the microsatellites to population genetic studies of *C. septempunctata*, PCR amplification was conducted for 20 samples collected from five natural populations in Korea, resulting in variability in nine pairs of primers. For size analysis, 0.2 µL of the PCR product was mixed with 9.8 µL of Hi-Di Formamide (Applied Biosystems, Foster City, CA) and 0.2 µL of a Liz-500 size standard. The samples were then denatured for 5 min at 95°C, placed on ice, and run on an ABI 3730xl sequencer (Applied Biosystems, Foster City, CA). GENEMAPPER ver. 4.0 (Applied Biosystems, Foster City, CA) was used to select the allele sizes. To determine the accuracy of size determinations, electrophoresis was conducted at least three times on two independent PCR products.

Statistical analysis

From each locus observed heterozygosity (H_O; Weir, 1996), expected heterozygosity (H_E;Nei, 1987), Polymorphic Information Content (PIC; Bostein et al., 1980), and the allelic and genotypic frequencies were calculated using PowerMarker ver. 3.25 (Liu and Muse, 2005). Allelic Polymorphism Information Contents (PIC) was calculated using the following formula: $PIC = 1- \sum (P_i)^2$, where P_i is the proportion of the strain carrying ith allele that was calculated for each microsatellite locus (Bostein et al., 1980). Also, deviation from Hardy-Weinberg equilibrium (HWE) was calculated using the PowerMarker program (Liu and Muse, 2005).

RESULTS AND DISCUSSION

Of the 34 loci isolated from *C. septempunctata*, nine pairs of primers successfully provided products (Figure 1). The nine loci selected revealed substantial allelic diversity, varying from 2 to 13 (Table 1). In particular, the locus CSBm4_25, which contains a tetra-nucleotide repeat motif provided the highest allele number, 13. The polymorphic information contents (PIC), which is an estimation of the probability that an individual is informative with regard to the segregation of its inherited alleles, had a value of 0.31 to 0.85 with an average of 0.55 (Table 1). The locus CSBm4_25, which provided the highest allele number of 13, had the highest recorded value of 0.85, and the loci CSBm2_3, CSBm2_5, CSBm2_9, CSBm4_20, and CSBm4_57 had PIC values higher than 0.50, thereby indicating a high individual discrimination power.

H_O and H_E at each locus ranged from 0.10 to 0.40 and 0.34 to 0.87, respectively, showing a lower H_O than H_E in all loci and a significant deviation from HWE was detected in eight loci following the Bonferroni correction (Table 1). Weir and Cockerham (1984) identified several biological factors, such as the Wahlund effects, inbreeding, and assertive mating that caused deficiency in heterozygotes, resulting in a deviation from HWE. Considering our microsatellite loci were genotyped only for four individuals collected from five localities in Korea, the exact reason for the lower H_O may be answered after the sample size and geographic regions covered are increased. Previous study on another ladybird, *Harmonia axyridis* (Coleoptera: Coccinellidae), also reported a lower H_O than H_E in 12 among 16 loci developed (Loiseau et al., 2009). Null alleles were detected at eight loci when calculated using Micro-Checker 2.2.3 (Van Oosterhout et al., 2004). The null allele is any allele at a microsatellite locus, which consistently fails to amplify the complete product or generates a reduced one (Kwok et al., 1990). The major cause for the null allele is the mutation, which is located within the DNA sequence complementary to the oligo-primers, preventing their binding during PCR.

The genotype frequencies of each microsatellite marker shows the greatest frequency in the 153/153 and 156/156 homozygote in CS.Bm2_3 (0.35), 134/134 homozygote in CS.Bm2_5 (0.40), 239/239 homozygote in CS.Bm2_9 (0.35), 239/239 homozygote in CS.Bm3-37 (0.40), 144/144 homozygote in CS.Bm4_14 (0.60), 330/330 homozygote in CS.Bm4_20 (0.40), 235/235 and 239/239

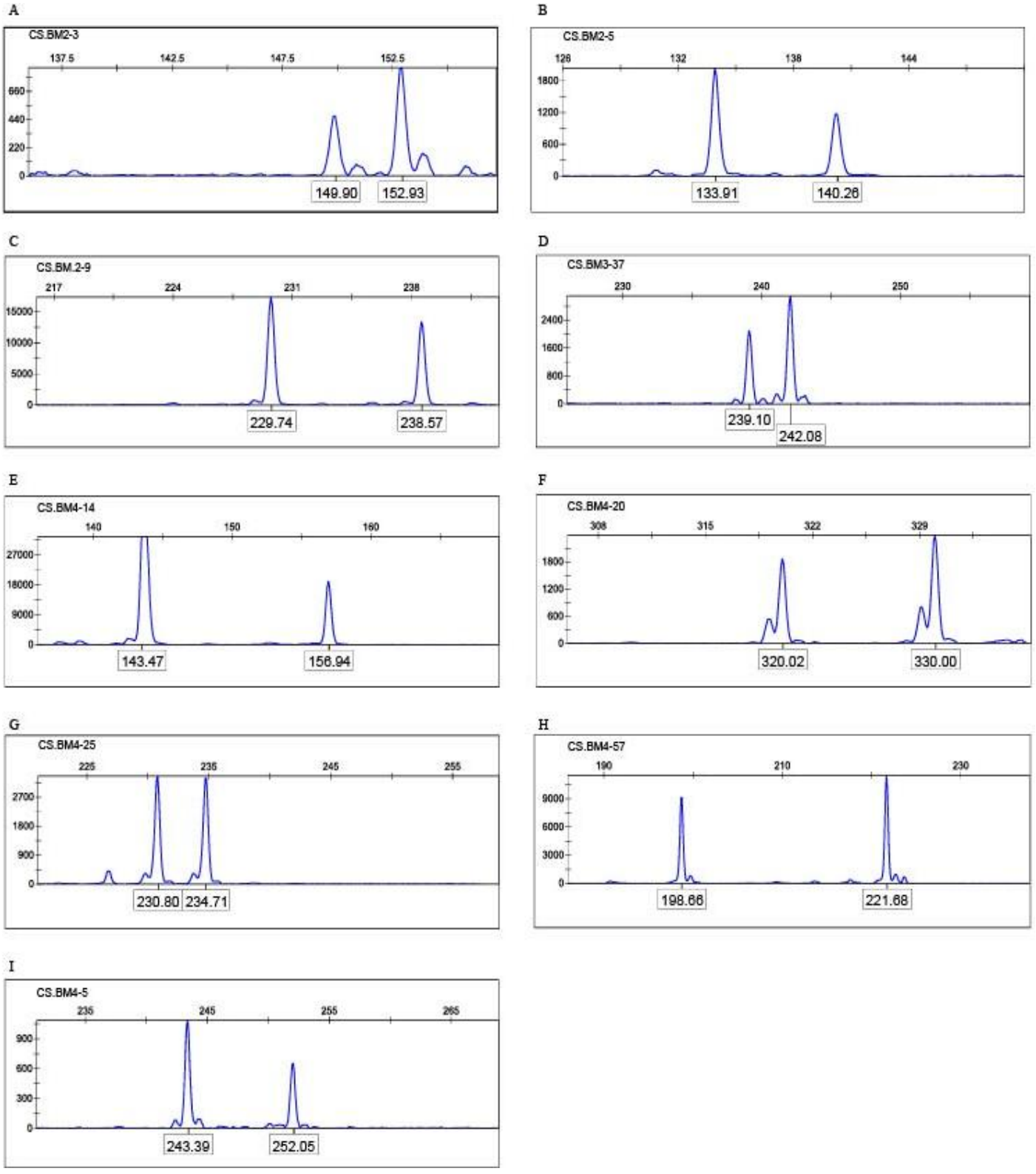

Figure 1. Exemplified electrograms of nine microsatellite markers used in this study. A, the locus CSBm2_3; B, the locusCSBm2_5; C, the locusCSBm2_9; D, the locusCSBm3_37; E, the locusCSBm4_14; F, the locusCSBm4_20; G, the locusCSBm4_25; H, the locusCSBm4_57; and I, the locusCSBm4_5.X-axis, DNA size; and y-axis, peak signal height.

Table 1. Primer sequences and characteristics of the 9 microsatellite loci in *Coccinella septempunctata*.

Locus	Repeat motif	Primer sequence (5'-3')	Size range (bp)	*n*	*a*	H_O	H_E	PIC	HWE† (*p*-value)	Estimated null allele frequency	GenBank Accession no.
CSBm2_3	$(CTG)_4(AAG)_7...(GAT)_5...(GAT)_3$	F: FAM-TGTTGCAACTACGGCTACCA R: GTTTCAAAGTCGCTTTCACCATCA	150-156	20	3	0.25	0.60	0.52	0.001**	0.2724	JX413377
CSBm2_5	$(CAT)_6...(CAG)_3$	F: FAM-TGCAGGAAATTAACGAGGATG R: GTTTCTTCTCAAAGCCACCAATCC	128-140	20	5	0.40	0.61	0.56	0.052	-	JX413378
CSBm2_9	$(TCA)_4C(CAT)_5$	F: FAM-TCAGCAACAACCTGTTCACC R: GTTTCCATTCTCGAATCCATTTCA	230-242	20	7	0.25	0.73	0.69	0**	0.3151	JX413379
CSBm3_37	$(ATC)_5$	F: FAM-TTGAAAAGGGTTCACGTTCC R: GTTTCGGTCAATAGCAGATGCAG	239-242	20	2	0.25	0.50	0.37	0.026*	0.2241	JX413380
CSBm4_14	$(TATG)_7$	F: FAM-GATTTGCGATTGTGAGCGTA R: GTTTGCAGCAAAATTCCCTTGAAA	138-157	20	3	0.15	0.47	0.39	0.001**	0.2853	JX413381
CSBm4_20	$(ATAC)_5$	F: FAM-TAAACACGGCTGCAGTTGAC R: GTTTCTCACGCATCGCTTGTAA	320-336	20	3	0.15	0.63	0.55	0**	0.3761	JX413382
CSBm4_25	$(TATC)_{23}$	F: FAM-GGGAGTTTGGCCCTCTAGTT R: GTTTGCCCCACTATGGGTATCTCC	235-291	20	13	0.26	0.87	0.85	0**	0.3392	JX413383
CSBm4_57	$(TATG)_5$	F: FAM-GGATCGAAACTCCAATCTGC R: GTTTCACCTGTGCATACGCATAAT	199-222	20	5	0.25	0.71	0.67	0**	0.3027	JX413384
CSBm4_5	$(CATA)_6$	F: FAM- CAATTTCATTCGTATCTCATCC R: GTTTCAACAACATAAGAACCGCACA	243-247	20	3	0.10	0.34	0.31	0.004*	0.2579	JX413385

n, number of tested individuals; *a*, number of observed alleles; H_O,observed heterozygosity; H_E, expected heterozygosity; PIC, polymorphic information contents; † significant deviation from Hardy-Weinberg equilibrium (*$p < 0.05$, ** $p < 0.01$).

homozygotes in CS.Bm4_25 (0.16), 222/222 homozygote in CS.Bm4_57 (0.35), and 243/243 homozygote in CS.Bm4_5 (0.75), indicating an excess of homozygosity in many loci (Table 2). In addition, the availability, which is defined as 1 - *Obs/n*, where *Obs* is the number of observations

Table 2. Genotype frequencies for each microsatellite marker.

Marker	Allele1	Allele2	Count	Frequency[a]	Availabilty[b]
CS.Bm2_3	150	150	1	0.05	1.00
	150	153	1	0.05	
	150	156	2	0.10	
	153	153	7	0.35	
	153	156	2	0.10	
	156	156	7	0.35	
CS.Bm2_5	128	128	1	0.05	1.00
	128	134	1	0.05	
	131	131	2	0.10	
	131	134	4	0.20	
	131	137	1	0.05	
	134	134	8	0.40	
	134	137	1	0.05	
	134	140	1	0.05	
	140	140	1	0.05	
CS.Bm2_9	230	230	1	0.05	1.00
	230	239	2	0.10	
	233	239	1	0.05	
	233	242	1	0.05	
	235	238	1	0.05	
	236	236	5	0.25	
	239	239	7	0.35	
	242	242	2	0.10	
CS.Bm3_37	239	239	8	0.40	1.00
	239	242	5	0.25	
	242	242	7	0.35	
CS.Bm4_14	138	138	5	0.25	1.00
	138	144	1	0.05	
	144	144	12	0.60	
	144	157	2	0.10	
CS.Bm4_20	320	320	5	0.25	1.00
	320	330	3	0.15	
	330	330	8	0.40	
	336	336	4	0.20	
CS.Bm4_25	231	235	1	0.05	0.95
	235	235	3	0.16	
	235	239	1	0.05	
	235	288	1	0.05	
	239	239	3	0.16	
	242	242	1	0.05	
	243	243	1	0.05	
	245	245	1	0.05	
	247	247	1	0.05	
	251	263	1	0.05	
	251	291	1	0.05	

Table 2. Continued.

	259	259	1	0.05	
	275	275	1	0.05	
	288	288	2	0.11	
	199	199	1	0.05	
	199	205	1	0.05	
	199	222	1	0.05	
	205	205	2	0.10	
CS.Bm4_57	210	210	4	0.20	1.00
	210	222	1	0.05	
	214	214	1	0.05	
	214	222	2	0.10	
	222	222	7	0.35	
	243	243	15	0.75	
CS.Bm4_5	243	247	1	0.05	1.00
	243	252	1	0.05	
	247	247	2	0.10	

[a]The sample allele frequencies are calculated as $\tilde{p} = n_u /(2n)$, with the variance estimated as $\mathrm{Var}(\tilde{p}_u) \widehat{=} \frac{1}{2n}(\tilde{p}_u + \tilde{p}_{uv} - 2\tilde{p}_u^2)$, where $\widehat{=}$means "estimated by". The sample genotype frequencies $\tilde{p}_{uv}$ are calculated as n_{uv}/n.Both the $\tilde{p}_u$ and $\tilde{p}_{uv}$ are unbiased Maximum Likelihood Estimates (MLEs) of the population frequencies. Confidence intervals for allele and genotype frequencies are formed by resampling individuals form the data set; [b],Availability is defined as1−*Obs*/ *n* , where *Obs*is the number of observations and *n* is the number of individuals sampled. And the number of observation for a marker locus is defined as the number of non-missing alleles (for haploid data) or non-missing genotypes (for diploid data) observed in the sample. A genotype is regarded as missing if one of its two alleles is missing.

of given markers and n is the number of individuals sampled, was 0.95 to 1.00 with an average of 0.99 per locus, providing generally high values (Table 2). Collectively, the microsatellite markers developed in this study will be very valuable for better understanding the population and species complexity of *C. septempunctata.*

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