



Linkage and mapping analyses of the normal marking gene *+P* in the silkworm (*Bombyx mori*) using SSR markers

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ABSTRACT. In the silkworm, *Bombyx mori*, normal markings are mainly controlled by the *+P* gene, which is located on the second chromosome. Due to a lack of crossing over in females, reciprocal backcrossed F_1 (BC_1) progenies were used for linkage analysis and mapping of the *+P* gene based on an SSR linkage map using silkworm strains P50 and H9, which are normal marking and sex-limited marking, respectively. The *+P* gene was found to be linked to 3 SSR markers. Using a reciprocal BC_1M cross, we constructed a linkage map of 22.5 cM, with *+P* mapped at 11.3 cM and the nearest SSR marker S0206 at a distance of 3.0 cM. Based on a fine genome map of domesticated silkworms, Kaikoblast analysis showed that the physical distance between the nearest markers (containing the *+P* gene) is 995 kb. Further analysis showed that BGIBMGA009689, BGIBMGA009688, and BGIBMGA009687 are closer to *+P*, and that BGIBMGA009689 is closest to *+P*, with a physical distance of 19.1 kb.

Key words: Silkworm; SSR markers; Linkage analysis; Gene location; *+P*

INTRODUCTION

The mulberry silkworm, *Bombyx mori*, has been domesticated for silk production for an estimated 5000 years (Xiang, 1995). With the development of biotechnology, *B. mori* has become the model organism for Lepidoptera, the second most numerous order of insects and one of the best genetically characterized insects. Silkworm larvae possess markings with various categories and are easy to identify, and hence, they are considered an extremely useful resource for research in genetics. Silkworm genetic studies treat normal marking as the normal type for larval marking, which is controlled by the *+P* gene. The *+P* gene was mapped to position 0.0 in the silkworm classical genetic linkage group 2.

With the development of molecular biotechnology, the molecular biology research of *B. mori* has also made considerable progress. Some molecular linkage maps in the silkworm have been constructed by using a variety of molecular markers (Yamamoto et al., 2006), including random amplified polymorphic DNA (RAPD) (Yasukochi, 1998; Yasukochi et al., 2006), restriction fragment length polymorphism (Nguu et al., 2005), amplified fragment length polymorphism (AFLP) (Tan, 2001; Mirhoseini et al., 2009), and simple sequence repeats (SSRs) (Miao et al., 2005).

Microsatellites or SSRs are tandemly repeated units of 1 to 6 nucleotides, which are abundant in prokaryotic and eukaryotic genomes (Field and Wills, 1996). They are PCR-based, highly reproducible, inherited in a Mendelian fashion and polymorphic, generally codominant, and abundant in animal and plant genomes. The SSR technique is a convenient and reliable tool to generate highly polymorphic molecular markers, which greatly facilitate building linkage maps (Litt and Luty, 1989; Tautz, 1989; Weber and May, 1989; Bornet and Branchard, 2001; Schlotterer, 2004). In addition, the use of SSR markers universally present in *B. mori* for map construction, as opposed to RAPD or AFLP, has the advantage of being non-strain-specific and is thus more suitable for positional cloning. An SSR linkage map has been constructed for the silkworm, which covers all its 28 chromosomes (Miao et al., 2005). By using SSR marking, Li et al. (2006) located the denonucleosis non-susceptible gene *nsd-Z* on chromosome 15 and Zhao et al. (2011) located the no-glue egg gene (*Ng*) on chromosome 12. In this study, our objectives were to locate the *+P* gene through screening-related SSR markers, and then to construct the genetic map by calculating the physical distance of *+P* in accordance with the silkworm database.

MATERIAL AND METHODS

Silkworm strains and genetic crosses

The P50 strain, whose larvae have normal marking, and the H9 strain, whose larvae have sex-limited marking, were obtained from the Silkworm Genetics and Breeding Laboratory, School of Life Sciences, Anhui Agricultural University. A single-pair cross between a female (H9) and male (P50) produced the F_1 offspring. Owing to the lack of crossing over in females, reciprocal backcrossed F_1 (BC_1) progeny were used for linkage analysis and mapping of the *+P* gene. For linkage analysis, BC_1 progeny from the cross $(H9 \times P50) \text{♀} \times H9 \text{♂}$, together with a second backcross, BC_1 progeny from $H9 \text{♀} \times (H9 \times P50) \text{♂}$, were used for recombination mapping.

DNA extraction

DNA samples were extracted from the single pupae of parents and the individuals

of the backcross population by the standard phenol-chloroform procedure as described by Sambrook et al. (1989). The fat body of pupae was ground with a mechanical homogenizer in a microcentrifuge tube and suspended in DNA extraction buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0; 0.5% SDS), which contained 100 µg/mL proteinase K. After digestion with proteinase K at 56°C for 3 to 5 h, phenol-chloroform extraction was carried out, and the DNA was recovered by isopropanol precipitation. The purified DNA was dissolved in TE buffer, pH 8.0. The DNA concentration was measured by spectrophotometry, and the samples were diluted to a concentration of 10 ng/µL for use in the PCR analysis.

PCR and SSR marker screening

Standard PCRs (25 µL) for SSR locus amplification contained 2.5 µL 10X buffer (Mg²⁺), 0.5 µL 10 mM dNTPs, 0.3 µL 5 U/µL Taq polymerase, 1 µL 10 µM of each primer, and 10 ng template DNA. The reactions were as follows: initial incubation at 95°C for 3 min, 63°C for 40 s, and 72°C for 1 min; 14 cycles of 94°C for 40 s [14-step touchdown decreasing the temperature by 0.5°C at each step to 56°C (40 s)] and 72°C for 1 min; 24 cycles of 94°C for 40 s, 56°C for 40 s, and 72°C for 1 min; and a final elongation step with extension at 72°C for 10 min. The amplified products (6 µL) from individual PCRs were resolved by polyacrylamide gel electrophoresis (PAGE) (8% acrylamide gels in 1X TBE buffer at 90 V for 6 h) followed by silver staining. We carried out polymorphism analysis of the parental using 15 SSR primers based on the SSR molecular linkage map (Miao et al., 2005) by PCR.

Linkage and recombination analysis

The primer pairs of SSR markers that showed polymorphism between H9 and P50 from 15 SSR markers were used for linkage analysis. We performed PCR amplification of genomic DNA from 22 offspring of the BC₁F progeny (11 individuals of normal marking and 11 individuals of sex-limited marking) and detected the primers that linked to the +P gene. To obtain a recombination rate, PCR amplification of genomic DNA of 158 offspring of BC₁M was performed. These products were detected for analyzing genotypes by PAGE.

Data analysis

The genetic relationship between markers was determined by maximum likelihood analysis, and the segregation pattern of marker data was analyzed using MAPMAKER version 3.0 (Lander et al., 1987) with the backcross data as an input file. The Kosambi mapping function was used to calculate the distances between marker loci in cM. The genome sequences of markers from the result of data analysis were blasted to analyze the physical distance between +P and SSR markers in the silkworm database (<http://silkworm.Genomics.Org.cn>).

RESULTS

Phenotype and genotype of the backcrosses

All F₁ progeny from a cross between an H9 female and a P50 male showed normal

marking. Two hundred and seventy-eight BC₁F offspring obtained from (H9xP50)♀xH9♂ had a different shade of marking. According to the differences, we separated the progeny into 4 types, where 79 larvae were with plain marking (ZZP/P), 67 with light marking (ZZ+P/P), 72 with normal marking (ZW·+P P/P), and 60 with dark marking (ZW·+P +P/P). As the difference in marking was not obvious in some individuals, the plain and light marking progeny, which can be clearly distinguished through their marking and sex, were used for analysis, yielding a 1:1 ratio (χ^2 test, $P > 0.05$) (Table 1) as expected for ZZP/P versus ZZ+P/P progeny. Similar results were obtained for 158 BC₁M offspring from H9♀x(H9xP50)♂; 71 individuals were with plain marking (ZZP/P) and 87 larvae with light marking (ZZ+P/P) ($P > 0.05$, ratio 1:1) (Table 1).

Table 1. Phenotypes of progeny from reciprocal backcross breeding.

Cross	Individual		Ratio	χ^2
	Plain marking	Light marking		
(H9xP50)♀xH9♂	79	67	1.18:1	0.99
H9♀x(H9xP50)♂	71	87	0.82:1	1.62

Screening for polymorphism of SSR markers

Figure 1 shows the result of some electrophoretograms of polymorphism screening between H9 and P50. To find polymorphic primers, the products from individual PCRs using the same primer pair in different DNA templates (H9 and P50) were resolved by PAGE, and the samples were placed in adjacent wells. The result of some primer pairs through PCR amplification was unsatisfactory, where polymorphism between H9 and P50 could not be determined by repeating several times, so we abandoned these primers to avoid influencing subsequent experiments. Finally, we obtained 10 polymorphic markers from 15 SSR markers, including S0202, S0204, S0205, S0206, S0208, S0209, S0210, S0211, S0213, and S0214.

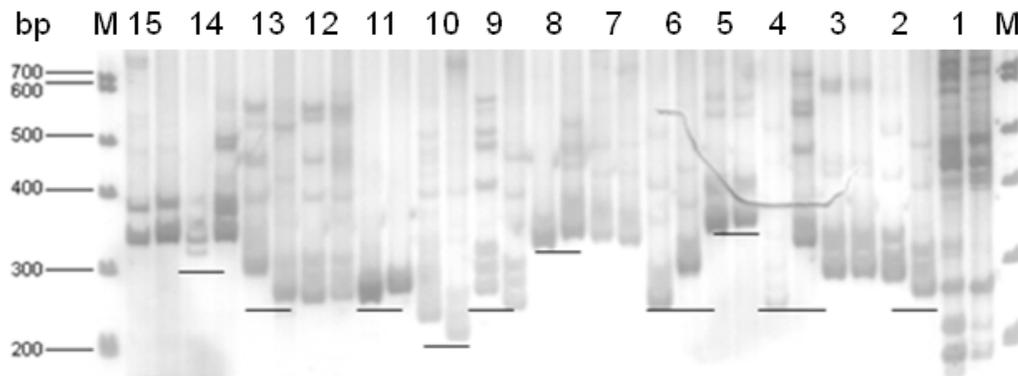


Figure 1. Electrophoretogram of polymorphism screening between H9 and P50. Lane M = DNA marker; lanes 1 to 15 = 15 SSR primer pairs.

Linkage analysis of SSR markers with the +P gene

In *B. mori*, linkage analysis can be carried out efficiently because no crossing over occurs in females. The polymorphic markers, which were found from H9 and P50 by polymorphism screening, were used to analyze the genetic inheritance pattern in 22 offspring from BC₁F progeny. Because SSRs are inherited in a Mendelian fashion and exhibit codominant alleles, we screened SSR markers for linkage to +P by comparing the pattern shown by groups of 22 individuals in BC₁F progeny with the F₁ and parent P50 pattern. If the individuals with light marking were all of the same F₁ pattern (heterozygous, +P/P), the SSR marker was confirmed to be linked to the +P gene; otherwise, the SSR marker was discarded (Figure 2). We continued with this type of analysis until we had analyzed all the polymorphic markers. Thus, a total of 3 SSR markers (S0202, S0206, and S0211) were identified to be linked to +P (Table 2, Figure 2).

1 2 3 M 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25

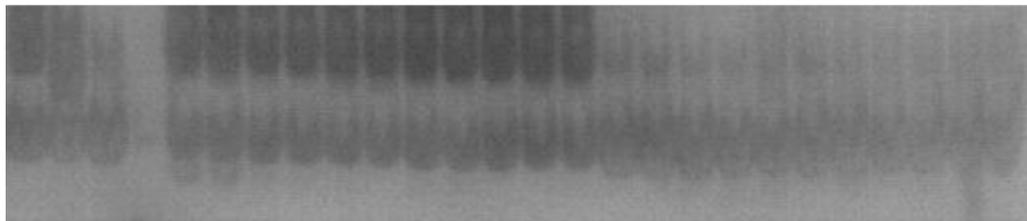


Figure 2. Amplification of individuals in BC₁F by S0202. Lane 1 = (H9xP50) F₁, female of the BC₁F population; lane 2 = parent P50; lane 3 = parent H9, male of the BC₁F population; lane M = DNA marker; lanes 4 to 14 = individuals with light marking type in BC₁F cross; lanes 15 to 25 = individuals with plain marking type in BC₁F cross.

Table 2. SSR loci linked to +P.

Locus symbol	Primer sequence (5'-3')	Allele size (bp)
S0202	TTATTGACCTCCCACATCCCC	228
	GGTGCAITTTGGGAAGATAAGTGTA	
S0206	TACTGGGTCGGAAGGATGGA	243
	AAGGTAGAAGGGGAAGAGGTCG	
S0211	CAGTTCTGGCAGTCGTTCTCC	271
	GCGAAGTAGTAGTGAGGACGGC	

Linkage map construction of the +P gene with SSR markers

Since there is no crossing over in females, the initial analysis was only done in the SSR markers that were linked to normal marking. To determine the marker order and relative distance with respect to +P, we carried out genotyping of the 3 SSR markers that were linked to +P using 158 surviving BC₁M progeny. As shown in Figures 3 and 4, we scored individual offspring in which the inheritance pattern showed either the same H9 pattern (homozygous, designated 1) or the same F₁ pattern (heterozygous, designated 2) for each SSR marker. The location of each marker was initially established through a 3-point comparative analysis of the number of recombinants in the sorted data, and the recombination fractions were then calculated from the whole data set using MAPMAKER 3.0.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 M 20

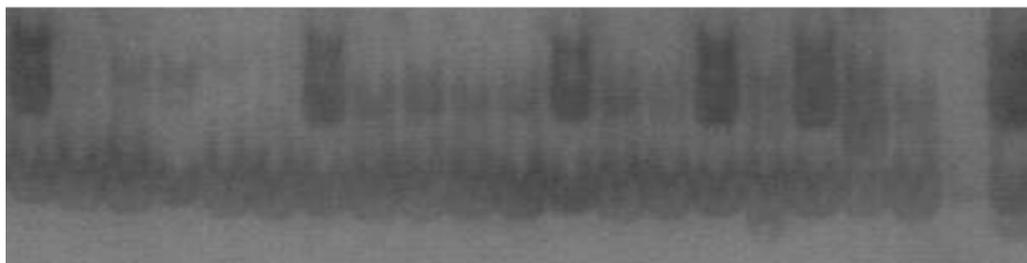


Figure 3. Amplification of individuals in BC₁M by S0206. Lanes 1 to 18 = plain marking individuals with BC₁M cross. Lane 19 = parent H9, female of the BC₁M populations; lane M = DNA marker; lane 20 = (H9xP50) F₁, male of the BC₁M populations.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

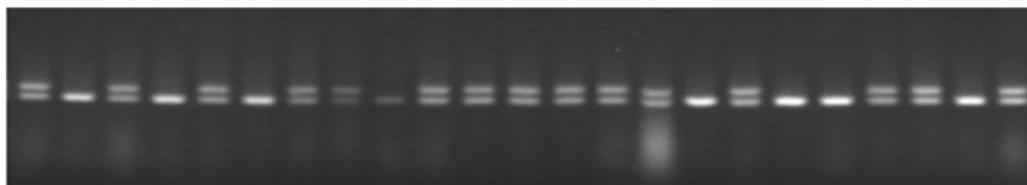


Figure 4. Amplification of individuals in BC₁M by S0211. Lane 1 = (H9xP50) F₁, male of the BC₁M populations; lane 2 = parent H9, female of the BC₁M populations; lanes 3 to 23 = light marking individuals with BC₁M cross.

The BC₁M progeny generated the recombination map shown in Figure 5. The order of the SSR markers in the linkage group was established as follows: S0202, S0206, +P, and S0211. The linkage map was 22.5 cM in length, and +P was mapped at 11.3 cM. S0206, which is the SSR marker identified as being most closely linked to +P, was mapped at a distance of 3.0 cM from +P.

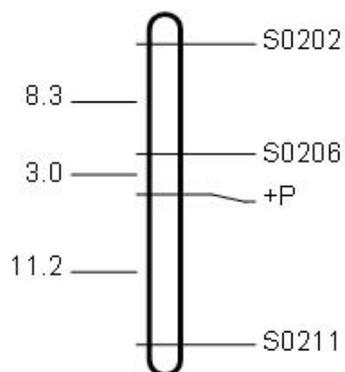


Figure 5. Mapping of the +P gene with SSR markers determined by analysis of the BC₁M progeny of the cross H9♀x(H9xP50)♂.

Analysis of physical distance between +P and SSR markers

According to the results of the genetic map, we blasted the genome sequences that were closest to +P. The Kaikoblast results showed that the physical distance between S0206 and S0211 is 995 kb (Table 3). The S0206 and S0211 were located in the nscaf27 in the fine genome map of the domesticated silkworm. Through further analysis of the up- and downstream sequences of S0206 and S0211 in the silkworm database, we found that 3 genes (BGIBMGA009690, BGIBMGA009689, and BGIBMGA009688) are closer to +P, and the physical distance of BGIBMGA005835, 44 kb, is closest to +P (Figure 6).

Table 3. Result of Kaikoblast in the silkworm database.

No.	Query ID	Hit scaffold ID	Hit length (hit rate)	E-value (score)	Hit query position	Hit scaffold position	Hit chromosome position
1	S0206	Bm_scaf27	574 (98.95%)	0.0 (1092)	183-756	1357760-1358332	chr2:5058263-5058835
2	S0211	Bm_scaf27	484 (99.38%)	0.0 (938)	5-488	362766-362283	chr2:4063269-4062786

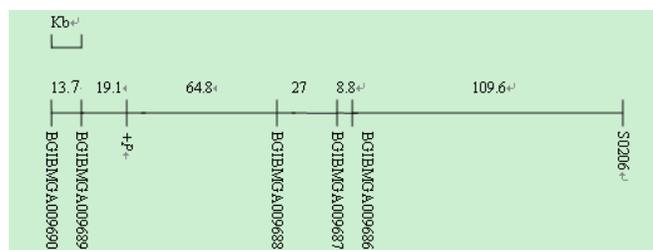


Figure 6. Physical distance between +P and molecular markers.

DISCUSSION

In this study, the +P gene was found to be linked to 3 SSRs. This was evident from the profile of the 3 SSRs in 22 BC₁F individuals. A linkage map of 22.5 cM for the linkage group was constructed, with +P mapped at 11.3 cM and with the closest marker being S0206. The order of the SSR markers and the +P gene was as follows: S0202, S0206, +P, and S0211. S0206 was most closely linked to +P with a distance of 3.0 cM from +P (Figure 5). The Kaikoblast results in the fine genome map of the domesticated silkworm (*B. mori*) showed that the physical distance between S0206 and S0211 is 995 kb (Table 3). The S0206 and S0211 were located in the nscaf27 in fine genome map of the domesticated silkworm. We also found that 3 genes (BGIBMGA009690, BGIBMGA009689, and BGIBMGA009688) are closer to +P, and that the physical distance of BGIBMGA005835, 44 kb, is closest to +P (Figure 6).

In screening SSR markers, some showed no polymorphism or could not be assessed for polymorphism because the genetic relationship was too close between parents and the differences could not be visualized by PAGE. The marker density of this molecular genetic map is relatively low and the backcross population is small. Due to the limited number of mapped SSR and related molecular markers, it is probably premature to establish a quantitative relationship between the physical and genetic maps; this conclusion is reinforced by the fact that only single matches were identified between SSR markers and sequenced contigs. With the

gradual increase in molecular markers on the genetic map, these genetic linkage groups will facilitate the estimation of marker density needed for map-based cloning, the ordering of genomic contigs, and the establishment of a complete physical map.

The identification of these markers represents an important step towards positional cloning of the gene with these markers as tags. It will be useful in the fine mapping and cloning of the *+P* gene based on the SSR linkage map. In the domesticated silkworm, sex-limited marking, which is widely used for male silkworm selection in silkworm breeding in China, is very important not only in evolution but also in the industry. Future research will focus on finding more closely linked molecular markers to facilitate marker-assisted selection with the long-term goal of fine mapping and cloning of the *+P* gene. It is useful for research on the regulatory mechanism of sex-limited marking silkworm and the breeding of sex-limited silkworm strains.

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REFERENCES

- Bornet B and Branchard M (2001). Nonanchored inter simple sequence repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting. *Plant Mol. Biol. Rep.* 19: 209-215.
- Field D and Wills C (1996). Long, polymorphic microsatellites in simple organisms. *Proc. Biol. Sci.* 263: 209-215.
- Lander ES, Green P, Abrahamson J, Barlow A, et al. (1987). MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* 1: 174-181.
- Li M, Guo Q, Hou C, Miao X, et al. (2006). Linkage and mapping analyses of the denonucleosis non-susceptible gene *nsd-Z* in the silkworm *Bombyx mori* using SSR markers. *Genome* 49: 397-402.
- Litt M and Luty JA (1989). A hypervariable microsatellite revealed by *in vitro* amplification of a dinucleotide repeat within the cardiac muscle actin gene. *Am. J. Hum. Genet.* 44: 397-401.
- Miao XX, Xub SJ, Li MH, Li MW, et al. (2005). Simple sequence repeat-based consensus linkage map of *Bombyx mori*. *Proc. Natl. Acad. Sci. U. S. A.* 102: 16303-16308.
- Mirhoseini SZ, Rabiei B, Potki P and Dalirsefat SB (2009). Linkage map construction for silkworm (*Bombyx mori* L.) based on amplified fragment length polymorphism markers. *Iran. J. Biotechnol.* 7: 28-36.
- Nguu EK, Kadono-Okuda K, Mase K, Kosegawa E, et al. (2005). Molecular linkage map for the silkworm, *Bombyx mori*, based on restriction fragment length polymorphism of cDNA clones. *J. Insect Biotechnol. Sericol.* 74: 5-13.
- Sambrook J, Fritsch EF and Maniatis T (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
- Schlötterer C (2004). The evolution of molecular markers - just a matter of fashion? *Nat. Rev. Genet.* 5: 63-69.
- Tan YD, Wan C, Zhu Y, Lu C, et al. (2001). An amplified fragment length polymorphism map of the silkworm. *Genetics* 157: 1277-1284.
- Tautz D (1989). Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Res.* 17: 6463-6471.
- Weber JL and May PE (1989). Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. *Am. J. Hum. Genet.* 44: 388-396.
- Xiang ZH (1995). *Genetics and Breeding of the Silkworm*. Chinese Agriculture Press, Beijing.
- Yamamoto K, Narukawa J, Kadono-Okuda K, Nohata J, et al. (2006). Construction of a single nucleotide polymorphism linkage map for the silkworm, *Bombyx mori*, based on bacterial artificial chromosome end sequences. *Genetics* 173: 151-161.
- Yasukochi Y (1998). A dense genetic map of the silkworm, *Bombyx mori*, covering all chromosomes based on 1018 molecular markers. *Genetics* 150: 1513-1525.

- Yasukochi Y, Ashakumary LA, Baba K, Yoshido A, et al. (2006). A second-generation integrated map of the silkworm reveals synteny and conserved gene order between lepidopteran insects. *Genetics* 173: 1319-1328.
- Zhao XM, Wei GQ, Liu CL, Zou CR, et al. (2011). Linkage and mapping analyses of the no glue egg gene Ng in the silkworm (*Bombyx mori* L.) using simple sequence repeats (SSR) markers. *Afr. J. Biotechnol.* 10: 9549-9556.