Int. J. Indust. Entomol. Vol. 25, No. 1, 2012, pp. 81~92

International Journal of *Industrial Entomology*

Microsatellite Analysis of the Silkworm Strains (*Bombyx mori*) Originated from China

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(Received 15 August 2012; Accepted 4 September 2012)

A total of 85 Chinese-origin silkworm strains preserved in Korea were genotyped for eight polymorphic microsatellite loci. We obtained per-locus number of alleles, ranging from 5 to 14 with an average value of 9.5, per-locus observed heterozygosity, ranging from 0.07 to 0.99, and per-locus polymorphic information content (PIC), ranging from 0.34 to 0.82, indicating that some loci are highly variable. Phylogenetic analysis with the eight concatenated microsatellite loci showed no clustering on the basis of known strain characteristics. A total of 22 strain-specific apomorphic alleles, which discriminate 19 among 85 silkworm strains were obtained from eight loci. These strain-specific alleles, thus, can casually be utilized for the discrimination of applicable strains without any further typing of other loci. Furthermore, a substantial number of homozygote strains, represented by 27 among 76 alleles in eight loci were found. These results collectively suggest that the silkworm microsatellite DNA is actually and potentially important molecular markers for the eventual discrimination of silkworm strains that are preserved as hundreds in Korea.

Key words: Silkworm strain, *Bombyx mori*, Microsatellite DNA, Allele, Simple sequence repeat

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http://dx.doi.org/10.7852/ijie.2012.25.1.081

Introduction

Silkworm strains are mainly defined by genetics, but also divided based on their geographic origin. Different climates and regional environment produce silkworm strains that possess different types of characteristics, due mainly by geographical isolation and local adaptation to a given environment. Consequently, one of the ways to classify world silkworm strains is geographic origin-based one, namely Japanese strain, Chinese strain, European strain, Tropical strain, and also Korean strain.

In Korea, approximately 85 China-originated strains are under conservation in the government institute, National Academy of Agricultural Science (NAAC; http://www.naas.go.kr/) and also in local government facilities. These strains are under continuous annual rearing to prevent genetic deterioration and maintain strain-specific characteristics. Due mainly to long inbreeding these silkworm strains possess relatively low level of genetic diversity compared to other insect species found in nature, except for the genes encoding for strain-specific characteristics, such as larval/cocoon color, larval marking, cocoon shape, voltinism, moltinism and so on (Kang *et al.*, 2009). However, these characteristics overlap substantially among strains, and the genes encoding for strain-specific characteristics are mostly polygene that have not well been characterized. Thus, discrimination of the silkworm strains on the basis of these characteristics may not be successful. Nevertheless, an enrichment of potential molecular markers for the discrimination of the silkworm strains is urgent for long-term conservation purpose.

The China-originated strains generally have characteristic features, such as a heavy feeding, a fast and uniform

larval growth, possession of plain larval marking in general, insensitivity to high temperature and muscardine (Lim *et al.*, 1996). Cocoon shapes are diverse in that some strains are oval (elliptical), spherical, or spindle (Lim *et*

Table 1. General information on the silkworm strains utilized in this study

Strain number	Strain	Origin	Voltinism	Moltinism	Egg color	Blood color	Cocoon color/shape
46	141	China	2	4	B	W	W/Oval
82	Kumkang	China	2	4	B	W	W/Oval
84	Gal H	China	2	4	B	W	W/Oval
85	Galwon	China	2	4	B	W	W/Peanut
86	Nakdong	China	2	4	B	W	W/Oval
87	Daedong	China	2	4	B	W	W/Oval
88	C3	China	2	4	B	W	W/Oval
89	C5	China	2	4	B	W	W/Oval
90	C7	China	2	4	B	W	W/Oval
91	C10	China	2	4	B	W	W/Oval
92	C11	China	2	4	B	W	W/Oval
93	C12	China	2	4	B	W	W/Oval
94	C14	China	2	4	B	W	W/Oval
95	C16	China	2	4	B	W	W/Oval
96	C17	China	2	4	B	W	W/Oval
97	C18	China	2	4	B	W	W/Oval
98	C25	China	2	4	B	W	W/Oval
99	C26	China	2	4	B	W	W/Oval
100	C27	China	2	4	Bb	W	W/Oval
101	C31	China	2	4	B	W	W/Oval
102	C42	China	2	4	B	W	W/Oval
103	C44	China	2	4	B	W	W/Oval
104	C45	China	2	4	B	W	W/Oval
105	C46	China	2	4	B	W	W/Oval
106	C48	China	2	4	B	W	W/Oval
107	C51	China	2	4	B	W	W/Oval
108	C53	China	2	4	B	W	W/Oval
109	C57	China	2	4	B	W	W/Oval
110	C60	China	2	4	B	W	W/Oval
112	C61	China	2	4	B	W	W/Oval
113	C66	China	2	4	B	W	W/Oval
114	C68	China	2	4	B	W	W/Oval
115	C70	China	2	4	B	W	W/Oval
116	C76	China	2	4	B	W	W/Oval
117	C78	China	2	4	B	W	W/Oval
118	C 79	China	2	4	B	W	W/Peanut
120	Sinchung 102	China	2	4	B	W	W/Peanut
121	Soyang	China	2	4	B	W	W/Oval
123	SC	China	2	4	B	W	W/Long peanut
124	4056	China	2	4	B	W	W/Peanut
126	Usungrokgyun	China	2	4	B	W	LG/Peanut
127	Woongjinhi	China	2	4	B	W	W/Oval

Table 1. Continued

Strain number	Strain	Origin	Voltinism	Moltinism	Egg color	Blood color	Cocoon color/shape
128	Yonggakjam	China	2	4	B	W	W/Peanut
129	UR	China	2	4	B	W	W/Oval
130	R Hwang	China	2	4	B	Y	Y/Oval
131	LY	China	2	4	B	Y	LYG/Peanut
132	Jam 104	China	2	4	B	W	W/Oval
133	Jam 116	China	2	4	B	W	W/Oval
134	Chung 14	China	2	4	B	Y	Y/Oval
135	Chung 17	China	2	4	B	Y	F/Peanut
136	Chung 112	China	2	4	B	W	W/Peanut
137	Chungjong	China	2	4	B	W	W/Oval
138	Chungchun	China	2	4	B	W	W/Oval
139	Chunmun	China	2	4	B	Y	LY/Oval
144	Hangang	China	2	4	B	W	W/Oval
164	ZO	China	2	4	B	W	W/Oval
165	KH	China	2	4	B	W	W/Peanut
187	Kumho	China	2	4	B	W	W/Short oval
195	Sinchung103	China	2	4	B	W	W/Peanut
196	Jam 110	China	2	4	B	W	W/Oval
199	Hansang 4ho	China	2	4	B	W	W/Oval
210	C Sugang	China	2	4	B	W	W/Oval
230	Chung 7	China	2	4	B	Y	Y/Oval
233	Suwonjam 102	China	2	4	B	W	W/Oval
241	Jam 106	China	2	4	B	W	W/Short oval
257	CH 1	China	2	4	B	W	W/Oval
258	CH 2	China	2	4	B	W	W/Oval
259	CH 3	China	2	4	B	W	W/Oval
260	CH 4	China	2	4	B	W	W/Oval
261	Yunil	China	2	4	B	W	W/Short oval
262	Jam 118	China	2	4	B	W	W/Short oval
275	Jam 108	China	2	4	B	W	W/Oval
281	Jam 122	China	2	4	B	W	W/Oval
282	Jam 128	China	2	4	B	W	W/Oval
283	Jam 130	China	2	4	B	W	W/Oval
284	Jam 132	China	2	4	B	W	W/Oval
285	Jam 136	China	2	4	B	W	W/Oval
286	Jam 138	China	2	4	B	W	W/Oval
288	NB 7	China	2	4	B	W	W/Oval
289	C 108	China	2	4	B	W	W/Oval
322	Jam 304	China	unknown	4	B	W	Y(♀), W(♂)/Oval
324	Jam 114	China	unknown	4	B	W	W/Oval
336	Jam 134	China	2	4	B	W	W/Oval
338	Jam 142	China	2	4	B	W	W/Oval
340	Jam 148	China	2	4	B	W	W/Oval

M, multi-voltine strain; B, black; R, red; DR, dark red; W, white; Bb, bright brown; Br, brown; Y, yellow; LYG, light yellow green; F, Flesh; LG, light green; PG, pale green; LPG, light pale green; LY, light yellow; PW, pale white; C, cream; and -, no rigid cocoon shape.

Table 2. Information of the eight microsatellite loci

Primer name	Primer sequence (5-3)[a]		Motifs[b]	Tem. (°C)[c]	Expected size (bp)	Size range (bp)	GenBank no.	References
K02	F	attgtaaccgatttgagaga	$(ga)_{23}$	46	107-122	105-121	DE376976	Unpublished
	R	attcgcacaataagttcact						
D49370	F	agcgcacccttatgacgat	$(cta)_2$-$(cta)_5$	48	220	202-235	D49370	Kawabata *et al.* (1995)
	R	gaaagtggaagtcgtgtact						
D90454	F	tgcgatgtctacatggtgg	$(ggc)_6$	54	183	181-184	D90454	Itoh *et al.* (1991)
	R	gtctctcgatagcttcct						
Bmsat127	F	aggcttagtgacgagccgt	$(tttga)_4$	50	193	180-193	DQ383507	Reddy *et al.* (1999)
	R	ggtgccaatcattcttatt						
D49948	F	acgcagacgagaagctcac	$(ca)_3$-$(ca)_5$	48	200	206-240	D49948	Ohta *et al.* (1995)
	R	actgcaccgtatatgcga						
Bmsat129	F	agttacccaggttgtctt	$(at)_{27}$	50	198	170-208	DQ383509	Reddy *et al.* (1999)
	R	cgacactggttctcatac						
X17219	F	gcaagccaccagttagatatgg	$(at)_9$	68	214	210-220	X17219	Michaille *et al.* (1990)
	R	cacgtacgttgcgttcaccg						
AF005384	F	caagatgtccaagagtg	$(gt)_2$-$(gtt)_4$-$(gc)_2$	46	225	177-181	AF005384	Unpublished
	R	ccggtgagagggacctt						

[a]Forward (F) and reverse (R) primer sequences for PCR. The forward primer was labeled with a fluorescent dye for genotyping.
[b]Sequences inside parenthesis indicate the motif sequence of the microsatellite DNA and subscripted numbers indicate number of repeats originally found at the microsatellite locus.
[c]Tem., annealing temperature for PCR.

al., 1996). Further, they are either univoltine, bivoltine or multivoltine, and some strains are three-moultering ones, although most strains are four molters (Lim *et al*., 1996). Therefore, strain discrimination based solely on morphological and physiological characters are hampered.

Microsatellite DNA is simple sequence repeats (one ~ six base pairs) that are abundantly present in all eukaryotic nuclear and some prokaryotic genomes (Tautz and Renz, 1984). Allelic hypervariability, conservation in flanking sequence, co-dominant mode of inheritance and abundance of microsatellites coupled with the advances in PCR technology made the molecules a useful marker for closely related species or population genetic studies (Huang *et al.*, 1998; Weber and May, 1989; Woodruff, 1993). Due by these merits microsatellite DNA has been used in many organisms for a diverse purpose (Edwards *et al.*, 1991; Morgante and Olivieri, 1993; Dallas *et al.*, 1995; Viard *et al.*, 1996; Mariat *et al.*, 1996; Pope *et al.*, 1996; Romero *et al.*, 2003). In the silkworm, abundant microsatellite primers have been published by several authors (e.g., Dharma Prasad *et al.*, 2005), but only some of the Chinese strains kept in Korea were genotyped using microsatellite DNA (Kim *et al.*, 2010).

In this study, we selected eight available microsatellite DNA from literatures and genotyped China-originated 85 silkworm strains preserved in Korea, in order to determine the utility of the markers in detecting DNA polymorphism and to assess their potential for use in strain discrimination.

Materials and Methods

Silkworm strains

Silkworm strains chosen for the present study are 85 China-originated strains that are under conservation in NAAC, Republic of Korea. Their voltinism, moltinism, egg color, blood color, and cocoon color/shape are presented in Table 1.

Genomic DNA extraction, amplification, and genotyping

Approximately 100 eggs of each *B. mori* strain were crushed in a glass grinder in liquid nitrogen, and genomic DNA was extracted using the DNA Extraction Kit, in accordance with the manufacturer's instruction (Qiagen, USA). Eight oligonucleotide primers either published or GenBank-registered were successfully adapted to amplify DNA segments containing microsatellite DNA (Table 2) These were previously utilized for the genotyping of silkworm strains kept in Korea (Kim *et al.*, 2010). In order to verify the presence of proper simple sequence repeats in the microsatellite loci PCR amplicons of each locus from one or two strains were cloned and sequenced. Locus

Table 3. Summary statistics of the eight microsatellite loci

Locus	Sample size	Availability[a]	Major allele frequency[b]	Genotype number	Allele number	*He*[c]	*Ho*[d]	PIC[e]
K02	85	1.00	0.78	9	6	0.37	0.07	0.35
D49370	85	1.00	0.62	11	8	0.56	0.45	0.52
D90454	85	1.00	0.66	5	5	0.46	0.67	0.38
Bmsat127	81	0.95	0.80	12	10	0.35	0.17	0.34
D49948	85	1.00	0.73	9	9	0.44	0.54	0.42
Bmsat129	82	0.96	0.30	19	14	0.83	0.79	0.80
X17219	85	1.00	0.41	15	10	0.75	0.99	0.72
AF005384	73	0.85	0.23	20	14	0.84	0.56	0.82
Mean	83	0.97	0.57	12.5	9.5	0.58	0.53	0.54

[a]Availability is defined as 1-*Obs*/*n*, where *Obs* is the number of observations and *n* is the number of individuals sampled (85 individuals).
[b]Major allele frequency indicate the sum of allele frequency with the most higher frequency.
[c]Expected heterozygosity (*He*) is defined as the probability that two randomly chosen alleles from the strains are different.
[d]Observed heterozygosity is simple the proportion of heterozygous individuals in the strains.
[e]PIC, polymorphic information contents.

information, such as repeat motif, annealing temperature, expected size and so on is presented in Table 2.

PCR was carried out in a 25 μl reaction volume containing ~30 ng of genomic DNA, each 200 nM of reverse and forward primers, 200 μM of each dNTP, 2.5 μl of 10X PCR buffer [50 mM KCL, 10 m M Tris-HCl (pH 8.8), 150 nM KCl, 1.5 mM $MgCl_2$], and 1 unit of FR-*Taq* DNA polymerase (Biomedic, Korea) using a ABI 2720 Thermo cycler (Applied Biosystems, USA). The condition for PCR amplification was as follows: the extracted DNA was initially denatured at 95°C for 3 min followed by 30 cycles (denaturation at 94°C for 30 sec, annealing at 46°C ~ 68°C for 30 sec, and extension at 72°C for 1 min). The final extension step was increased from 1 min to 6 min. Each one primer per locus was tagged with 6-FAM fluorescent dye (Yue *et al.*, 2000) to detect the fragment peak later in the automatic DNA sequencer, ABI 3703xl (Applied Biosystems, USA). In order to confirm successful DNA amplification, electrophoresis was carried out in 1.4% agarose gel for 1 hr. For the eventual size estimation, 0.2 μl of PCR product was mixed with 9.8 μl of Hi-Di Formamide (Applied Biosystems, USA) and 0.2 μl of Liz-500 size standard (Applied Biosystems, USA). This mixture was then denatured at 95°C for 5 min, placed on ice, and ran on a ABI 3703xl (Applied Biosystems, USA). GENEMAPPER ver. 4.0 (Applied Biosystems, USA) was used to decide allele sizes. For the accuracy of size determination electrophoresis was carried out at least for three times mostly using two independent PCR products.

Variation and phylogenetic analyses

From each locus observed heterozygosity (H_o; Weir, 1996), expected heterozygosity (E_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 PIC was calculated using the following formula: $PIC = 1-\Sigma (P_i)^2$, where P_i is the proportion of the strain carrying i^{th} allele, calculated for each microsatellite locus (Bostein *et al.*, 1980). The relationships among silkworm strains were constructed by Neighbore-Joining (NJ) method using the shared allelic methods (Jin and Charkaborty, 1994) as a distance matrix. This analysis also was performed using PowerMarker (Liu and Muse, 2005).

Results and Discussion

Characteristics of alleles

Size estimation of the 85 Chinese silkworm stains from eight microsatellite loci was mostly successful, but 12 strains at AF005384 were not successful either by none or multiple amplification. Thus, 82.6 among 85 strains per locus were genotyped on average (Table 3). Total of alleles and the average allele number were respectively 76 and 9.5 from eight loci (Table 3). The locus Bmsat129 and locus AF005384, which are supposed to be consisted of each di- and composition of di- and tri-nucleotide repeats in original study (Reddy *et al.*, 1999; Unpublished, GenBank accession number AF005384) provided the highest allele number,14 (Table 3). On the other hand, the loci K02 and D90454 that are supposed to be each di- and tri-nucleotide repeats (Unpublished, GenBank accession number DE376976; Itoh *et al.*, 1991), respectively, pro-

Table 4. Genotypes of 85 China-originated silkworm strains at each microsatellite locus

Strain number	Strain	Microsatellite locus (bp) :															
		K02		D49370		D90454		Bmsat127		D49948		Bmsat129		X17219		AF005384	
46	141	104	104	202	251	177	180	190	190	199	265	171	175	215	223	188	246
82	Kumkang	104	104	202	251	177	180	190	190	191	199	171	175	215	225	188	246
84	Gal H	117	117	202	257	177	180	190	190	199	265	169	169	215	221	188	188
85	Galwon	104	104	202	257	177	180	182	190	199	199	169	169	217	223	188	246
86	Nakdong	104	104	251	257	177	180	190	190	199	199	169	169	217	223	174	218
87	Daedong	104	104	251	251	156	180	160	190	199	199	171	175	215	225	188	246
88	C3	104	104	251	251	177	180	190	190	191	199	171	177	215	225	188	246
89	C5	104	104	251	251	177	180	190	190	199	209	171	171	215	221	188	246
90	C7	104	104	257	257	177	180	190	190	191	199	171	177	215	219	188	246
91	C10	110	110	215	251	177	180	190	190	191	199	169	169	215	223	174	174
92	C11	104	104	251	251	177	180	190	190	199	199	171	177	215	223	174	218
93	C12	104	104	202	251	177	180	190	190	191	199	-	-	215	221	174	174
94	C14	104	104	251	251	177	180	190	190	199	199	173	175	215	223	174	218
95	C16	104	104	251	251	177	180	190	190	191	199	171	175	215	223	-	-
96	C17	104	104	251	251	177	180	190	190	199	199	171	175	205	223	174	174
97	C18	104	104	251	257	177	180	190	190	199	265	-	-	215	223	172	172
98	C25	104	104	202	257	177	180	190	190	191	199	171	175	215	223	160	160
99	C26	104	104	251	251	177	180	190	190	199	243	171	175	215	223	-	-
100	C27	104	104	251	251	177	180	190	190	199	199	171	171	215	248	-	-
101	C31	104	115	202	257	174	180	190	190	199	199	171	177	215	215	-	-
102	C42	104	104	251	251	177	180	190	190	199	199	171	175	215	248	-	-
103	C44	104	104	251	251	177	180	184	190	199	199	177	177	215	223	174	174
104	C45	104	104	215	251	177	180	190	190	199	265	163	169	217	223	172	172
105	C46	104	104	251	251	177	180	160	190	199	199	-	-	215	223	172	172
106	C48	110	110	202	251	177	180	190	190	199	209	171	177	217	223	-	-
107	C51	104	104	202	261	177	180	190	190	199	199	171	177	215	221	160	160
108	C53	115	115	215	257	177	180	190	190	199	265	163	169	215	221	-	-
109	C57	104	104	251	251	180	180	190	190	199	199	165	171	219	221	200	200
110	C60	115	115	251	251	174	180	-	-	191	199	171	175	217	219	172	188
112	C61	104	104	215	251	177	180	190	190	199	199	163	169	217	223	172	172
113	C66	104	104	257	257	177	180	190	190	199	199	171	175	215	223	172	172
114	C68	104	104	257	257	177	180	190	190	199	199	169	171	217	248	-	-
115	C70	104	104	202	251	177	180	190	190	191	199	155	171	215	248	-	-
116	C76	104	104	202	251	177	180	190	190	191	199	155	171	215	223	-	-
117	C78	104	104	251	251	180	180	190	190	199	199	175	177	215	223	216	237
118	C 79	110	115	251	257	177	180	190	190	199	221	171	177	217	223	-	-
120	Sinchung 102	110	110	257	257	177	180	190	190	199	199	175	177	217	219	174	174
121	Soyang	104	104	257	257	177	180	190	190	191	199	169	171	217	223	174	188
123	SC	104	104	215	257	174	180	190	190	199	199	169	187	215	221	218	218
124	4056	115	115	215	257	177	180	190	190	199	265	169	169	215	223	174	218
126	Usun-grokgyun	104	104	202	251	177	180	-	-	191	199	173	173	197	217	174	218
127	Woongjinhi	104	104	257	257	177	180	190	190	191	199	153	153	207	215	174	188
128	Yonggakjam	104	104	251	251	177	180	160	190	199	199	153	153	217	223	172	188

Table 4. Continued

Strain number	Strain	Microsatellite locus (bp) :															
		K02		D49370		D90454		Bmsat127		D49948		Bmsat129		X17219		AF005384	
129	UR	104	104	251	251	177	180	190	190	191	199	171	175	215	221	204	246
130	R Hwnag	115	115	251	251	177	180	190	190	199	281	177	177	215	223	186	204
131	LY	115	115	251	257	177	180	190	190	199	265	171	177	215	223	174	218
132	Jam 104	104	104	251	251	177	180	190	190	199	243	175	177	215	221	186	204
133	Jam 116	104	104	251	251	177	180	190	190	199	199	171	175	215	221	204	246
134	Chung 14	104	104	224	251	177	180	160	164	199	265	153	153	215	223	188	188
135	Chung 17	104	104	215	251	180	180	194	194	199	265	171	177	215	223	218	218
136	Chung 112	110	110	251	251	177	180	190	190	199	199	171	175	215	221	-	-
137	Chungjong	104	104	202	251	180	180	190	190	199	199	171	177	215	223	188	218
138	Chungchun	104	104	202	251	180	180	190	190	199	199	171	177	215	223	188	255
139	Chunmun	104	104	215	259	177	180	190	190	199	271	169	169	215	223	188	188
144	Hangang	104	104	251	251	180	180	182	190	199	199	153	173	215	219	188	188
164	ZO	104	104	251	251	180	180	175	190	199	199	171	175	217	225	188	188
165	KH	110	110	251	257	180	180	190	190	199	265	171	177	215	223	174	174
187	Kumho	104	104	251	251	180	180	190	190	199	199	171	175	215	221	246	246
195	Sinchung103	110	115	251	251	180	180	182	190	199	265	169	169	217	223	188	188
196	Jam 110	104	104	251	251	180	180	190	190	199	265	171	175	215	219	188	218
199	Hansang 4ho	104	104	251	251	180	180	190	190	199	199	171	175	215	219	246	246
210	C Sugang	120	120	202	257	177	180	188	188	199	265	169	169	215	223	246	246
230	Chung 7	104	104	251	257	180	180	-	-	199	243	153	153	215	223	218	218
233	Suwonjam 102	104	104	251	251	180	180	164	190	199	199	169	187	217	223	188	246
241	Jam 106	104	104	195	202	180	180	190	190	199	199	171	175	215	221	188	246
257	CH 1	104	104	251	257	177	180	190	190	199	265	173	173	215	221	188	246
258	CH 2	115	115	202	251	177	180	192	192	199	199	171	177	215	221	172	172
259	CH 3	104	117	251	257	177	180	192	192	199	265	171	175	215	221	174	188
260	CH 4	110	113	202	257	177	180	192	192	199	265	171	175	215	219	172	172
261	Yunil	104	104	202	251	177	180	192	192	199	199	157	173	215	221	172	172
262	Jam 118	104	104	202	251	177	180	184	190	199	199	171	175	215	221	174	174
275	Jam 108	104	104	251	251	180	180	190	190	199	265	171	175	215	221	191	246
281	Jam 122	104	104	251	251	180	180	186	186	199	199	171	175	215	221	246	246
282	Jam 128	104	104	251	251	180	180	184	190	199	199	169	187	215	221	188	246
283	Jam 130	104	104	251	251	180	180	188	192	199	199	169	187	215	221	246	246
284	Jam 132	104	104	251	251	180	180	190	190	199	265	183	185	215	221	191	246
285	Jam 136	104	115	251	251	180	180	190	190	199	199	171	175	215	223	191	241
286	Jam 138	104	104	251	251	180	180	190	190	199	265	169	187	215	221	191	246
288	NB 7	115	115	251	257	180	180	190	190	199	239	171	175	215	223	191	218
289	C 108	104	104	257	257	180	180	184	190	199	265	169	187	205	215	191	246
322	Jam 304	104	104	251	251	180	180	186	186	199	199	153	171	215	219	191	246
324	Jam 114	104	104	251	251	171	180	-	-	199	265	171	175	215	219	188	246
336	Jam 134	104	104	251	251	180	180	190	190	199	199	183	185	205	215	188	246
338	Jam 142	104	104	251	251	180	180	190	190	191	199	169	187	215	221	172	246
340	Jam 148	104	104	251	251	180	180	190	190	199	265	183	185	215	221	188	246

vided only six and five alleles, respectively (Table 3). Thus, the allele number at each locus is unlikely to have a direct relation to the length of repeat unit as has previously been suggested (Kim *et al.*, 2010). Previously, Li et al. (2005) assayed 26 microsatellite markers from 31 silkworm strains and found a total of 188 alleles with a mean value of 7.2 alleles, ranging in number from two to 17.

Heterozygosity

The number of genotype also is roughly proportional to allele number (Table 3). For example, the locus D90454, which provided five alleles, only resulted in five genotypes in 85 strains, whereas the loci Bmsat129 and AF005384, which provided both 14 alleles resulted in 19 genotypes in 82 strains and 20 genotypes in 73 strains, respectively (Table 3). The frequency of the most common allele in each locus ranged from 0.23 (the locus AF005383) to 0.80 (the locus Bmsat127) (Table 3). Thus, some loci provided very high frequency of a particular allele, but others are not. For example, the allele 180 found in the locus D90454 is represented by all strains, but the remaining four alleles, 156, 171, 174 and 177, found in the locus D90454 were observed as a heterozygote, in combination with the allele 180 (Table 4).

The expected (H_e) and the observed heterozygosity (H_o) over all microsatellite loci ranged from 0.35 to 0.84 and 0.07 to 0.99, respectively (Table 3). The loci D90454, D49948, Bmsat129, and X17219 revealed somewhat higher or equivalent estimates of H_o compared to H_e, but the remaining loci K02 (0.37 vs. 0.07), D49370 (0.56 vs. 0.45), Bmsat127 (0.35 vs. 0.17), and AF005384 (0.84 vs. 0.56) have shown substantially lower estimates of H_o, may indicating long-held inbreeding within the strains. The PIC value, which is the estimation of the probability that a strain is informative with respective to segregation of its inherited alleles ranged from 0.34 to 0.82 with an average of 0.54 per locus (Table 3). The locus AF005384, which provided the high allele number as 14 has recorded the highest value of 0.82, and the D49370, Bmsat129, and X17219 recorded the PIC values higher than 0.50, indicating the high power of strain discrimination in those loci. Previous work of Kim *et al.* (2010) with 54 silkworm strains originated from several countries has shown the mean PIC in overall loci as 0.47, ranging from 0.06 to 0.86 at each locus.

Analysis of relationships among silkworm strains

To test whether or not the microsatellite DNA reflects any known general characteristics of silkworm strains (Table 1) phylogenetic relationships among the silkworm strains were constructed by NJ method (Fig. 1). From the careful examination of the tree we found no clear grouping of

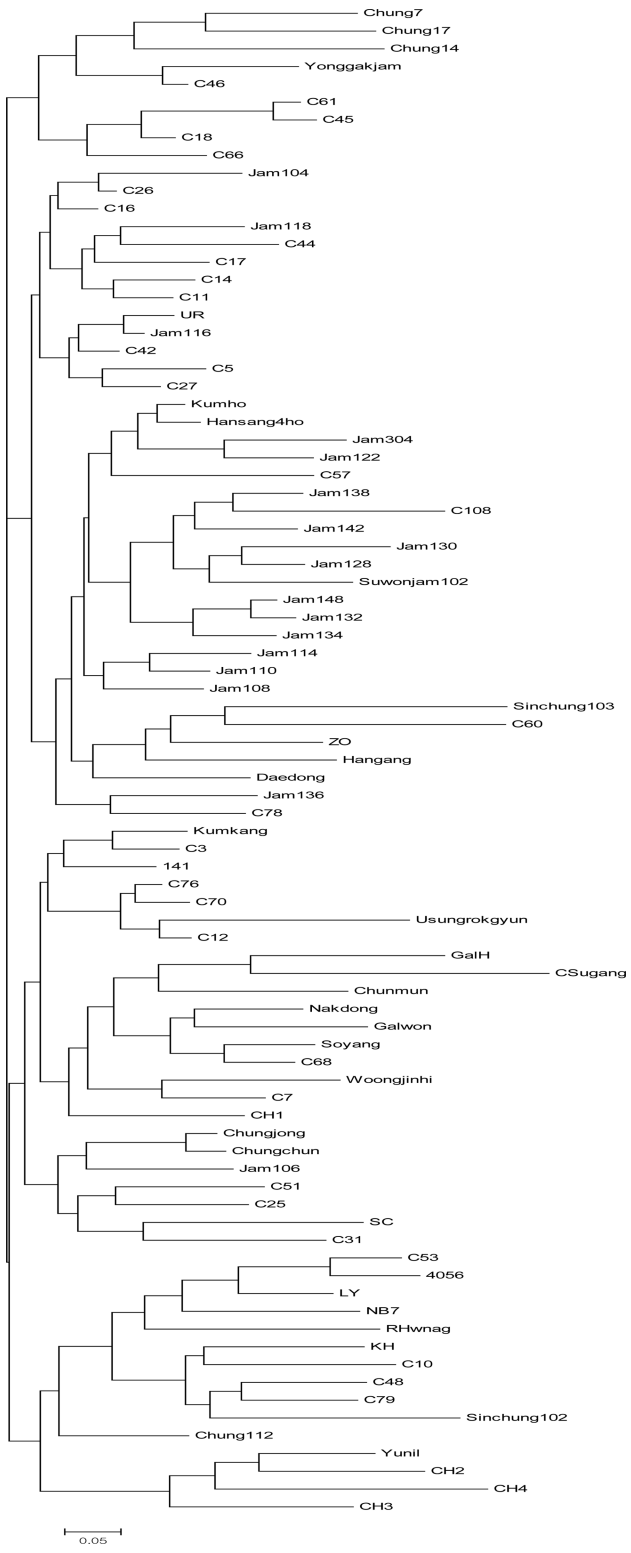

Fig. 1. Neighbor-Joining tree for genetic relationships among 85 silkworm strains. The genetic distances between strains were calculated based on the shared allelic methods (Jin and Chakraborty, 1994) using the PowerMarker v3.25 program. The scale bar indicates the branch length.

Table 5. Genotype frequency in each microsatellite marker among 85 silkworm strains

Locus	Allele 1	Allele 2	No. of strains	Frequency	Locus	Allele 1	Allele 2	No. of strains	Frequency
K02	104	104	65	0.765		155	171	2	0.024
	104	115	2	0.024		157	173	1	0.012
	104	117	1	0.012		163	169	3	0.037
	110	110	5	0.059		165	171	1	0.012
	110	113	1	0.012		169	169	8	0.098
	110	115	2	0.024		169	171	2	0.024
	115	115	7	0.082		169	187	7	0.085
	117	117	1	0.012		171	171	2	0.024
	120	120	1	0.012		171	175	26	0.317
						171	177	13	0.159
D49370	195	202	1	0.012		173	173	1	0.012
	202	251	12	0.141		173	175	1	0.012
	202	257	6	0.071		173	176	1	0.012
	202	261	1	0.012		175	177	3	0.037
	215	251	4	0.047		177	177	2	0.024
	215	257	3	0.035		183	185	3	0.037
	215	259	1	0.012					
	224	251	1	0.012	X17219	197	217	1	0.012
	251	251	40	0.471		205	215	2	0.024
	251	257	9	0.106		205	223	1	0.012
	257	257	7	0.082		207	215	1	0.012
						215	215	1	0.012
D90454	156	180	1	0.012		215	219	7	0.082
	171	180	1	0.012		215	221	25	0.294
	174	180	3	0.035		215	223	26	0.306
	177	180	52	0.612		215	225	3	0.035
	180	180	28	0.329		215	248	3	0.035
						217	219	2	0.024
Bmsat127	160	164	1	0.012		217	223	10	0.118
	160	190	3	0.037		217	225	1	0.012
	164	190	1	0.012		217	248	1	0.012
	175	190	1	0.012		219	221	1	0.012
	182	190	3	0.037					
	184	190	4	0.049	AF005384	160	160	2	0.027
	186	186	2	0.025		172	172	8	0.110
	188	188	1	0.012		172	188	2	0.027
	188	192	1	0.012		172	246	1	0.014
	190	190	59	0.728		174	174	7	0.096
	192	192	4	0.049		174	188	3	0.041
	194	194	1	0.012		174	218	6	0.082
						186	204	2	0.027
D49948	191	199	15	0.176		188	188	6	0.082
	199	199	39	0.459		188	218	2	0.027
	199	209	2	0.024		188	246	14	0.192

Table 5. Continued

Locus	Allele 1	Allele 2	No. of strains	Frequency	Locus	Allele 1	Allele 2	No. of strains	Frequency
D49948	199	221	1	0.012		188	255	1	0.014
	199	239	1	0.012		191	218	1	0.014
	199	243	3	0.035		191	241	1	0.014
	199	265	22	0.259		191	246	5	0.068
	199	271	1	0.012		200	200	1	0.014
	199	281	1	0.012		204	246	2	0.027
						216	237	1	0.014
Bmsat129	153	153	4	0.049		218	218	3	0.041
	153	171	1	0.012		246	246	5	0.068
	153	173	1	0.012					

strains on the basis of any known characteristics, such as voltinism, moltinism, egg color, blood color, and cocoon color/shape (Fig. 1). Similar examples can be found in many other branches of the tree. Thus, it suggests that the microsatellite loci do not reflect any well-known strain characteristics. Instead, it seems that the microsatellite DNA reflect the product of neutral evolution which might add the resolving power for the discrimination of silkworm strains, rather than for the illustration of long-term evolutionary relationships among strains. Our result is similar to previous work for silkworm strains kept in Korea (Kim *et al.*, 2010), but is sharply contradict to the previous microsatellite study of Li *et al.* (2005) in that the 31 silkworm strains originated from a diverse countries were clustered on the basis of their origin. Furthermore our result is sharply different from that of Reddy *et al.* (1999) in that microsatellite markers that they used have partially been successful in discriminating some silkworm strains on the basis of diapause (Reddy *et al.*, 1999). The difference among ours and them may be due to different microsatellite loci utilized and silkworm strains involved in the analyses.

Strain discrimination

The eight microsatellite loci utilized in this study evidenced the presence of strain-specific alleles (Table 5). The locus K02 provided the alleles 113 and 120 that are unique each to the strain CH4 (strain no. 260) and C Sugang (no. 210), respectively (Table 4). In the same manner, the locus D49370 provided the allele 195, 224 and 259 unique to the strain Jam106 (no. 246), Chung14 (no. 134), and Chunmun (no. 139), respectively; the locus D90454 provided the allele 156 unique to Daedong (no. 87), and 171 unique to Jam114 (no. 324); the locus Bmsat127 provided the allele 175 unique to ZO (no. 164), and 194 unique to Chung17 (no. 135); the locus D49948 provided the allele 221 unique to C79 (no. 118), 239 unique to NB7 (no. 288), 271 unique to Chunmun (no. 139), and 281 unique to R Hwang (no. 130); the locus Bmsat129 provided the allele 157 unique to Yunil (no. 261), 165 unique to C57 (no. 109), and 176 unique to Usungrokgyun (no. 126); the locus X17219 provided the allele 197 unique to Usungrokgyun (no. 126), and 207 unique to Woongjinhi (no. 127); and the locus AF005384 provided the allele 200 unique to C57 (no. 109), 237 unique to C78 (no. 117), 241 unique to Jam136 (no. 285), and 255 unique to Chungchun (no. 138) (Table 4). Resultantly, a total of 22 apomorphic alleles, which discriminate 19 among 85 silkworm strains were obtained. These strain-specific alleles, thus, can casually be utilized for the discrimination of applicable strains without any further typing of other loci.

More importantly, each locus provided substantial number of homozygote strains. The locus K02 provided 65 strains with the homozygote of allele 104, five strains with the homozygote of allele 110, seven strains with the homozygote of allele 115, one strain with the homozygote of allele 117, and one strain with the homozygote of allele 120 (Table 5). Likewise, the locus D49370 provided a total of 47 strains possessing homozygote in two alleles; the locus D90454 provided a total of 28 strains possessing homozygote in one allele; the locus Bmsat127 provided a total of 67 strains possessing homozygote in five alleles; the locus D49948 provided a total of 39 strains possessing homozygote in one allele; the locus Bmsat129 provided a total of 17 strains possessing homozygote in five alleles; the locus X17219 provided a total of one strain possessing homozygote in one allele; and the locus AF005384 provided a total of 32 strains possessing homozygote in seven alleles (Table 5). In a total, 27 among 100 alleles in eight loci were typed into homozygote in a substantial number of silkworm strains. Considering that we used ~ 100 eggs per strains for the extraction of DNA no hidden allele is

expected from each locus. Thus, most of silkworm strains are expected to be unambiguously discriminated from each other by the eight microsatellite loci, which are consisted of the homozygote, apomorphic alleles, and remaining genotyping results.

Acknowledgement

This research was supported by the research grant (PJ0069572012) of Rural Development Administration (RDA) of Korea.

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