African Journal of Biotechnology Vol. 10(47), pp. 9549-9556, 24 August, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2389
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Linkage and mapping analyses of the no glue egg gene *Ng* in the silkworm (*Bombyx mori* L.) using simple sequence repeats (SSR) markers

Xiao-ming Zhao#, Guo-qing Wei#, Chao-liang Liu*, Chang-rui Zou and Bao-jian Zhu

School of Life Science, Anhui Agricultural University, Hefei Anhui 230036, China.

Accepted 23 May, 2011

In the silkworm, *Bombyx mori,* no glue egg is mainly controlled by *Ng* (No glue) gene, which is located on the 12th chromosome. Owning to a lack of crossing over in females, reciprocal backcrossed F_1 (BC_1) progenies were used for linkage analysis and mapping of the *Ng* gene based on the simple sequence repeats (SSR) linkage map using silkworm strains H9 and P50, which are *Ng* mutant and normal to egg, respectively. The *Ng* gene was found to be linked to three SSR markers. Using a reciprocal BC_1M cross, we constructed a linkage map of 36.4 cM, with *Ng* mapped at 15.9 cM and the nearest SSR marker at a distance of 7.4 cM. Based on fine genome map of domesticated silkworm (*B. mori*), the result of Kaikoblast show that the physical distance between the near markers (containing *Ng* gene) is 181.7 Kb. Further analysis show that BGIBMGA005833, BGIBMGA005835 and BGIBMGA005836 are closer to *Ng*, and the BGIBMGA005835 is nearest to *Ng,* which physical distance is 44 Kb.

Key words: Gene location, linkage analysis, microsatellite, *Ng*, silkworm.

INTRODUCTION

The mulberry silkworm, *Bombyx mori*, is an agriculturally important insect that has been domesticated for silk production for an estimated 5,000 years (Xiang, 1995). With the development of biotechnology, *B. mori* is the model organism for *Lepidoptera*, the second most numerous order of insects and has been used as an important bioreactor for the production of recombinant proteins (Tomita et al., 2003). It has many mutations, more than 400 of them have been identified and more than 200 visible and biochemical mutations have been placed on silkworm linkage maps (Fujii et al., 1998; Miao et al., 2007). These mutations affect many fundamental aspects of the insect life cycle, including egg and egg shell formation, early embryonic pattern formation, development and diapause, larval feeding behavior, and molting (Nagaraju and Goldsmith, 2002). Mutation at the mucus gland in *B. mori* produces no glue eggs which are mainly controlled by *Ng* gene. The *Ng* gene was mapped at 28.0 of the silkworm classical genetic linkage group 12 (Xiang, 1995).

In recent years, molecular biology has made considerable progress in silkworm. Molecular linkage maps were constructed employing random amplified polymorphic DNA (RAPD), restriction fragment length polymorphic (RFLP), an amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSRs) (Tan, 2001; Miao et al., 2005; Yamamoto et al., 2006; Seyed, 2009), which lay a solid foundation for the location of silkworm. RAPDs, AFLPs and RFLPs are convenient tools for constructing molecular linkage maps. However, their disadvantages restrain its application in the relevant field (Sharma et al., 1996). 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; Weber and May, 1989; Bornet

*Corresponding author. E-mail: cyschx@163.com.

Abbreviations: RAPD, Random amplified polymorphic DNA; **RFLP,** restriction fragment length polymorphic; **AFLP,** amplified fragment length polymorphism; **SSRs,** simple sequence repeats; **PCR,** polymerase chain reaction.

#Both authors contributed equally to this work.

and Branchard 2001; Schlötterer, 2004). Microsatellites, or SSRs are randomly repeated units of one to six nucleotides and are abundant in prokaryotic and eukaryotic genomes (Santana et al., 2009).

Now, because of polymerase chain reaction (PCR)-based, highly reproducible and polymorphic, generally co dominant and abundant in animal and plant genomes, SSRs have become the most widely used of second generation molecular markers (Yoshiaki, 2000; Gupta and Varshney, 2000; Li, 2002; Viruel, 2004). Miao et al. (2005) constructed a genetic linkage map employing 518 SSR markers for *B. mori*, which covered all 28 chromosomes of silkworm, the greatest progress in the research of SSR markers in silkworm or even *Lepidoptera*. Li et al. (2006) located the densonucleosis non-susceptible gene *nsd-Z* on chromosome 15 using SSR markers. In addition, the silkworm genome research team of Southwest University (China, 2008) successfully completed the first fine genome map of domesticated silkworm (*B. mori*) in the world, it provides a great help to fine map and clone mutant gene. In this study, we located the No glue (*Ng*) gene using SSR markers, then calculated and analyzed genetic distance and physic distance of *Ng* in accordance with the silkworm Database.

MATERIALS AND METHODS

Silkworm strains and genetic crosses

The P50 strain, which lay glue eggs, and the H9 strain, which lay no glue eggs (*Ng* mutant), 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 F1 offspring. Owing to lack of crossing over in females, reciprocal backcrossed F_1 (BC_1) progeny were used for linkage analysis and mapping of the *Ng* gene. For linkage analysis, BC_1 progeny from the cross (H9 × P50)♀ × P50♂, together with a second backcross, BC_1 progeny from P50♀ × (H9 × P50)♂, were used for recombination mapping.

DNA extraction

DNA samples were extracted from the single pupae of parents and the individuals of backcross population as described by Sambrook et al. (1989). The pupae was grounded with a mechanical homogenizer in a microcentrifuge tube and suspended in DNA extraction buffer (10 mmol/L Tris-HCl pH 8.0; 0.1 mol/L EDTA pH8.0; 0.5% SDS) that contained 100 µg/mL proteinase K. After digestion with proteinase K at 56℃ 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.

PCR and SSR markers screening

Standard PCRs (25 µl) for SSR locus amplification contained 10×buffer (Mg^{2+}) 2.5 µl, dNTPs (10 mmol·L^{-1}) 0.5 µl, Taq polymerase 0.3 µl (5 units/µl), each primer (10 µmol·L^{-1}) 1 µl, template DNA 10 ng. The reactions were initiated at 95℃ for 3 min, 63℃ for 40 s and 72℃ for 1 min; 14 cycles of 94℃ for 40 s (14-step touchdown decreasing the temperature by 0.5℃ at each step to 56℃ (40 s)) and 72℃ for 1 min; 24 cycles of 94℃ for 40 s, 56℃ for 40 s, and 72℃ for 1 min; a final elongation step with extension at 72℃ for 10 min. The amplified products (6 µl) from individual PCRs were resolved on polyacryalmide gel electrophoresis (PAGE) (8% acryl amide gels in 1×TBE buffer at 90 volts for 6 h) followed by silver staining. We carried out polymorphism analysis of the parental using 20 SSR primers which chose 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 20 SSR markers were used for linkage analysis. We performed PCR amplification of genomic DNA from 22 offspring of BC_1F progeny (11 individuals of no glue egg and 11 individuals of glue egg) and detected the primers which linked to *Ng* gene. To obtain recombination rate, PCR amplification of genomic DNA of 161 offspring of BC_1M were performed. These products were detected for analyzing genotypes by acryl amide gels electrophoresis.

Analysis of genetic distance and physical distance between *Ng* and SSR markers

The genome sequences of markers from the result of data analysis were blasted to analyze the genetic and physical distance between *Ng* and SSR markers in the silkworm Database (http:// silkworm.Genomics.Org.cn).

Data analysis

The genetic relationship among 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 markers loci in cM.

RESULTS

The phenotype and genotype of the backcrosses

All F_1 progeny from a cross between a H9 female and a P50 male are no glue egg. One hundred and fifty-eight BC_1F offspring were obtained from (H9 × P50)♀ × P50♂, among which 76 larvae were from no glue eggs ($Ng+^{Ng}$) and 82 larvae were from glue eggs ($+^{Ng}+^{Ng}$), yielding a 1:1 ratio ($\chi 2$ test, $P > 0.05$) (Table 1) as expected for $Ng+^{Ng}$ versus $+^{Ng}+^{Ng}$ progeny. Similar results were obtained for 161 BC_1M offspring from P50♀ × (H9 × P50)♂; 83 individuals were from no glue eggs ($Ng+^{Ng}$) and 78 larvae were from glue eggs ($+^{Ng}+^{Ng}$) ($P > 0.05$, ratio 1:1) (Table 1).

Polymorphism of SSR markers screening

Figure 1 shows the result of some electrophoretogram of polymorphism screening between H9 and P50. To find

Table 1. Phenotypes of progeny from reciprocal backcross breeding.

Cross	Individual		Ratio	χ^2
	No glue eggs	Glue eggs		
(H9 × P50)♀ × P50 ♂	76	82	0.93:1	0.23
P50♀ × (H9 × P50)♂	83	78	1.06:1	0.18

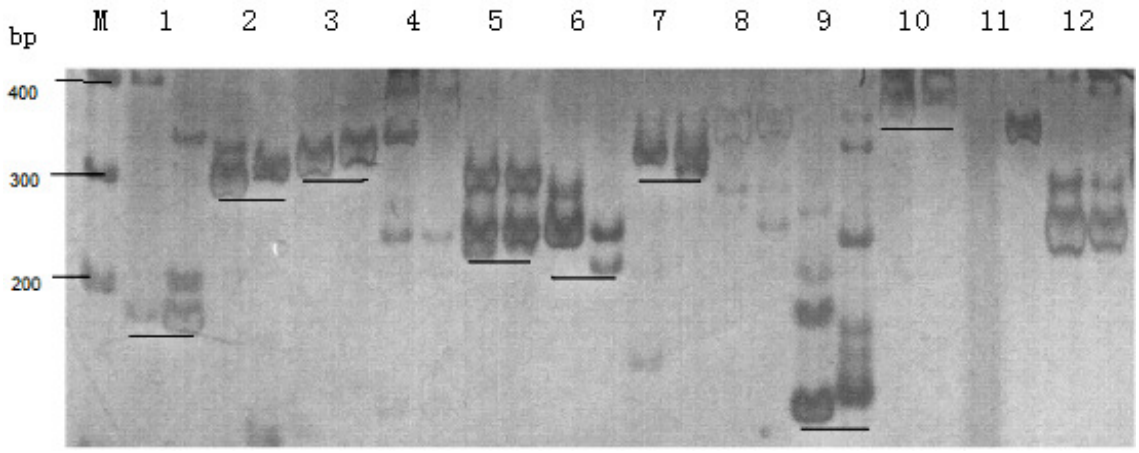

Figure 1. The electrophoretogram of polymorphism screening between H9 and P50. M: DNA marker; 1 to 12: twelve SSR primer pairs.

polymorphic primers, the products from individual PCRs using the same primer pair in different DNA templates (H9 and P50) were resolved on PAGE, and the samples were pointed adjacently. The result of some primer pairs through PCR amplification is unsatisfactory and cannot judge polymorphism between H9 and P50 by repeating several times, so we abandoned these primers to avoid influencing subsequent experiment. Finally, we obtained 10 polymorphic markers from 20 SSR markers, including S1201, S1202, S1203, S1205, S1209, S1211, S1217, S1218, S1219 and S1220.

Linkage analysis of SSR markers with *Ng* 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 BC_1F progeny. Because SSRs are inherited in a Mendelian fashion and exhibit co dominant alleles, we screened SSR markers for linkage to *Ng* by comparing the pattern shown by groups of 22 individuals in BC_1F progeny with the F_1 and parent P50 pattern. If the individuals of no glue eggs were all the same F_1 pattern (heterozygous, $Ng+^{Ng}$), the SSR marker was confirmed to be linked to *Ng* 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 (S1202, S1203 and S1211) were identified to be linked to *Ng* (Table 2, Figures 3 and 4).

Linkage map construction of *Ng* gene with SSR markers

Since there is no crossing over in silk moth females, the initial analysis was only done in the SSR markers that were linked to the mutation. To determine the marker order and relative distance with respect to *Ng*, we carried out genotyping of the 3 SSR markers that were linked to *Ng* using 161 surviving BC_1M progeny. As shown in Figures 5 and 6, we scored individual offspring in which the inheritance pattern showed either the same P50 pattern (homozygous, designated 1) or the same F_1 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.

The BC1M progeny generated the recombination map shown in Figure 7. The order of the SSR markers in the linkage group was established as follows: S1203, *Ng*, S1202 and S1211. The linkage map was 36.4 cM in length, and *Ng* was mapped at 15.9 cM. S1202, which is the SSR marker identified as being most closely linked to

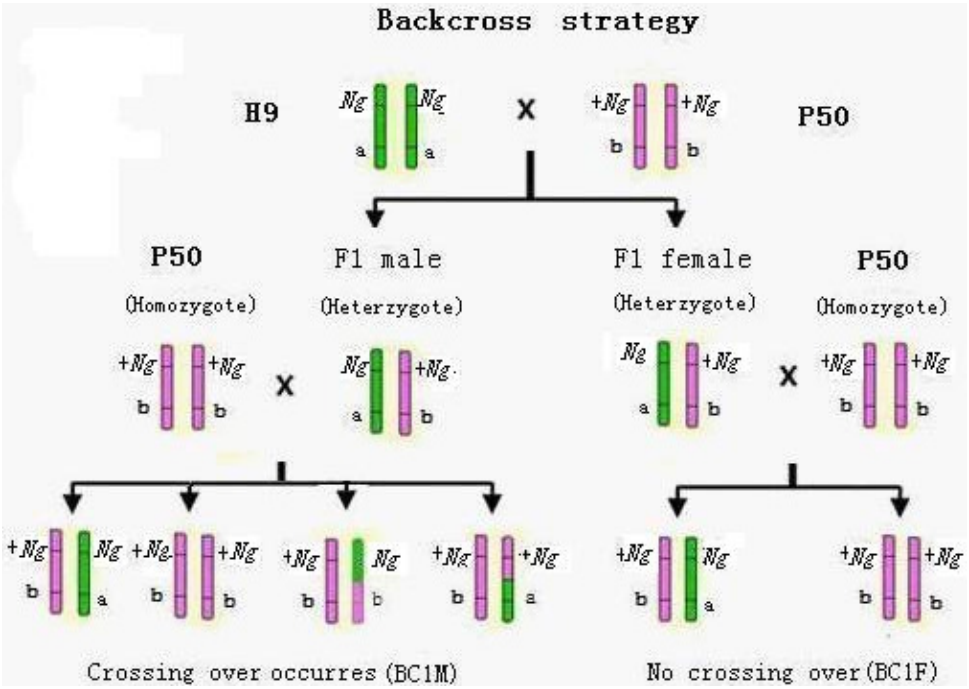

Figure 2. Inheritance pattern of simple sequence repeat (SSR) markers and *Ng*: a, one SSR marker in H9; b, one SSR marker in P50.

Table 2. The SSR loci linked to *Ng*.

Locus symbol	Primer sequence (5'--3')	Allele size (bp)
S1202	CCTCGGAATTGAAGGACGATAT ATGGTAGTGCAAGGTAGAGGGG	309
S1203	AGGCGGGCTCTGGCAAT AGAAACAAAAGCTTGCAAAATTTCT	286
S1211	CTGGGATGTTTAGTGTTAAGTGCG GCGATAAGACCGCCAATTGT	136

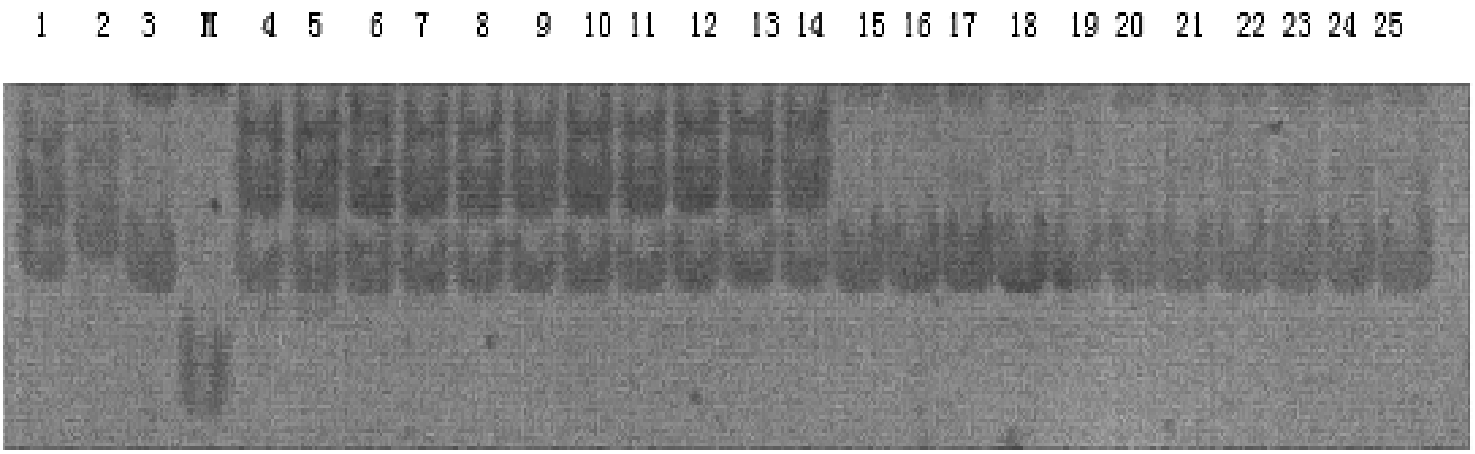

Figure 3. The amplification of individuals in BC_1F by S1211. 1: (H9×P50) F_1, the female of the BC_1F population; 2: Parent H9; 3: Parent P50, the male of the BC_1F population; M: DNA marker; 4 to 14: individuals with mutant type in BC_lF cross; 15 to 25: individuals with normal type in BC_lF cross.

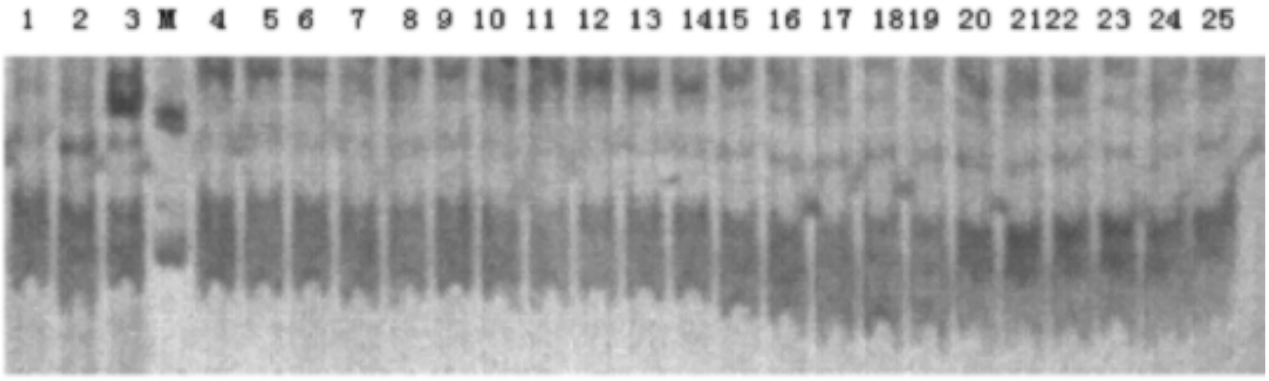

Figure 4. The amplification of individuals in BC_1F by S1202. 1: Parent H9; 2: Parent P50, the male of the BC_1F populations; 3: (H9 × P50) F_1, the female of the BC_1F populations; M: DNA marker; 4 to 14: individuals with mutant type in BC_lF cross; 15 to 25: individuals with normal type in BC_lF cross.

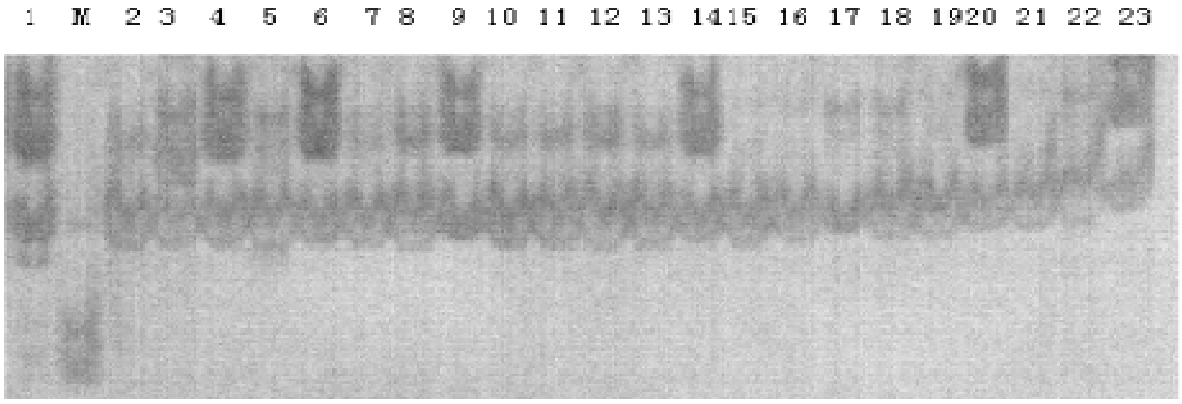

Figure 5. The amplification of individuals in BC_1M by S1211. 1: (H9 × P50) F_1, the male of the BC_1M populations; M: DNA marker; 2: Parent P50, the female of the BC_1M populations; 3 to 23: individuals with BC_lM cross

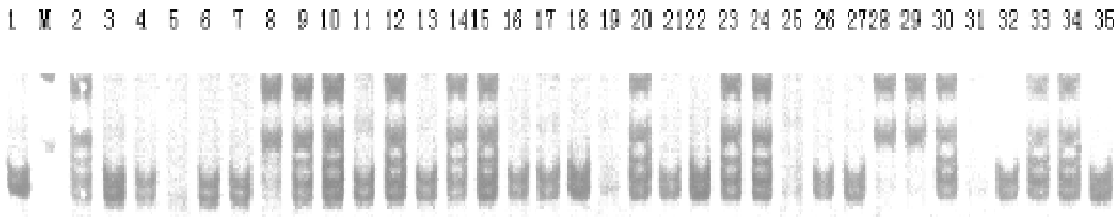

Figure 6. The amplification of individuals in BC_1M by S1211. 1: Parent P50, the female of the BC_1M populations; M: DNA marker; 2: (H9 × P50) F_1, the male of the BC_1M populations; 3 to 35: individuals with BC_lM cross.

Ng, was mapped at a distance of 7.4 cM from *Ng*.

Prediction of ORFs and analysis of physical distance between *Ng* and SSR markers

According to the result of genetic map, we blasted the genome sequences which are mostly close to *Ng.* The result of Kaikoblast show that the physical distance between S1202 and S1203 is 181.7 Kb (Table 3); If one ORF has 100 amino acids at least, with ATG as start codon, we tested 95 ORFs by the Clonemanager software. Meanwhile, the S1203 and S1202 were located in the nscaf2839 in fine genome map of domesticated silkworm. Through further analysis of the up and down-stream sequences of S1202 and S1203 in the silkworm

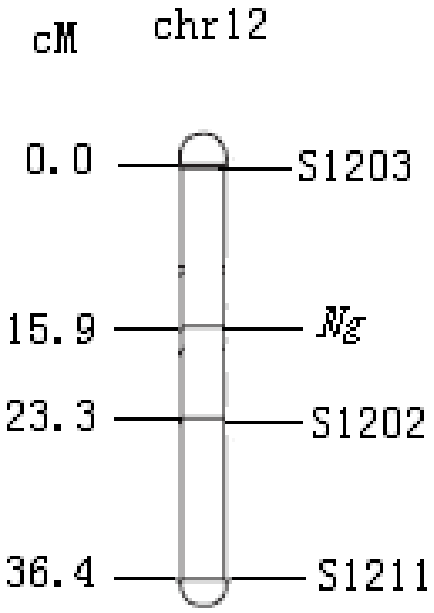

Figure 7. Mapping of *Ng* gene with SSR markers determined by analysis of the BC_1M progeny of the cross P50♀ × (H9 × P50)♂.

Table 3. The result of Kaikoblast in the silkworm Database.

S/N	Query ID	Hit scaffold ID	Hit length (hit rate)	e-value (score)	Hit query position	Hit scaffold position	Hit chromosome position
1	S1202	Bm_scaf125	380 (97.63%)	2e-177 (624)	119-498	383302-383679	chr12:2019239 3-20192770
2	S1203	Bm_scaf125	639 (99.69%)	0.0 (1197)	567-1204	564345-564983	chr12:20373436-20374074

database, we found that three genes (BGIBMGA 005833, BGIBMGA 005835 and BGIBMGA 005836) are closer to *Ng*, and the physical distance of BGIBMGA 005835, 44 Kb, is closest to *Ng* (Figure 8).

DISCUSSION

In the present study, the *Ng* gene was found to be linked to 3 SSR markers. A linkage map of 36.4 cM for linkage group was constructed, with *Ng* mapped at 15.9 cM, and with the closest marker being S1202. The order of the SSR markers in the linkage group is as follows: S1203, *Ng,* S1202 and S1211 (Figure 7). Based on fine genome map of domesticated silkworm (*B. mori*), the result of Kaikoblast shows that the physical distance between S1202 and S1203 is 181.7 Kb. Further analysis show that BGIBMGA005833, BGIBMGA005835 and BGIBMGA-005836 are closer to *Ng*, and the BGIBMGA005835 is nearest to *Ng*, with physical distance of 44 Kb.

Ten polymorphic markers were screened from 20 SSR markers between H9 and P50 in this experiment, but, possibly, because the genetic relationship is too close between parents or small allele fragment which cannot be segregated by PAGE, some SSR markers have no polymorphism or are not able to judge polymorphism. In addition, we also found that some markers have polymorphism in parents, but no linkage to *Ng* when analysis of linkage was performed using individuals of BC_1F progeny. The order of S1203 showed from the linkage map is different from the SSR linkage map (Miao et al., 2005). We conclude that the backcross population is too small or occur as double crossover.

In this study, the *Ng* gene was mapped using a small backcross population. However, fine mapping and cloning of the gene are not completed. Meanwhile, the regulatory mechanism of producing no glue egg which is caused by *Ng* gene is not yet known. Thus, mapping of the *Ng* gene will result in a better understanding of the mechanism of producing no glue egg in *B. mori*. The identification of these markers represents an important step towards positional cloning of the gene with these markers as tags. Now, a near-isogenic line which is set up using *Ng* mutant strain H9 and normal strain P50 is in progress in our

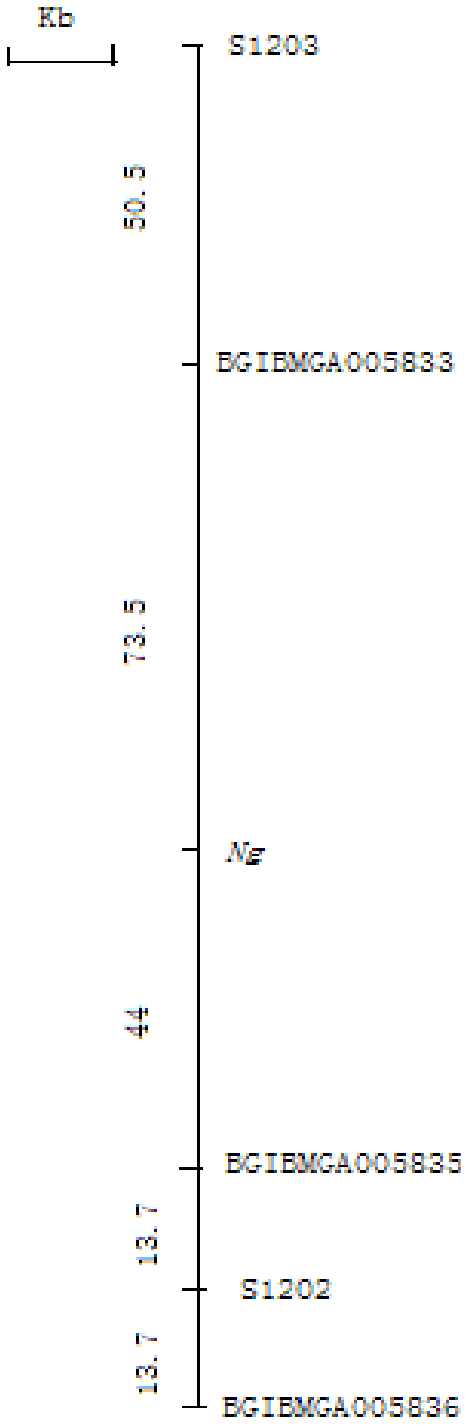

Figure 8. The physical distance between Ng and molecular markers.

laboratory. It will be useful in fine mapping and cloning of the *Ng* gene based on the SSR linkage map. In addition, *Ng* mutant is one of the most important gene resources of silkworm, so the identification of *Ng* gene opens up the possibility of using marker-assisted selection to accelerate the breeding of no glue egg strains.

ACKNOWLEDGEMENTS

Contract grant sponsor: Anhui international cooperation project (08080703017), China agriculture research system (CARS-22-SYZ10) and Anhui talent special fund (2008Z022).

REFERENCES

Bornet B, Branchard M (2001). Nonanchored inter simple sequence repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting. Plant Mol. Biol. Rep., 19: 209-215.

Fujii H, Banno Y, Doiar H, Kihara H. & Kawaguchi Y (1998). Genetical Stocks and Mutations of *Bombyx mori*: Important Genetic Resources. 2nd ed. Institute of Genetic Resources, Kyushu Univ., Fukoka, pp. 54.

Goldsmith MR (2002). Genetics of the silkworm: revisiting an ancient model system. In Molecular Model Systems in the *Lepidoptera* & New York: Cambridge University Press. pp. 21-76.

Gupta PK, Varshney RK (2000). The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis in bread wheat. Euphytica, 13: 163-185.

Lander ES, Green P, Abrahamson J, Barlow A, Daly K (1987). MAPMAKER: an interactive computer package for constructive primary genetic linkage maps of experimental and natural populations. Genomics, 16: 224-230.

Li MW, Guo Q, Hou C, Miao X, Xu A, Guo X, Huang Y (2006). Linkage and mapping analyses of the den-sonucleosis non-susceptible gene nsd-Z in the silkworm, *Bombyx mori*, using SSR markers. Genome, 49(4): 397–402.

Li YC, Korol AB, Fahima T, Beiles A, Nevo E (2002). Microsatellites: genomic distribution, putative functions and mutational mechanisms: Rev. Mol. Ecol., 11(12): 2453-2465.

Litt M, 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, Li MW, Dai FY, Lu C, Gold Smith MR and Huang YP (2007). Linkage analysis of the visible mutations *Sel* and *Xan* of *Bombyx mori* (*Lepidoptera: Bombycidae*) using SSR markers, Eur. J. Entomol., 104: 647–652.

Miao XX., Xu SJ, Li MH, Li MW, Huang JH, Dai FY, Marino SW, Mills DR, Zeng P, Mita K, Jia SH, Zhang Y, Liu WB, Xiang H, Guo QH, Xu AY, Kong XY, Lin HX, Shi YZ, Lu G, Zhang X, Huang W, Yasukochi Y, Sugasaki T, Shimada T, Nagaraju J, Xiang ZH, Wang SY, Goldsmith MR, Lu C, Zhao GP, Huang YP (2005). Simple sequence repeat-based consensus linkage map of *Bombyx mori*. Proc. Natl. Acad. Sci. USA, 102(45): 16303-16308.

Nagaraju J, Goldsmith MR (2002). Silkworm genomics-Progress and prospects. Curr. Sci., 83: 415-425.

Sambrook J, Fritsch EF, Maniatis T (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p.1659.

Santana Q, Coetzee M, Steenkamp E, Mlonyeni O, Hammond G, Wingfield M, Wingfield B (2009). Microsatellite discovery by deep sequencing of enriched genomic libraries. Biotechniques, 46(3): 217-223.

Schlotterer C (2004). The evolution of molecular markers-just a matter of fashion? Nat. Rev. Genet., 5: 63-69.

Seyed ZM, Babak R, Seyed BD (2009). Linkage map construction for silkworm (*Bombyx mori* L.) based on amplified fragment length polymorphism markers. Ir. J. Biotechnol., 7(1): 28-36.

Sharma SK, Knox MR, Ellies THN (1996). AFLP analysis of the diversity and phylogeny of Lens and its comparison with RAPD analysis. Theor. Appl. Genet., 93: 751-758.

Tan YD, Wan C, Zhu Y, Lu C, Xiang Z, Deng HW (2001). An amplified fragment length polymorphism map of the silkworm. Genetics, 157(3): 1277-1284.

Tomita M, Munetsuna H, Sato T, Adachi T, Hino R, Hayashi M, Shimizu K, Nakamura N, Tamura T, Yoshizato K (2003). Transgenic silkworms

produce recombinant human type I II procollagen in cocoons. Nat. Biotechnol., 21(1): 52-56.
Viruel MA. and Hormaza JI (2004). Development, characterization and variability analysis of microsatellites in lychee (*Litchi chinensis* Sonn, Sapindaceae). Theor. Appl. Genet., 108(5): 896-902.
Weber JL, 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). In Genetics and Breeding of the Silkworm (Chinese Agriculture Press, Beijing), pp. 273-289.
Yamamoto K, Narukawa J, Kadono-Okuda K, Nohata J, Sasanuma M, Suetsugu Y, Banno Y, Fujii H, Gold smith MR, Mita k (2006). Construction of a single nucleotide polymorphism linkage map for the silkworm, *Bombyx mori*, based on bacterial artificial chromosome and sequences. Genetics, 173(1): 151-161.
Yoshiaki K, Yuji I, Kazuaki N and Nobukazu N (2000). Microsatellite analysis of pollen flow in *Rhododendron metternichii* var. *hondoense*. Ecol. Res., 15(3): 263-269.