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Studies on the Hereditary Properties of SSR Marker in Silkworm (*Bombyx mori* L.)

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Two BC1 group, c108 × (p50 × c108) and p50 × (p50 × c108), one group of F_2 progeny, (p50 × c108) F_2, and 3 SSR markers, Fl0539, Fl0626 and Fl0618 were used to test the hereditary properties of SSR markers in silkworm. Fl0539, Fl0626 were proved to be linkage, and Fl0618 was proved to be independent to those two markers. According to Mendel's law, the recombinant value between Fl0539, Fl0626 was calculated in all of these groups, and they were 8.55% (c108BC_1), 8.02% (p50BC_1) and 7.81% (F_2) respectively. There was dominant difference among the crossing-over value using paired-samples tests by SPSS 10.0 software. This research proved that SSR markers were co-dominant in *B. mori* too, and F_2 progeny could be used to construct SSR linkage map although *B. mori* lacked of crossing over in females.

Key words: Silkworm, *Bombyx mori* L., SSR, Hereditary properties

Introduction

In the past decade, several key advances in molecular biology have greatly advanced the impact of molecular genetics in biology. Many kinds of molecular markers, such as restriction fragment length polymorphism (RFLP) (Botstein *et al.*, 1980), random amplified polymorphic DNA (RAPD)(Williams *et al.*, 1990), amplified fragment length polymorphisms (AFLP)(Vos *et al.*, 1995) and simple sequence repeats (SSR)(Tauze *et al.*, 1989) had been developed, and these markers had been used in studying genetic diversity, race identification, constructing genetic linkage map, molecular assistant selection in many species including the silkworm (Shi *et al.*, 1995; Abe *et al.*, 2000; Li *et al.*, 2001; Tan *et al.*, 2001; Chatterjee and Mohandas, 2003; Yao *et al.*, 2003; Lu *et al.*, 2004). Recently, simple sequence repeats (SSRs) or microsatellites have been often and increasingly used. SSRs, consisted of short tandem repeats (usually 1 – 6 nucleotide) have shown advantages over other markers. Microsatellites are very abundant in eukaryotic organisms and relatively spaced throughout genome, therefore, it is a good source of polymorphism (Tauze, 1989; Edwards *et al.*, 1991; Morgante *et al.*, 1993). In addition, SSRs often have flanking regions highly conserved in related species, which allows the use of the same primer pairs in related genomes (Huang *et al.*, 1998).

Microsatellites have been isolated and characterized in many important species, and had been proved to be co-dominant. This property was very benefit for constructing linkage map and research on genetic diversity in germplasms (Goldstein *et al.*, 1995; Xiang *et al.*, 2001; Yu *et al.*, 2004). Reddy *et al.* (1999) obtained 28 SSR markers and used 15 of them to estimate the relationships among 13 strains of silkworm; they classified those strains into diapause and non-diapause groups. Li *et al.* (2005) used 26 SSR markers to study the genetic diversity among 31 strains of silkworm. Prasad *et al.* (2005) estimated that microsatellites account for 0.31% of the genome of *B. mori*, and mapped 37 microsatellites into 8 linkage groups.

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It is common to use backcross population for linkage mapping in *B. mori* because of lack of crossing over in females, and it was impossible to distinguish homozygotes from heterozygotes when the dominant markers were present (Zhao *et al.*, 1995; Tan *et al.*, 2001). But if an F_2 cross was used, it would yield eight-fold savings in labor with only about a two-fold decrease in information content, and would yield maximum efficiency in the initial phases of linkage mapping. RAPDs, AFLPs, and selective amplification DNA fragments (SADFs) can be amplified by PCR, facilitating rapid large-scale genotyping. However, these markers are usually strain specific and, because of their dominant inheritance, limit integration of independently constructed genetic maps. The F_2 progeny raised from a single pair mating of F_1 sibs have been used for molecular map construction and various molecular markers (RAPD, SADF) are being integrated to this common F_2 mapping population, and two sub linkage map were constructed respectively considering the markers source (Promboon *et al.*, 1995; He *et al.*, 2001; Goldsmith *et al.*, 2005). Shi *et al.* (1995) used two programs to analyse RFLP linkage map in silkworm using a F_2 population, and construct an RFLP linkage group covering 15 linkage group and 8 ungrouped loci. Yasukochi (1998) converted F_2 intercross data to BC_1 backcross data on the base of hypothesis that absence of a maternal marker in a certain F_2 progeny meant the nonrecombinant autosome of the progeny was paternal, and then calculated genetic distances between markers using mapmaker software.

Because F_2 progeny contains more information than backcross population, it is very useful and convenient if they can be used in constructing linkage map. In this research, hereditary property of SSR markers were studied and whether F_2 progeny could be used to construct SSR linkage map directly in silkworm was discussed too.

Materials and Methods

Silkworm strains

Silkworm strains c108 and p50 were reared in single batch in Sericultural Research Institute, Chinese Academy of Agricultural Sciences (SRI-CAAS). Two F_1 male moths of the cross p50 × c108 was crossed to a female moth of the recurrent parent c108 or p50 respectively to generate BC1 populations, they were c108 × (p50 × c108) (c108BC_1) and p50 × (p50 × c108) (p50BC_1). An F_2 intercross was performed using F_1 sibs from the same single pair mating of a c108 female by a p50 male. Three hundred individuals of each population were used for DNA isolation. At the same time, an F_1 female of p50 × c108 was crossed to a male of c108 to group the SSR makers, which took the advantage of no crossing over occurs in silkworm females.

DNA extraction

Genomic DNA was prepared from larval posterior silk glands following analysis. In each of the strains, DNA was extracted from 12 individuals. A pair of whole silk glands from last stage larvae was ground with a mechanical homogenizer in a microcentrifuge tube and suspended in DNA extraction buffer (50 mM Tris-HCI, pH 8.0, 100 mM NaCl, 20 mM EDTA) containing 100 μg/ml proteinase K. After digestion with proteinase K at 50°C for 8 – 10 hrs, phenol/chloroform extraction was carried out and DNA was recovered by isopropanol precipitation. Purified DNA was dissolved in 0.1 × TE buffer (pH 8.0). DNA concentration was measured spectrophotometerically (BioPhotometer, Eppendorf).

Microsatellites and PCR amplification

Genomic DNA from silkworm strain-p50 was digested with two enzymes, Sau3AI, or Tsp509I (Takara). Size-selected DNA (7 – 10 kb) was cloned into the pUC18 vector. DNA from the recombinant clones was transferred to nylon membranes (Hybond N, Amersham) by the dot-blot method. Membranes were soaked in 2 × SSC for 5 min, then air-dried and used for hybridization. Probes of the repeat sequences, $(CA)_{15}$ and $(CT)_{15}$, were hybridized with the genomic libraries and positive clones were selected. These selected positive clones were sequenced with a ABI 3700 DNA automatic sequencer using the ubiquitous primers $(GT)_7$ or $(GA)_7$. Forward primers of the microsatellite loci were designed from the first sequence of the positive clones. Reverse primers of the microsatellite loci were designed from the second sequencing using the forward primers as the primers for the second sequencing of the same clone. The microsatellite primers were designed from the repeat flanking sequences using the program Primer Premier 5.00 (Premier Biosoft International, Palo, Alto, Calif. http://www.premierbiosoft.com). Three microsatellites (Fl0539, Fl0618 and Fl0626) (Table 1) were used in this research; they had been proved to be homozygous in c108 and p50 respectively, but be polymorphic between them. Two of them (Fl0539 and Fl0626) had been proved to be linkage, another one had been proved to be independent from them by detecting 24 (p50 × c108) × c108 individuals.

Primers were synthesized by Bioasia Co. Ltd. PCR reactions were performed by a Techne Flexigene Cycler under standard conditions: pre-denaturing at 95°C for 3 min, denaturing at 95°C for 40 sec; extension at 72°C for 1 min except annealing temperature was step touchdown from 63°C to 56°C, each step down 0.44°C, for the first

Table 1. Microsatellite loci, repeat motif, forward and reverse flanking primer sequences

Locus symbol	Repeat motif	Primer sequence
Fl0539	$(CA)_{12}$	5'- GCCGCAAAACAATCAAGTGG-3' 5'- CGTATTTTAGTGTATGACTCGGATGA-3'
Fl0618	$(CT)_6$	5'- CAGTGCGGAGGCAGAAGATAG-3' 5'- CGGTTAGCGTGGTATGGACAT-3'
Fl0626	$(CT)_7$	5'- TGGGCGACGGTAACCACATA-3' 5'- CGATAAGATCGCCTATTTTACAATG-3'

fifteen cycles, then kept at 56°C for the rest 24 cycles. The last cycle was 10 min final polymerization at 72°C. PCR was performed in a total volume of 15 µl containing 1 × PCR buffer (10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl, pH 8.3), 200 µM dNTPs, 10 pM of each primer, 0.5 U Taq polymerase (Dingguo Biotechnology) and 20 ng genomic DNA.

Electrophoresis

The PCR-amplified fragments were detected by an automated laser fluorescence sequencer (ABI, 377) with a 36 cm long gel cassette. Denaturing gels were prepared with 5% readysol DNA/PAGE, 7 M urea in 1 × TBE buffer. After filtered, TEMED and ammonium persulphate were added just before filling the cassette. For each sample, 0.4 ng PCR products were mixed in 4 µl loading buffer containing 5 mg/ml of blue dextran in 5% formamide. Fluorescent-labelled internal markers-Genescan 400HD (rox) (ABI) and PCR products were denaturated at 95°C for 3 min and then kept on ice before loading. Half microliters of marker solution and 0.5 µl of the mixed sample were loaded to ABI 377 sequencer at the same time. Sequencing gel was run in 1 × TBE buffer at 51°C for 2 hrs under 3000 V, 50 mA and 150 W with a sampling interval of 2 m.

Data analysis

The SSR marker could amplify one or two band in these three populations. If the locus was heterozygous, two bands could be amplified; otherwise, only one band could be amplified if this locus was homozygous. If genomic DNA of an individual was amplified by two markers (1 and 2), each marker could amplify one (homozygous) or two (heterozygous) bands as their co-dominant character, Fig. 1 showed the genotype of F_1 gamete and the individuals of F_2 and BC_1. For marker 1, "1" was recorded as homozygous for 1-*a* allele (1-a1-a), "3" was recorded as homozygous for 1-*b* allele (1-b1-b), "2" was recorded when it was heterozygous (1-a1-b); in the same way, "1'" was recorded as homozygous for 2-*a* allele (2-a2-a), "3'" was recorded as homozygous for 2-*b* allele (2-b2-b), "2'" was recorded when it was heterozygous (2-a2-b) for marker 2. According to Mendel's law, the ratio of the four

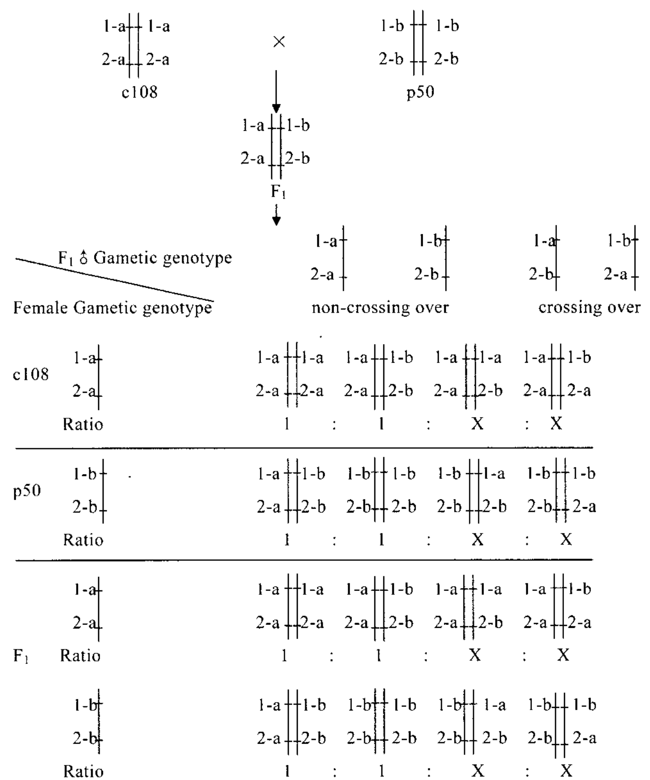

Fig. 1. The genotype of F_1 gamete and the individuals of F_2, $c108BC_1$ and $p50BC_1$. The parents, c108 and p50, could only produce one kind of gamete, and F_1 female could produce two kinds of gametes, but F_1 male could produce four kinds of gametes, they were two kind of non-crossing over gametes and two kinds of crossing over gametes. So the ratio of c108BC1 and p50BC1 individuals genotype was 1 : 1 : X : X (X < 1) respectively, and ratio of F_2 individuals genotype was 1 : 2 : 1 : X : X : X : X (X < 1).

phenotype (11': 22': 12': 21' or 33': 22': 32': 23') of two markers in the two BC1 populations should be 1 : 1: X : X (X < 1) if they were linked, and 1 : 1 : 1 : 1 if they were independent (Table 2). In F_2 population, 7 phenotypes (11': 22': 33': 12': 23': 21': 32') would express if two markers were linked because of lack of crossing over in females, and their ratio was 1 : 2 : 1 : X : X : X : X (X < 1). If they were independent, 9 phenotypes would express (11': 13': 23': 12': 22': 21': 32': 31': 33'), and their ratio was

Table 2. Gametic genotype of parents and phenotype of BC_1 individuals

♀ \ F_1 ♂		1-a2-a	1-b2-b	1-a2-b	1-b2-a
c108BC_1	1-a2-a	1-a1-a 2-a2-a	1-a1-b2-a2-b	1-a1-a 2-a2-b	1-a1-b2-a2-a
	phenotype	11'	22'	12'	21'
		Non-crossing over		Crossing over	
p50BC_1	1-b2-b	1-a1-b2-a2-b	1-b1-b2-b2-b	1-a1-b2-b2-b	1-b1-b 2-a2-b
	phenotype	22'	33'	23'	32'
		Non-crossing over		Crossing over	

Table 3. Gametic genotype of F_1 and phenotype of F_2 individuals

♂ \ ♀		1-a2-a	1-b2-b	1-a2-b	1-b2-a
Non-crossing over	1-a2-a	1-a1-a2-a2-a	1-a1-b2-a2-b	1-a1-a2-a2-b	1-a1-b2-a2-a
	Phenotype	11'	22'	12'	21'
	1-b2-b	1-a1-b2-a2-b	1-b1-b2-b2-b	1-a1-b2-b2-b	1-b1-b2-a2-b
	Phenotype	22'	33'	23'	32'
Crossing over	1-a2-b	1-a1-a2-a2-b	1-a1-b2-b2-b	1-a1-a2-b2-b	1-a1-b2-a2-b
	Phenotype	12'	23'	13'	22'
	1-b2-a	1-a1-b2-a2-a	1-b1-b2-a2-b	1-a1-b2-a2-b	1-b1-b2-a2-a
	Phenotype	21'	32'	22'	31'

1 : 1 : 2 : 2 : 4 : 2 : 2 : 1 : 1 (Table 3). Therefore, two phenotype, 13' and 31', would not present if these two markers were linked. The crossing value was calculated as: Crossing value = Number of crossing over individuals/ Total individuals × 100%

Results

Results of amplification

Two hundred and sixty-nine of 285 individuals in c108BC_1 group, 262 out of 285 individuals in p50BC_1 group, and 255 out of 285 individuals of (p50 × c108)F_2 group could be amplified by these three markers and clear DNA bands generated by them could be detected easily in the 377 sequencer. Their phenotypes ratio was shown in Table 4 and Table 5, all accorded with the law of co-dominant marker by *chi*-square statistical analysis.

Linkage analysis of SSR markers in three populations

Table 4 showed the number of the individuals that expressed different phenotype in the three groups combined by any two of the SSR markers. All of these markers were hypothesized to be independent from each other

Table 4. Phenotype of the individuals and their ratio in the three groups

Group	c108 BC_1			Ratio	p50 BC_1			Ratio	(p50 × c108) F_2			Ratio
Marker	Fl0539	Fl0626	Fl0618		Fl0539	Fl0626	Fl0618		Fl0539	Fl0626	Fl0618	
Homozygous individuals (c108 pattern)	139	132	129	1					70	67	61	1
Heterozygous individuals	130	137	140	1	130	134	123	1	121	125	119	2
Homozygous individuals (p50 pattern)					132	128	139	1	64	63	75	1
Total	269	269	269		262	262	262		255	255	255	
chi-square	0.3	0.09	0.45		0.02	0.14	0.98		0.47	0.11	1.33	

Table 5. Phenotype of the individuals combined with two SSR markers

Group	Phenotype	11'	22'	12'	21'	33'	23'	32'	31'	13'	Total	Chisquare	Crossing over value
	Fl0539-Fl0626	122	124	10	13						269	45.96	8.55
c108BC$_1$	Fl0539-Fl0618	71	67	70	61						269	0.30	-
	Fl0626-Fl0618	68	65	69	67						269	0.04	-
	Fl0539-Fl0626		118			123	12	9			262	61.66	8.02
p50 BC$_1$	Fl0539-Fl0618		68			59	66	69			262	0.31	-
	Fl0626-Fl0618		65			69	66	62			262	0.13	-
	Fl0539-Fl0626	61	124	3	8	50	5	4	0	0	255	47.33	7.81
(p50 × c108) F$_2$	Fl0539-Fl0618	15	68	28	31	13	37	28	18	17	255	0.385	-
	Fl0626-Fl0618	13	59	38	37	14	32	26	19	17	255	0.61	-

Table 6. Paired samples test results of the crossing-over value among the three groups analyzed with SPSS 10.0

		Paired differences					t	df	Significance (2-tailed)
		Mean	Std. deviation	Std. error mean	95% Confidence interval of the difference				
					Lower	Upper			
Pair 1	CBC1-PBC1	0.5143	0.9456	0.5460	-1.8347	2.8634	0.942	2	0.446
Pair 2	CBC1-F2	0.7805	2.6498	1.5298	-5.8019	7.3629	0.510	2	0.661
Pair 3	PBC1-F2	0.2661	3.5308	2.0385	-8.5048	9.0371	0.131	2	0.908

first, and two pairs were proved to be true by *chi*-square statistical analysis, they were Fl0539-Fl0618 and Fl0626-Fl0618, and Fl0539 and Fl0626 were proved to be linkage. This had been proved by *chi*-square statistical analysis by supposing that the phenotype of the individuals should accord with 1: 1 in BC$_1$ generation, and 1 : 2 : 1 in F$_2$ generation. The X^2 value was 1.98 (c108BC$_1$), 1.78 (p50BC$_1$) and 1.06 (F$_2$) respectively, and was lower than 3.84 (df = 1, c108BC$_1$ and p50BC$_1$), and 5.99 (df = 2, F$_2$ group).

The crossing-over value was calculated according the ratio of the recombinant individuals. Because recombination only happened in silkworm males, Due to the merit of co-dominant property, recombinant gametes could be judged easily by detecting the phenotype of each individual, including F$_2$ group. The individual in F$_2$ that expressed 12', 21', 13' or 31' was combined by a parental gametes and a recombinant gametes. The crossing-over value was 8.55 in (c108BC$_1$), 8.02 (p50BC$_1$) and 7.81 (F$_2$) respectively.

As PCR reaction was performed in three 96-hole plastic plates, each 96 individuals were treated as a group. Paired-samples tests were analyzed using SPSS 10.0 software (http://www.spss.com/) to test whether there was dominant difference among the crossing-over value calculated from three groups. *t* value of each pair was lower than the tabulated *t* value for 2 df ($t = 4.303$, $p = 0.05$) (Table 6), indicated that there was not dominant difference among the crossing-over values gained from the three groups, and the differences among them were caused by the random samples.

Discussion

When a new gene was to be located in silkworm, F$_2$ populations were investigated when the aim gene and marker were repulsion, and BC$_1$ (F$_1$ female was crossed to the double recessive individual) was used when they were coupling because of lacking crossing-over in females. There would be no double recessive individuals present and phenotype ratio would be 1 : 2 : 1 in F$_2$ population. According to BC$_1$ population, only 2 types of phenotype (no recombinant ones) would be present, and their ratio was 1 : 1. Gene site was calculated using three recessive individuals crossed to the F$_1$ (obtained by crossing a double recessive parent to a recessive parent). It was very difficult to breed three recessive lines because of the lacking crossing over in females of silkworm. The efficiency would be improved remarkably if codominant molecular

markers could be used in locate morphological markers.

The F_2 progeny raised from a single pair mating of F_1 sibs have been used for constructing two dominant molecular map (RAPD and SADF) and two sub linkage map were constructed respectively. Yasukochi (1998) converted F_2 intercross data to BC_1 backcross data on the base of hypothesis that absence of a maternal marker in a certain F_2 progeny meant the nonrecombinant autosome of the progeny was paternal, and then calculated genetic distances between markers using mapmaker software because it was impossible to distinguish homozygotes from heterozygotes directly when the dominant markers were present. But F_2 populations contain more information than backcross population, and would yield maximum efficiency in the initial phases of linkage mapping. RAPDs, AFLPs, and SADFs could be amplified by PCR, facilitating rapid large-scale genotyping. However, these markers were usually strain specific and, because of their dominant inheritance, limit integration of independently constructed genetic maps. But as codominant markers, the recombinant individuals could be detected easily, and the crossing-over value could be calculated. On this base, F_2 population could be used in constructing linkage map of SSR markers and other codominant markers. These phenomena would be the same to drosophila too, in which there was no crossing-over in males.

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