Int. J. Indust. Entomol. Vol. 6, No. 1, 2003, pp. 45~48

International Journal of *Industrial Entomology*

The Use of Fibroin Light Chain Gene Sequence for the Genetic Marker of the Silkworm Races

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(Received 1 October 2002; Accepted 14 February 2003)

We have previously cloned and characterized the complete fibroin L-chain gene from one of the silkworm races Baekok-Jam (*Bombyx mori*) and found two variable regions (I, intron 2 ~ exon 3; II, intron 6) with the primer sets designed to cover these variable regions. We tested the utility of these regions as genetic markers among silkworm races. For the purpose of study, Japanese race (Jam 123), Chinese race (Jam 124) and their F_1 hybrid Baekok-Jam were used. The PCR product size of region I was 787 bp in Jam 123, 770 bp in Jam 124 and 768 bp in Baekok-Jam. The size of region II was 470 bp in Jam 123, 428 bp in Jam 124 and 429 bp in Baekok-Jam. In the extended experiment, Jam 125 (Japanese race), Jam 126 (Chinese race) and their F_1 hybrid Daeseong-Jam were also analyzed. The sizes of region I and II in Jam 125, Jam 126 and Daeseong-Jam were similar to those of Jam 123, Jam 124 and Baekok-Jam. DNA sequence divergence between the two geographic races of Jam 123 or Jam 125 and Jam 124 or Jam 126 was substantial. The result suggests that the fibroin L-chain gene of F_1 hybrids were inherited from Chinese races. These results are concordant with cocoon shapes of tested animals, and suggested that Baekok-Jam or Daeseong-Jam is more closely related to Jam 124 or Jam 126 than to Jam 123 or Jam 125. Taken these data together, the primer sets designed from two variable regions of fibroin L-chain gene would be highly useful, as the genetic markers for silkworm races, at least in Japanese and Chinese races, although an extended study including more geographic races is required.

Key words: Silkworm, Fibroin L-chain gene, Polymorphism, Genetic marker

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Introduction

Nuclear spliceosomal introns are nucleotide sequences that are transcribed but spliced out of the precursor mRNA and they generally do not encode any other polypeptide (Berget *et al.*, 1977; Chow *et al.*, 1977). Some introns play regulatory roles in transcription or translation processes (Cohen *et al.*, 1989; Chapman and Walter, 1997). Introns can also be involved in the maintenance of secondary structure of immature messenger RNAs (pre-mRNAs) (Kirby *et al.*, 1995; Schaeffer and Miller, 1993). Moreover, the detection of a negative correlation between intron length and recombination rates both in *Drosophila* and in humans suggests that intron length might be under weak selective constraint, with a minimum intron size determined by strong selection (Carvalho and Clark, 1999; Comeron and Kreitman, 2000). These constraints may be associated with either transcriptional costs or the evolutionary advantage that longer introns may provide in genomic regions with reduced recombination (Comeron and Kreitman, 2000).

Silkworm, *Bombyx mori*, is a Lepidopteran insect with a long history of significant agricultural value. Understanding its genome organization using molecular markers is important for genetic studies and breeding purpose. In general, silkworms are classified into geographic races on the basis of their origin such as Japanese, Chinese, European, and tropical. Although many studies have attempted

to classify silkworm races by molecular approaches (Hwang *et al.*, 1995; Kim *et al.*, 2000; Seong, 1997) decisive molecular markers are yet available and still more effort is need to better classify silkworm races. In previous study, we completely sequenced fibroin L-chain gene from Baekok-Jam (Choi *et al.*, 2002) and found the existence of two variable regions in the Baekok-Jam fibroin L-chain gene against that of J139, Japanese race. The I and II regions exist in intron 2-exon 3 and intron 6 on the fibroin L-chain gene, respectively. In the present study, therefore, the variable regions from the fibroin L-chain gene were determined from several geographic silkworm races to test if these regions of the gene can be utilized as genetic markers for silkworm races.

Materials and Methods

Silkworm

The silkworm races employed in this study were three geographic races: Japanese race (Jam 123, peanut shape cocoon), Chinese race (Jam 124, elliptical shape cocoon) and their F_1 hybrid Baekok-Jam (Jam 123 × Jam 124, elliptical shape cocoon). For the additional evidence, Jam 125 (Japanese race, peanut shape cocoon), Jam 126 (Chinese race, elliptical shape cocoon) and their F_1 hybrid Daeseong-Jam (Jam 125 × Jam 126, elliptical shape cocoon) were also analyzed.

Primer

The complete fibroin L-chain gene of Baekok-Jam, *B. mori,* was analyzed in previous study (Choi *et al.*, 2002). Two variable regions were observed in the fibroin L-chain gene against that of J139, Japanese race. The region I and II exist in the region spanning from intron 2 and exon 3 and in intron 6 on the fibroin L-chain gene, respectively. The primer sets were synthesized for amplification of the variable region I and II according to the published sequence data for the J139 L-chain gene (GenBank accession number M76430; Kikuchi *et al.*, 1992) and Baekok-Jam L-chain gene (GenBank accession number AF 541967; Choi *et al.*, 2002). The primer set I was; primer 1 as a sense primer (5'-GGCCTTAACTCATAGTCAGTTACGTCA-3') and primer 2 as an antisense primer (5'-ATTGGTAATTACCCTAACCATTGA-3'). The primer set II was; primer 3 as a sense primer (5'-TTGCTCAAGTGTTCCACCAATCAGC-3') and primer 4 as an antisense primer (5'-TCGCAAACGTGTAGGCAGTTACGAA-3').

Genomic DNA and PCR amplification

Genomic DNAs were extracted from silkworm eggs using a Wizard™ Genomic DNA Purification kit, according to the manufacturers instructions (Promega). The primer set I and II were used for amplification of the two variable regions, respectively. PCR reaction was conducted for 500 ng of each genomic DNA with 2.5 U of Taq DNA polymerase, 200 nM each deoxynucleotide triphosphate, 10 pM each primer and 3 mM $MgCl_2$ in a final volume of 20 μl with the DNA Thermal Cycler (TaKaRa). The PCR amplification condition consisted of an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 52°C for 30 sec and 72°C for 1 min, and a final extension step of 72°C for 6 min. Amplified PCR products were analyzed by 1.5% agarose gel electrophoresis. The PCR products were inserted into pGem-T-Easy vector (Promega).

Nucleotide sequencing and multiple sequence alignment

For nucleotide sequencing of the variable regions, pGem-T plasmid clones were purified by a Wizard mini-preparation kit (Promega). Sequence of each clones were determined using SP6 and T7 primers (Promega) by an automatic sequencer (Perkin Elmer, Watsonville, CA, ABI 377). Each purified DNA sample (300 ng) was mixed with the primer (3.2 pmol) and Termination Reaction Mix (Perkin Elmer), and sequenced following 25 cycles of PCR reaction (30 sec at 96°C, 15 sec at 50°C, and 4 min at 60°C). The resulting PCR products were separated on 4.5% denatured polyacrylamide gel and analyzed by DNA Sequencing Analysis Software (Perkin Elmer). The sequence data were compared using the BLAST programs provided by the NCBI using the option Nucleotide query-protein DB [blastx]".

Results and Discussion

Silkworms are classified into geographic races on the basis of their origin. In previous study, nucleotide sequence of Baekok-Jam fibroin L-chain gene was analyzed (Choi *et al.*, 2002). Interestingly, two sequence variable regions were unexpectedly detected on the fibroin L-chain gene against that of J139, Japanese race. The two regions were located in intron 2~ exon 3 and intron 6 on the L-chain gene, respectively. To survey the usefulness of these regions as genetic marker for silkworm races, DNA sequence divergence was analyzed on the fibroin L-chain gene among Japanese races, Chinese races and their F_1 hybrids by PCR and nucleotide sequencing analysis.

Figure 1a shows the PCR products of the variable region I and II from Jam 123, Jam 124 and their F_1 hybrid Baekok-Jam. The PCR product size of region I was 787 bp in Jam 123, 770 bp in Jam 124 and 768 bp in Baekok-Jam, respectively. The size of region II was 470 bp in Jam 123, 428 bp in Jam 124 and 429 bp in Baekok-Jam, respectively.

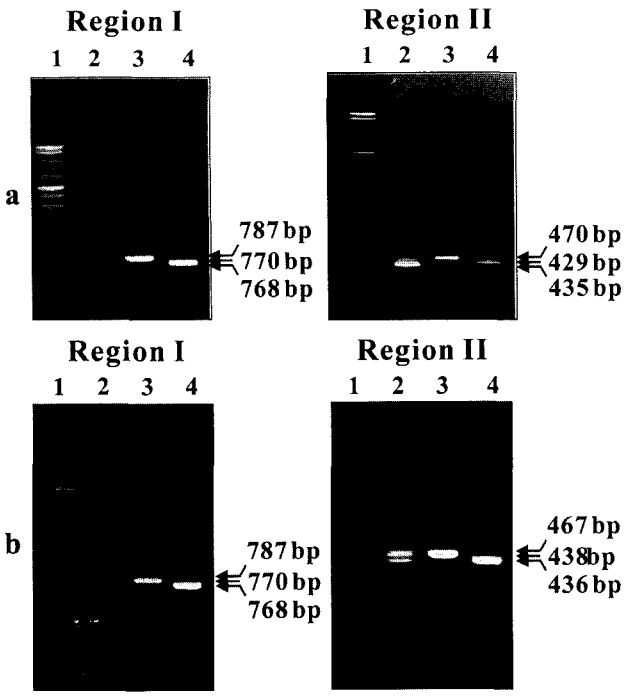

Fig. 1. PCR products of the variable region I and II on fibroin L-chain gene among the geographic silkworm races and their F_1 hybrids. Panel a, Jam 123 (lane 3), Jam 124 (lane 4) and their F_1 hybrid Baekok-Jam (lane 2); panel b, Jam 125 (lane 3), Jam 126 (lane 4) and their F_1 hybrid Daeseong-Jam (lane 2); Lane 1, 1 kb DNA size marker.

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JAM 123 CATCGACGTAGTCCCATGCACGTGAGATTACGGAACTAAAATTATAATAATTATTGAATA
JAM 125 CATCGACGTAGTCCCATGCACGTGAGATTACGGAACTAAAATTATAATAATTATTGAATA
JAM 124 CATCGACGTAGTCCCATGCACGTGAGATTACGGAACTAAAATTATAATAATTATTATATG
JAM 126 CATCGACGTAGTCCCATGCACGTGAGATTACGGAACTAAAATTATAATAATTATTATATG
Baekok  CATCGACGTAGTCCCATGCACGTGAGATTACGGAACTAAAATTATAATAATTATTATATG
Daeseong CATCGACGTAGTCCCATGCACGTGAGATTACGGAACTAAAATTATAATAATTATTATATG

JAM 123 TATAATTATTGAAAACACTTGAACCATGATTGAAAAGTTTTGTGCAATAAAAACTTTAGT
JAM 125 TATAATTATTGAAAACACTTGAACCATGATTGAAAAGTTTTGTGCAATAAAAACTTTAGT
JAM 124 ----------------ACTTGAACCATGATTGAAAAGTTTTGTGCAATAAAAACTT-AGT
JAM 126 ----------------ACTTGAACCATGATTGAAAAGTTTTGTGCAATAAAAACTT-AGT
Baekok  ----------------ACTTGAACCATGATTGAAAAGTTTTGTGCAATAAAAACT--AGT
Daeseong ----------------ACTTGAACCATGATTGAAAAGTTTTGTGCAATAAAAACT--AGT

JAM 123 TTAAAGAAAGGATACTGGTGAGCTTTGCCTGTTACAGATTTGAAGCAGCCAAACGAATTT
JAM 125 TTAAAGAAAGGATACTGGTGAGCTTTGCCTGTTACAGATTTGAAGCAGCCAAACGAATTT
JAM 124 TTAAAGAAAGGATACTGGGGAGCTTTGCCTGGTACAGATTTTGAACACCAAAACGATTTC
JAM 126 TTAAAGAAAGGATACTGGGGAGCTTTGCCTGGTACAGATTTTGAACACCAAAACGATTTC
Baekok  TTAAAGAAAGGATACTGGGGAGCTTTGCCTG-TACAGATTTTGAACACCAAAACGATTTC
Daeseong TTAAAGAAAGGATACTGGGGAGCTTTGCCTG-TACAGATTTTGAACACCAAAACGATTTC
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Fig. 2. Sequence comparison of the variable region I on fibroin L-chain gene among the geographic silkworm races and their F_1 hybrids. In shaded boxes are the nucleotides that are different to each other.

Figures 2 and 3 showed mismatches on the region I and II on the fibroin L-chain gene among Jam 123, Jam 124 and Baekok-Jam. Nucleotide sequences of the region I showed insertion/deletion between Jam 123 (Japanese race) and Jam 124 (Chinese race). However, the region I sequence of Baekok-Jam is almost similar to that of Jam 124 (Fig. 2). Also, the region II of Baekok-Jam is similar to that of Jam 124, Chinese race (Fig. 3). The result indicates that Baekok-Jam fibroin L-chain gene is more

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JAM 123 AACGTGTTTTGGAGTTGATAGATATGGAAACTTCTTATCAAAAAAATATATATACATAGA
JAM 125 AACGTGTTTTGGAGTTGATAGATATAGAAACTCCTTATCAAAAAAAAACATATACATAGA
JAM 124 AACGTGTTTTGGAGTTGATAGAAATAGAAACTCCTTATCAAAAAAAAA---ATACATAGA
JAM 126 AACGTGTATTGGAGTTGATAGAAATAGAAACTCCTTATCAAAAAAAAA---ATACATAGA
Baekok  AACGTGTTTTGGAGTTGATAGAAATAGAAACTCCTTATCAAAAAAAAA---ATACATAGA
Daeseong AACGTGTATTGGAGTTGATAGAAATAGAAACTCCTTATCAAAAAAAAACA-ATACATAGA

JAM 123 CAACAATTACAATAGGTGTCTTAAAAAAAAGTGAAAAATATGTCTGTCGGTTCGAGGAGT
JAM 125 CAGCAGTGAGAATAGGTGTCTTAAAAAAAAGAGAAAAAAATGTCTGTCGGTTCGAGAAGT
JAM 124 CAGTAGTGACAATAGGTGTCTTACAAAAAAVGTGAAAAATATGT----------------
JAM 126 CAGTAGTGACAATAGGTGTCTTACAAAAACGTGAAAAATATGT----------------
Baekok  CAGTAGTGACAATAGGTGTCTTACAAAAAVGTGAAAAATATGT----------------
Daeseong CAGTAGTGACAATAGGTGTCTTACAAAAACGTGAAAAATATGT----------------

JAM 123 AAAACGTAAAACAAAATAACAAAACTGTGATTTTGATAAAAAA-AATCTAAAGCCAT-CC
JAM 125 AAAACAAAATAACAAAACTGTGATTTTGATTTAAAAAAGTATAAAGCCA----ACATAAT
JAM 124 ----------------GACTGTGATTTTGATAAAAAACAATGTAAAGCCAT-CC
JAM 126 ----------------GACTGTGATTTTGATAAAAAGCAATGTAAAGCCATGCCACGTAAT
Baekok  ----------------GACTGTGATTTTGATAAAAAACAATGTAAAGCCATGCC
Daeseong ----------------GACTGTGATTTTGATAAAAAGCAATGTAAAGCCATGCCACGTAAT
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Fig. 3. Sequence comparison of the variable region II on fibroin L-chain gene among the geographic silkworm races and their F_1 hybrids. In shaded boxes are the nucleotides that are different to each other.

closely related to the Chinese race Jam 124 than to the Japanese race Jam 123.

For the additional evidence, Jam 125 (Japanese race), Jam 126 (Chinese race) and their F_1 hybrid Daeseong-Jam were also analyzed. Figure 1b shows the PCR results of the region I and II from Jam 125, Jam 126 and their F_1 hybrid Daeseong-Jam. PCR product size of region I was 787 bp in Jam 125, 770 bp in Jam 126 and 768 bp in Daeseong-Jam, respectively. The size of region II was 467 bp in Jam 125, 436 bp in Jam 126 and 438 bp in Daeseong-Jam, respectively. The nucleotide sequence alignment results of the region I and II were described in Fig. 2 and 3. The nucleotide sequences of Jam 125 and Jam 126 were also different to each other, and the region I of Daeseong-Jam was similar to that of Jam 126 (Fig. 2). In the region II, nucleotide sequence of Daeseong-Jam was very similar to that of Jam 126 (Fig. 3). In the results of additional experiment, the sizes of region I and II were similar to the results of Jam 123, Jam 124 and Baekok-Jam. Presence of insertion/deletion between the two geographic races of Jam 123 or Jam 125 and Jam 124 or Jam 126 was obvious. The results strongly suggested that in the fibroin L-chain gene, Baekok-Jam or Daeseong-Jam is more closely related to Jam 124 or Jam 126 than to the Jam 123 or Jam 125, respectively. In the present study, it was observed that the F_1 hybrid silkworms have a natural mutational bias for tend to be inherited the shorter length intron of fibroin L-chain gene from the previous generations. The phenomenon is in good agreement with that the natural mutational tendencies appear to be biased toward the production of shorter introns (Comeron and Kreitman, 2000; Petrov *et al.*, 1996; Petrov and Hartl, 1998). The phenomenon is not convinced yet in the silkworm races, because only four silkworm races (two geographic groups) were observed in this study. For the confirmation of the phenomenon, an extended study including more geographic races is required.

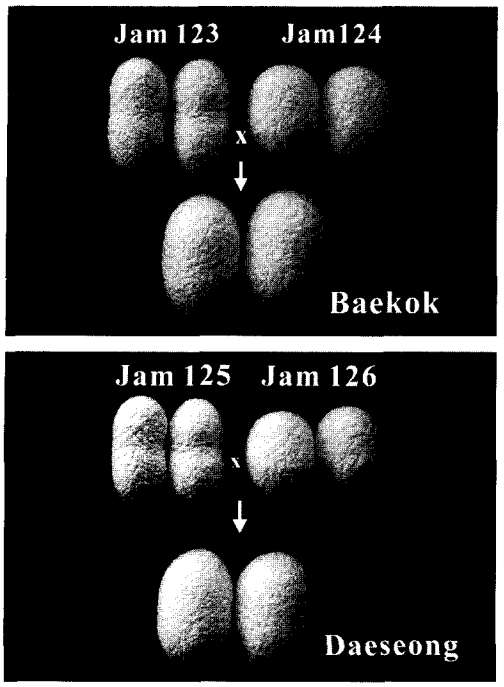

Fig. 4. Photographs of silkworm cocoon. Japanese races (Jam 123 and Jam 125, peanut shape), Chinese races (Jam 124 and Jam 126, elliptical shape) and their F_1 hybrids (Baekok-Jam and Daeseong-Jam, elliptical shape) were observed. The F_1 hybrids are more resemble to the Chinese races in cocoon shapes.

Additionally, the cocoon shapes of tested animals were observed. Figure 4 shows the cocoon shapes of Jam 123 and Jam 125 (peanut shape cocoon), Jam 124 and Jam 126 (elliptical shape cocoon) and their F_1 hybrids Baekok-Jam and Daeseong-Jam (elliptical shape cocoon). In the pictures, Baekok-Jam and Daeseong-Jam are more similar to the Chinese races (♂) than to the Japanese races (♀). Contrarily, opposite crossing manners were conducted with Jam 123 and Jam 125 to be males, Jam 124 and Jam 126 to be females, respectively. Also, the cocoon shapes of F_1 hybrids were resemble to that of Chinese races cocoon shapes, suggesting that the F_1 hybrids inherited their cocoon shapes from Chinese races in the crossing manners between Chinese race and Japanese race. However, this phenomenon is not convinced yet, because the phenotypes are not entirely correlated with their genotypes (Kim *et al.*, 2000).

In conclusion, the primers sets designed in this study from the two variable regions on the fibroin L-chain gene divergence region would be highly useful, at least in Japanese and Chinese races, for the genetic marker among silkworm races and/or breeds. The fibroin L-chain gene and cocoon shape of Baekok-Jam or Daeseong-Jam are more closely related to the Jam 124 or Jam 126 than to Jam 123 or Jam 126.

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