Int. J. Indust. Entomol. Vol. 8, No. 2, 2004, pp. 161~167

International Journal of *Industrial Entomology*

Screening and Cloning of RAPD Markers from the W Chromosome of Silkworm, *Bombyx mori* L.

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(Received 28 December 2003; Accepted 7 May 2004)

Silkworms sex determination drew high attention from researchers. Sex chromosomes on the silkworm are of ZW type for females and ZZ type for males. Chromosome W plays an important role in sex determination. Although several molecular linkage maps have been constructed for silkworm, very few markers are discovered on the W chromosome. In order to look for molecular markers and to further locate the *Fem* gene on chromosome W, we used genomic DNA from both female and male larvae of a silkworm strain named 937 as PCR templates for RAPD amplification with 200 arbitrary 10-mer primers. The amplification results showed three female-specific bands, namely OPG-07_{496}, OPC-$15_{1,660}$ and OPE-$18_{1,279}$. Further verification, however, revealed no band from OPG-07 and OPC-15 in either sex in the strain 798, but OPE-18 provided female-specific band in the strains Suluan7 and C108, and absent in both males and strain 798. This indicates that the bands from OPG-07_{496} and OPC-$15_{1,660}$ are probably female-specific in strain 937, and the band from OPE-18 was probably amplified from a common segment shared by most strains. The genomic DNAs from OPG-07 and OPC-15 were cloned and sequenced. Sequence analysis showed that the DNAs from OPG-07 and OPC-15 have high identities with the retrotransposable elements, and DNA from OPC-15 contains a portion of sequence which probably encodes an eukaryotic translation initiation factor 4E binding protein (eIF4EBP).

Key words: *Bombyx mori*, RAPD marker, Female-specific markers, W chromosome

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Introduction

Silkworm (*Bombyx mori* L.) is a good model animal for entomological studies. Its sex is determined by the presence of chromosome W. Its sex chromosomes are of ZW type for females and ZZ type for males. The presence of chromosome W determines individuals sex as female even in a triploid or tetraploid silkworm. One of the female determining genes named *Fem* has been reported and it is supposed to be located on chromosome W since 1933 (Hasimoto, 1933).

Molecular markers of silkworm genome have been extensively studied in recent years. Yasukochi (1998) constructed a dense genetic map of the silkworm, covering all 27 autosomes and chromosome Z, based on 1,018 RAPD markers. Tan *et al.* (2001) prepared an AFLP (Amplified Fragment Length Polymorphism) linkage map of silkworm based on 1,248 AFLP markers. Although a considerable number of molecular markers have been found on autosomes and chromosome Z of silkworm, very few ones have been found on chromosome W. Abe *et al.* (1998) found four RAPD markers which showed homology to retrotransposable elements and a complete full-length non-LTR retrotransposon (BMC1) on chromosome W. Compared with chromosome Z, on which 14 genes have been found and BAC contigs constructed (Koike *et al.*, 2003), no single gene has been found on chromosome W, even the *Fem* gene.

The main reason could be that the chromosomes of silkworm are small, holocentric and numerous. It is difficult to identify the sex chromosome especially chromosome W from the whole genome. Furthermore, the chromosome W is recombinationally isolated from the Z chromosome and autosomes during meiosis, thereby making it impossible to analyse the chromosome W by recombination way.

Molecular biological methods are useful to study chromosomes of silkworm. Beyond AFLP and RAPD techniques, a number of other methods have been widely used.

Table 1. Characteristics of silkworm strains used in this study

Strain	Chromosome W	Autosome	Female characteristics	Male characteristics
937	T (w, 2)$^{+p}$	*p/p*	Spotted skin	White skin
Suluan7	T (w, 10)$^{+w3}$	*w3/w3*	White egg	Black egg
798	Normal	*+p/+p*	Spotted skin	Spotted skin
C108	Normal	*p/p*	White skin	White skin

Note: +p: larval marker gene, epistatic, spotted skin; p: allele of +p, white skin; +w_3: the 3rd white egg marker gene, epistatic, white egg; w_3: allele of + w_3, and normal colour of egg.

These include RFLP (Restriction Fragment Length Polymorphism)(Cheng *et al.*, 2000), SSR (Simple Sequence Repeat) (Reddy *et al.*, 1999), and SADF (Selective Amplification of DNA Fragment) (He *et al.*, 2001).

In this study, we used two translocation-carrying strains: 937, Suluan7, and two normal strains: 798 and C108. The translocation carrying strains were prepared by means of radiation treatment, through which some marker genes on the autosome were translocated onto the chromosome W. That is to say, some segments of the chromosome W of these strains were displaced by the translocated genes (The Sericultural Research Institute, 1991). Strain 937 has the [T (w, 2)+^{p} chromosome W], which carries visible larval marker of the +^{p} loci [T (w, 2)+^{p} chromosome]. Suluan7 has the T (w, 10)+$^{w\text{-}3}$ chromosome W, which carries the 3rd white egg marker gene (Tazima *et al.*, 1951). The normal strains have a natural chromosome W. All strains autosomes are normal. RAPD technique was used to find the difference between the females and males. Yokoyama *et al.* (2003) used a translocation-carrying strain, TWPB, found a female specific RAPD marker (named female-218). The female larvae of strain TWPB has black skin due to the larval marker gene *p*(B) (T (W, 2) *p*(B), *+p/+p*), while male larvae has whitish skin (*+p/+p*). It was verified that the female-218 band was amplified from the translocated part of chromosome W. This means that the RAPD technique is workable and reliable.

Materials and Methods

Preparation of materials

Strain 937 was the major material used in this study. Meanwhile, three other strains, C108, Suluan7, and 798, were also used. Each strain was highly inbred and had been maintained by sister-brother mating respectively at Institute of Life Sciences, Jiangsu University. Strains 937 and Suluan7 have translocation carring chromosome W, C108 and 798 have a normal chromosome W. Strain 798 was used as a productive strain and has the same normal larval marking gene +^{p} on autosome, same as that of strain 937 on chromosome W, the characteristics of the four strains are shown in Table 1. All silkworms were raised under favorable environment and fed on fresh mulberry leaves.

DNA extraction

For each strain, 30 males and 30 females from the same pregnancy were used. The posterior silk glands were separated from the larvae at the 3rd day of the 5th instar, frozen with liquid nitrogen and ground into powder. For each sample, 5 ml of the DNA extraction buffer [10 mM Tris-HCl (pH 8.0), 100 mM EDTA (pH 8.0), 0.5% SDS] was added to the powder and the mixture was incubated with proteinase K (100 μg/ml) overnight at 56°C. The further purification procedure was as that of Yao *et al.* (2003). Finally, the samples inspected with Gene spec (Naka Instruments Co., Ltd.), electrophoresed the DNA on 0.8% agarose gel to see the integrality, and stored them at –20°C for further analysis.

PCR amplification

We used 200 arbitrary 10-mer primers for the PCR amplification using a thermocycler (GeneAmp PCR System 9600, PE, USA). Total reaction volume was 25 μl containing 2.5 μl 10× *Taq* polymerase buffer, 150 μM of dNTPs, 2 mM of Mg^{2+}, 0.5 μM of 10-mer primers, 10 – 20 ng of template DNA and 0.75 unit of *Taq* polymerase (TaKaRa). The amplification procedure was as follows: one cycle of 94°C for 180 sec, 40 cycles of 94°C for 30 sec, 40°C for 60 sec, and 72°C for 90 sec, followed by final extension of 10 min at 72°C. PCR products were analyzed by electrophoresis on 1.0% agarose gel, stained with ethidium bromide, and photographed with AlphaImager™ 1220 gel image analyzing system (Alpha Innotech, USA).

Cloning and sequencing

The RAPD specific bands obtained by OPG-07 and OPC-15 primers were cut from 1.0% low-melting point agarose gel (Sigma, Spanish)and extracted according to Sambrook *et al.* (1989). The derived DNA was linked using pMD18-T Vector Kit (TaKaRa) according to the manufactory's instruction.

The linkage production was transformed into competent DH-5α cells (TaKaRa) by the following steps: on ice for 30 min, 42°C for 2 min and on ice for 2 min. Cells were cultured on LB medium with 50 μg/ml Ampicillin Amin overnight. The transformants were selected and cultured respectively in 3 ml of liquid LB medium with 50 μg/ml Ampicillin Amin overnight. Next morning, the plasmids were extracted according to Sambrook *et al.* (1989), digested with endonucleases (*Hind* and *EcoR*) at 37°C for 3 – 5 hrs, and electrophoresed to see whether the DNA fragment was inserted into the vector. The cloned target bands were subjected to PCR to confirm the presence of the clones in the plasmid, analyzed the PCR products and selected the best clone for sequencing. The sequencing were performed using an automatic sequencer: 4200L NEN Global IR^2 DNA Sequencer System (Li-Cor, John Morris Scientific Pty Ltd., USA).

GenBank accession number

Sequences obtained from the OPG-07 and OPC-15 primers were registered in the GenBank, EMBL, DDBJ and PD nucleotide sequence databases under the accession numbers: AY378098 for the OPG-07$_{496}$ RAPD of strain 937 and AY378099 for the OPC-15$_{1,660}$ RAPD.

Results and Discussion

RAPD amplification and verification of the molecular markers

Among 200 10-mer primers (Operon Kits A, B, C, D, E, F, G, H, I, S) used, three showed female-specific bands. Using OPG-07 (5-GAACCTGCGG-3'), a female-specific band of 496 bp (Fig. 1A) was obtained (named OPG-07$_{496}$). Using OPC-15 (5-GACGGATCAG-3'), a female-specific band of 1,660 bp (Fig. 1B) was obtained (named OPC-15$_{1,660}$). Using OPE-18 (5-GGACTGCAGA-3'), a female-specific band of 1,279 bp was obtained (named OPE-18$_{1,279}$).

Strain 798 was used to verify the OPG-07$_{496}$ band, but not found the band in either sex of strain 798 (Fig. 1A). Using strain 798 to verify the OPC-15$_{1,660}$ band, it appeared only in the female of strain 937, but not found in either sex of strain 798 (figure not shown). Using strain Suluan7 to verify the OPE-18$_{1,279}$, the same female-specific band was found in both strains (Fig. 1C). Using strains 798 and C108 to verify the OPE-18$_{1,279}$, the same female-specific band was found in strain C108, but not found in either sex of strain 798 (Fig. 2).

RAPD adopts a positive amplifying strategy. Any primer-associated sequence in the genome can be the primer's targets and be amplified. On the other hand, the sample size we used (silk glands from 30 individuals of each sex) can minimize diversity among the individuals. This ensures that any difference observed in the amplification results were genuinely from difference of sex. That is to say, the female-specific bands were amplified from chromosome W.

For band OPG-07$_{496}$ appearing in females of strain 937, there are two possibilities. First, it was from the translocation region (carrying $+^p$ loci) of the W chromosome. Second, it was from the region of the chromosome W beyond the translocation loci (carrying $+^p$ loci). The verification amplification indicated the latter was what happened, because OPG-07$_{496}$ was not found in either sex of strain 798 (Fig. 2), while strain 798 has the $+^p$ loci in both sex. For band OPC-15$_{1,660}$, the same conclusion can be drawn. These results imply that OPG-07$_{496}$ and OPC-15$_{1,660}$ are probably female-specific only in strain 937. OPE-18$_{1,279}$ RAPD did not appear in either sex of strain

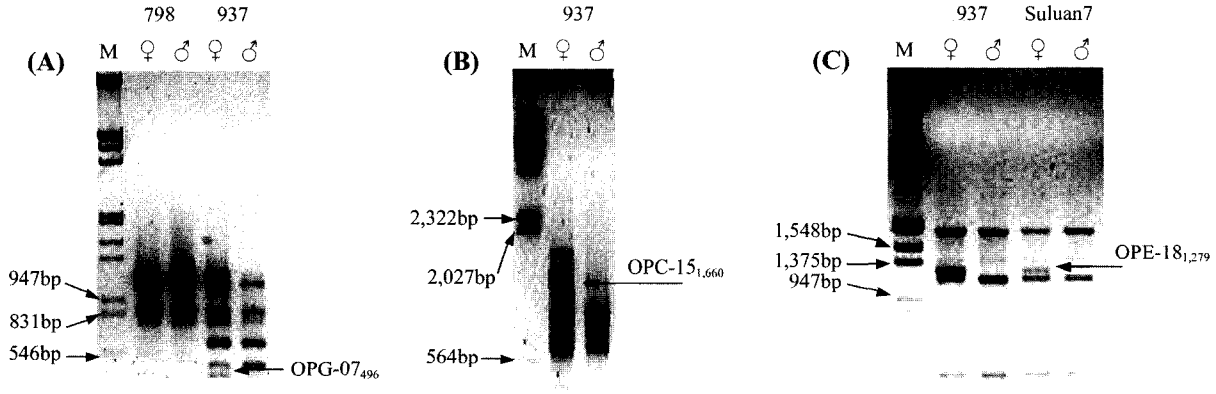

Fig. 1. Amplification patterns of genomic DNA from females and males of strains of 798, 937 and Suluan7 using 10-mer primers: A, OPG-07; B, OPC-15; C, OPE-18. Arrows indicate female-specific RAPDs: OPG-07$_{496}$, OPC-15$_{1,660}$, and OPE-18$_{1,279}$ respectively. M, Melecular marker (TaKaRa): A,C, λ DNA *HindIII/EcoRI*; B, λ DNA *HindIII*.

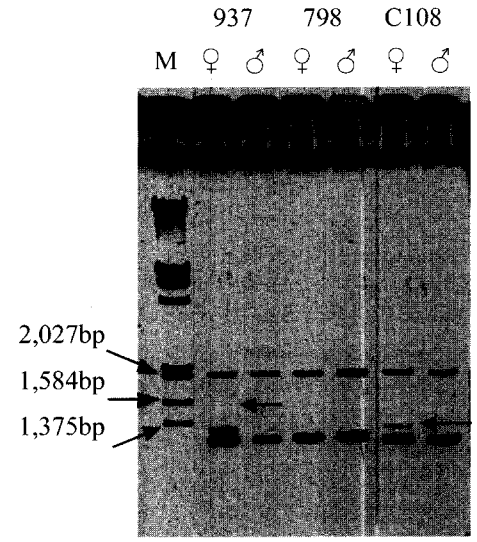

Fig. 2. Amplification patterns of genomic DNA from females and males of strains of 937, 798 and C108 using 10-mer primer OPE-18. Arrows indicate female-specific RAPD OPE-$18_{1,279}$. M, Melecular marker(TaKaRa): λ DNA *HindIII/EcoRI*.

798. This indicates that it was not amplified from the translocation loci (carrying $+^{p}$ loci). On the other hand, OPE-$18_{1,279}$ RAPD was present in all females of strains 937, Suluan7 and C108 but absent in all males. These results strongly suggest that chromosome W of different strains probably share a same nucleotide fragment. Whether OPE-$18_{1,279}$ RAPD has any relationship with the *Fem* gene or any other unknown sex determining element on the chromosome W, it requires further analysis and research work.

In addition, all three bands (OPG-07_{496}, OPC-$15_{1,660}$ and OPE-$18_{1,279}$) did not appear in all females and males of strain 798. We regard that it was probably because that strain 798 was used as a productive strain and some segments of its chromosome W had been lost during the construction.

Sequence analysis

OPG-07_{496} RAPD of strain 937

OPG-07_{496} RAPD of strain 937 consists of 496 bp (Fig.

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CGTTAGTATT TTTGTGCTAC ATCTCCAGCC GTTGAATCCT AATGTATTTT ATGATTATAA   120
TAATCTATCA ATTTTTTTTT TTTCCTACCT AAGCTGAGAG CCTTGAGAGG CTCTTTCAGC   180
GTAACCCTAA CGTTTGTAGG TGAGCTCACG GGGCTCAAAC CTGATGACGT TGCTAACACG   240
AACCCTAGCG AGAGCCGTGC TTCGCAGAAT CTACCACCGG ATCGGAAACG CGACCCACTG   300
AGAAGATCCG GCGAGAAACT CAGTGGGCTG TGTCTGAGAG TTAATTTACT CGTCGAGCCC   360
TTTGTCGCTT GCGACGGGTT CAACGAGAAC GGTGACCGGT GCTTGAAGTA CCTAGAAGCA   420
CTGTTAGTGG ATCGGGAGGA TCCGAGATGA CGTGTTTTGG GCGACGTCGA CTGCTTTCCA   480
TTCTGTCCGC AGGTTC                                                   496
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(A)

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L1Bm DNA:625tgcggacagaatggaaagcagtcgacgtcgcccaaaacacgtcatctcggatcctcctga 684
         ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
OPG-07496:491tgcggacagaatggaaagcagtcgacgtcgcccaaaacacgtcatctcggatcctcccga 432

L1Bm DNA:685 tccactaacggtgcttttaggtacttcaagcaccggtcaccgttctcgtcgaacccgtcg 744
             ||||||||| |||||| ||||||||||||||||||||||||||||||||| ||||||||||
OPG-07496:431 tccactaacagtgcttctaggtacttcaagcaccggtcaccgttctcgttgaacccgtcg 372

L1Bm DNA:745 cttgcgacgaagggctcgacgaataaattaactctcagacacagcccactgagtttctcg 804
             | ||||| |||||||||||||| ||||||||||||||||||||||||||||||||||||
OPG-07496:371 caagcgacaaagggctcgacgagtaaattaactctcagacacagcccactgagtttctcg 312

L1Bm DNA:805 ccggatcttctcagtggntcgcg-ttccgatccggtggtagattctgcgaagcacggctc 863
             |||||||||||||||||| ||||| ||||||||||||||||||||||||||||||||||
OPG-07496:311 ccggatcttctcagtgggtcgcgtttccgatccggtggtagattctgcgaagcacggctc 252

L1Bm DNA:864 ttgctagggttcgtgttagcaacatcgtcaggtttgagccccgtgag 910
             | |||||||||||||||||||| || ||||||||||||||||||||
OPG-07496:251 tcgctagggttcgtgttagcaacgtcatcaggtttgagccccgtgag 205
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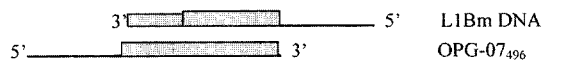
(B)



(C)

Fig. 3. Nucleotide of OPG-07_{496} of strain 937 and the alignment of OPG-07_{496} with L1Bm DNA of *B. mori*. A, Nucleotide of OPG-07_{496} of strain 937. B, bl2seq alignment of OPG-07_{496} RAPD of strain 937 and L1Bm DNA of *B. mori* (Ichimura *et al.*,1997, at GenBank number: AB002276.1). C,The superposition of L1Bm DNA and OPG-07_{496}. The numbers on the right correspond to the nucleotide numbers, "|" indicates similarity.

3A). GenBank search using blastn showed that the highest identity was found with a non-LTR retrotransposon L1Bm DNA of *B. mori* (Ichimura *et al.*, 1997, GenBank number: AB002276.1). The L1Bm DNA was 910 bp long and at the nucleotide position from nt 910 to nt 625 (with one gap) has 95% identities with OPG-07_{496} RAPD at the nucleotide position from nt 205 to nt 491 (Fig. 3B). OPG-07_{496} RAPD has an end overlapped with one end of L1Bm DNA (Fig. 3C).

GenBank search using blastx revealed that the sequence of OPG-07_{496} RAPD has a high identity with many sequences of reverse transcriptase (data not shown). The highest one is an endonuclease and RT-like protein of *B. mori* (Abe *et al.*, 1998; GenBank number: BAA76304.1) at the nucleotide position from nt 911 to nt 936 and it has 57% identities at the nucleotide position from nt 475 to nt 398 of OPG-07_{496} RAPD. However, GenBank search using rpsblast for conserved domain, not any bits found. The search results indicate strongly that the band of OPG-07_{496} RAPD of strain 937 probably belongs to the same retrotransposon as the L1Bm DNA of *B. mori*. However, this needs further verification.

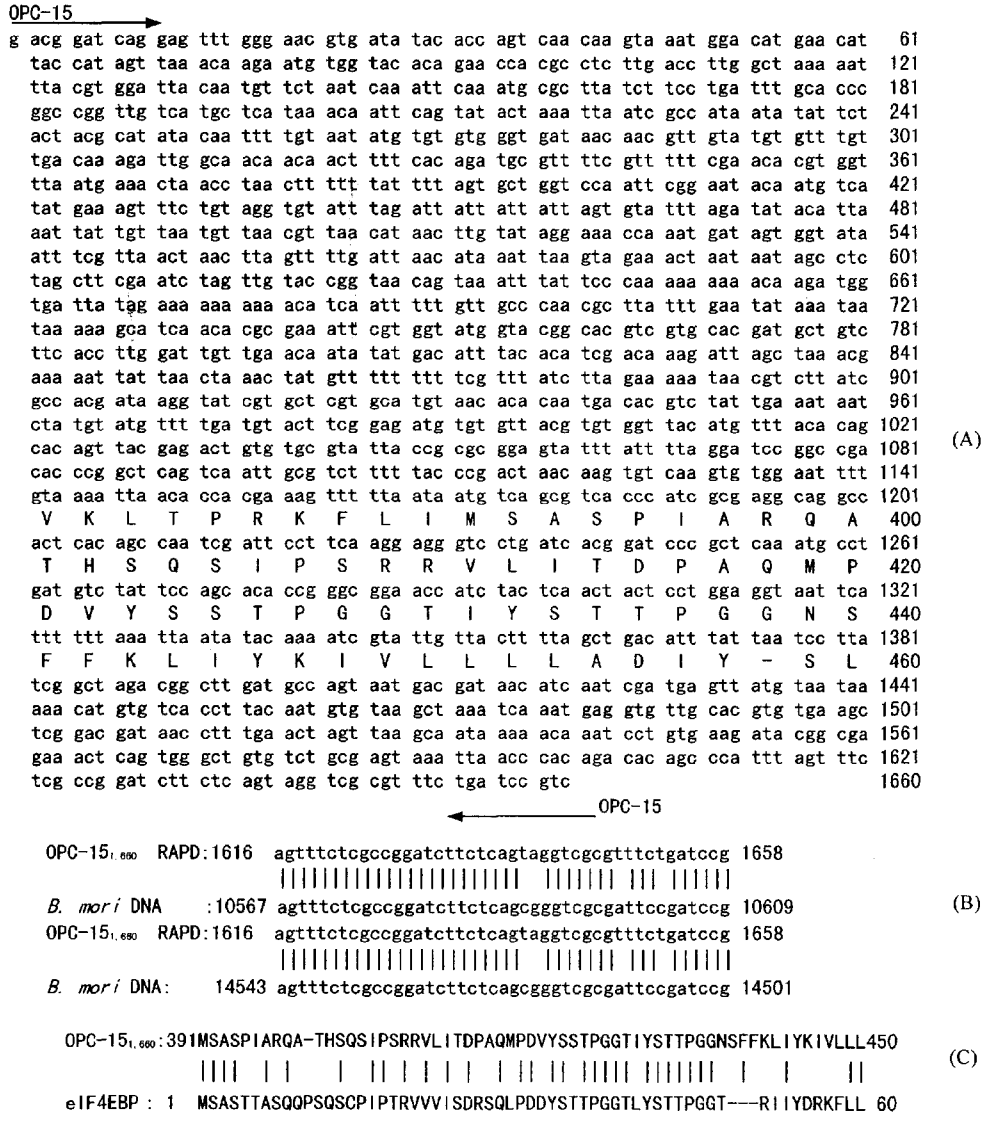


Fig. 4. Nucleotide and deduced amino acid sequence of OPC-$15_{1,660}$ RAPD and their blast results.A, Nucleotide and deduced amino acid sequence of OPC-$15_{1,660}$ RAPD. B, Alignment of CPC-$15_{1,660}$ RAPD and *B. mori* DNA on chromosome W (Unpublished, GenBank number: AB055391.1,). C, Alignment of deduced amino acid sequence of OPC-$15_{1,660}$ RAPD and an eIF4EBP (GenBank number: 23682). The deduced amino acid sequence is indicated below the nucleotide sequence, numbers on the right correspond to the nucleotide and amino acid numbers, arrows top and bottom indicate locations of primers used, underlined amino acid residues indicates the conserved domain compared with eIF4EBP, "|" indicates similarity, "–" was introduced to align sequence.

OPC-$15_{1,660}$ RAPD of strain 937

OPC-$15_{1,660}$ RAPD of strain 937 consists of 1,660 bp, and has two long ORFs (Open Reading Frames). The first is at the nucleotide position from nt 1,034 to nt 1,119. The second is 177 bp long, at the nucleotide position from nt 1,172 to nt 1,348. The nucleotide and deduced amino acid sequence are shown in Fig. 4A.

GenBank search using blastn showed that short portion of less than 80 bp of OPC-$15_{1,660}$ RAPD has identifies with many species. Such as: *Bombyx mori*, *Drosophila melanogaster*, *Danio rerio* (Zebrafish), *Homo sapiens*, *Mus musculus* (House mouse), etc. However, it has stronger identities with sequences from *B. mori*. For example, OPC-$15_{1,660}$ RAPD of strain 937 at the nuleotide position from nt 1,588 to nt 1,649 has 90% identities with a non-LTR retrotransposon L1Bm DNA of *B. mori* (Ichimura *et al.*, 1997, GenBank number: AB002276.1) at the nucleotide position from nt 768 to nt 829. The fragment of OPC-$15_{1,660}$ RAPD of strain 937 at the nucleotide position from nt 1,616 to nt 1,658 appears in more than 90% of the sequences of all these retrotransposons and repetitive elements of *B. mori*. A non-LTR retrotransposons on chromosome W of *B. mori* DNA (GenBank number: AB055391.1), which Abe *et al.* (1998) found in female with a T (w, 2)+*p* chromosome W of silkworm, at the nucleotide position from nt 10,567 to nt 10,609 (forward) and from nt 14,543 to nt 14,501 (reverse) has 90% identities with OPC-$15_{1,660}$ RAPD from nt 1,616 to 1,658 (Fig. 4B).

GenBank search using rpsblast for reserved domain found 60 residues in the second ORF of OPC-$15_{1,660}$ RAPD produced a significant alignment with an eukaryotic translation initiation factor 4E binding protein (eIF4EBP, GenBank number: 23682), shown in Figure 4C. The eIF4EBP is a family consists of several eIF4EBPs (eIF4EBP1, 2 and 3). Tomek *et al.* (2002) found the eIF4E-BP[1] in Bovine Oocytes regulating the maturation. The eIF4EBP homologue was also found in *H. sapiens* (Poulin *et al.*, 1998), *M. musculus* (Lin *et al.*, 1996), *Dictyostelium discoideum* (Morio *et al.*, 1998), *D. rerio* (Unpublished, GenBank number: AAG50053), *Rattus norvegicus* (Norway rat) (Lin *et al.*, 1994) and *Drosophila melanogaster* (unpublished, GenBank number: AAF 51100) etc., but the similar report of *B. mori* hasn't appeared.

The search results of OPC-$15_{1,660}$ RAPD showed a high sequence similarity in a portion in many species, and this portion probably encodes the eIF4EBP. However, the presence of this eIF4EBP homologue in *B. mori*, its function and specificity in female all need further research.

Acknowledgments

This study was supported by Jiangsu Agricultural High-Tech Research Fund (No.: BG2001317) and Student Sciences Research Fund of Jiangsu University (No.: A014).

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