Studies on the molecular structure, organization, and expression of novel bombyxin genesof the silkmoth Bombyx mori

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Studies on the molecular structure, organization, and expression of novel bombyxin genes of the silkmoth *Bombyx mori*

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ABSTRACT

Insulin family peptide members play a pivotal role in regulation of growth, metabolism, and reproduction in vertebrates. Insulin-like peptides (ILPs) have also been identified from invertebrates, including several insect species. Bombyxin, isolated from the silkmoth *Bombyx mori* was the first ILP identified in insects. To gain insight into the role of bombyxin genes, the full genome of *B. mori* was analyzed, identified six novel bombyxin genes and grouped to five novel families: bombyxin-V through Z. All of these genes encoded preprobombyxin, the precursor molecule for bombyxin. Amino acid sequence comparison showed that these prepropeptides have 21 to 50% and 21 to 26% identities with previously characterized preprobombyxins and human preproinsulin, respectively. Genomic organization and chromosomal location of the genes were characterized. Each of *bombyxin-V1* and -*V2* had one intervening intron at 5' untranslated region (UTR), whereas *Z1* had three introns - two at 5' UTR and one in C- peptide region. Human insulin and relaxin genes have intervening introns at almost the same position of these bombyxin genes.

Reverse transcription-PCR and whole-mount *in situ* hybridization in different tissues and developmental stages were performed to observe temporal and spatial expression pattern. Newly identified bombyxin genes were expressed in diversified tissues. *Bombyxin-V1, -W1* and -*Y1* genes were predominantly expressed in four pairs of the medial neurosecretory cells of brain, which are the expression sites of other bombyxin genes also. The appearance of a short C-peptide, an extended A chain, appearance of high expression in non-feeding stages fat body, and induction upon starvation of *bombyxin-X1* indicates its growth function and some structural similarities with insulin-like growth factors (IGFs). *Bombyxin-Z1* was prejudiced to female insect. High level expression of *Z1* in the follicular cells of ovary may suggest its function in reproduction. These results therefore demonstrate that bombyxin gene might have been originated from a vertebrate insulin-like common ancestral gene, and evolved into a diverse gene family with multiple functions.

INTRODUCTION

Insulin is the most widely studied peptide hormone, as its malfunction to regulate carbohydrate metabolism in human causes most common metabolic disorder worldwide – diabetes. Mammals including humans produce upto 10 insulin-like peptides (ILPs) that are further subdivided into insulin, insulin-like growth factors (IGFs), and relaxins (Wu and Brown, 2006). Insulin and IGFs constitute a fundamental family of hormone polypeptides common to all metazoans. These hormones control essential functions including cell growth, metabolism, development, reproduction, and longevity (Efstratiadis, 1998; Nakae *et al*., 2001; Saltiel and Kahn, 2001; Holzenberger *et al*., 2003; Nef *et al*., 2003) in vertebrate. Members of the insulin superfamily are present in both vertebrate and invertebrate species where they are usually designated as ILPs (De Meyts, 2004; Badisco *et al*., 2008). ILPs have also been characterized in very few insect species of order Orthoptera (Lagueux *et al*., 1990), Coleoptera (Li *et al*., 2008), Lepidoptera (Nagasawa *et al.,* 1984, 1986; Iwami *et al*., 1989; Ishizaki, 2004), Diptera (*Brogiolo et al., 2001*; Riehle and Brown, 2002; Krieger *et al*., 2004; Riehle *et al*., 2006), and Hymenoptera (Wheeler *et al*., 2006; de-Azevedo and Hartfelder, 2008). Among these, bombyxin, a family of peptides produced by the brain of the silkmoth *Bombyx mori*, was the first ILP to be isolated from insect (Nagasawa *et al.,* 1984, 1986). Bombyxin was originally identified as the *B. mori* small prothoracicotropic hormone (4K-PTTH), as it exerted prothoracicotropic activity in a related silkworm species, *Samia cynthia ricini* (saturniid silkworm), but unexpectedly not in *B. mori* itself. (Ishizaki *et al*., 1983)

Subsequently, many putative ILPs from a number of non-vertebrate animals have been deduced (Nassel, 2002; Wu and Brown, 2006). The organization of insect ILP precursors is very similar to that of vertebrate insulin. Mammalian insulin is very well known for its key role in glucose homeostasis. For insects on the contrary, reports on the involvement

of ILPs in carbohydrate metabolism are scarce (Satake *et al*., 1997; Broughton *et al*., 2005). Most research has focused on the role of ILPs in controlling cell, organ and total body size, and growth (Brogiolo *et al*., 2001; Ikeya *et al*., 2002; Nijhout and Grunert, 2002; Nijhout, 2003; Stern, 2003; Goto *et al*., 2005). Moreover, ILPs thought to be involved in controlling life-span (Tatar *et al*., 2001). Role for ILPs in insect reproduction physiology (Iwami *et al*., 1996a; Fullbright *et al*., 1997a, b; Tatar *et al*., 2001; Broughton *et al*., 2005) has also suggested. Additionally, effect of ILPs on ecdysteroidogenesis at prothoracic gland and ovary has been described in few insect species (Nagasawa *et al*., 1984; Tu *et al*., 2002; Maniere *et al*., 2004). From physiological experiment of *B. mori*, bombyxin has been thought as a critical factor regulating insect molting and metamorphosis (Nagasawa *et al.,* 1984), homeostasis of glucose level (Masumura *et al.,* 2000), lipid metabolism (Satake *et al.,* 1999), and ovarian development in the female pupae (Fullbright *et al*., 1997b). But the exact physiological function of bombyxin in the *B. mori* is still considered obscure and further study was felt necessary for the elucidation of their real function (Iwami *et al.,* 1996a; Iwami, 2000; Garde and Hoffmann, 2005).

Most insect ILP genes encode a single prepropeptide with a secretory signal peptide, a B chain, an interconnecting C-peptide, and an A chain (Adachi *et al*., 1989; Lagueux *et al*., 1990; Iwami *et al*., 1996b; Brogiolo *et al*., 2001; Krieger *et al*., 2004). The conserved structural motifs of the insulin superfamily in vertebrates and invertebrates are based on limited amino acid identity (Claeys *et al*., 2002). Bombyxins have structural similarity to vertebrate insulin and amino acid sequence identity to mammalian ILPs (Iwami, 2000; Truman, 2006; Okamoto *et al*., 2009a), which suggest a probable role of bombyxin in the regulation of growth and development.

Timing, location, and amount of gene expression can have a profound effect on the functions (actions) of the gene in the organism. A detailed understanding of how genes

are expressed in different tissues can help to elucidate the molecular mechanisms of tissue development and function (Liu *et al.*, 2008). Bombyxin genes are expressed predominantly in the brain (Iwami *et al.,* 1989, Kawakami *et al.*, 1989; Iwami, 1990; Iwami, 2000) and at low level in a number of other larval tissues (Iwami *et al*, 1996a), in contrast to the insulin gene which is expressed in the gastroenteromic organ and is almost silent in the brain. Although insulin is a gut hormone, IGFs are found in diverse tissues and relaxin in the brain and reproductive tract (Ivell, 1997; Ehrenborg *et al.*, 1999; Wu and Brown, 2006). Recently, two ILPs of *B. mori* and *Drosophila melanogaster* have found to be highly produced in fat body and suggested as IGF-like peptides, IGFLPs (Okamoto *et al.*, 2009a, b; Slaidina *et al.*, 2009). Possibility of finding more IGFLP homologs by examining characteristic very high level of expression in the fat body during pupa-adult development was suggested by these authors.

Compared with the limited structural variation of vertebrate insulins (Steiner *et al.*, 1985), bombyxin has a large diversity in structure. So far, thirty-two bombyxin genes belonging to 7 families have been isolated from the *B. mori* genome. (Kawakami *et al.,* 1989 ; Iwami *et al.*, 1989, 1990; Kondo *et al.*, 1996; Tsuzuki *et al.*, 1997; Yoshida *et al.*, 1997, 1998 ; Iwami, 2000 ; Wu and Brown, 2006). This suggests that very high degree of diversification in structure and genomic organization of bombyxin genes occurred through long time evolution.

Bombyxin comprises highly heterogenous molecular forms, I, II, III, IV, and V have so far been identified from *B. mori* head (Nagasawa *et al.*, 1984, 1986, 1988; Jhoti *et al.*, 1987; Maruyama *et al.*, 1988). The amino acid sequences deduced from the bombyxin genes showed that bombyxin II is the product of genes A6 and /or A7 (Kondo *et al.*, 1996) and bombyxin IV is that of gene E1 (Tsuzuki *et al.*, 1997). On the other hand, bombyxins III and V do not coincide with any bombyxins deduced from the genes. Based on these

analyses Iwami (2000) assumed that still large number of bombyxin gene copies remain undetected in the *B. mori* genome.

Although insulins and structurally related peptides are found in vertebrates as well as in invertebrates, it is not clear whether the genes encoding these hormones have emerged from a single ancestral (insulin-type) gene or, alternatively, have arisen independently through convergent evolution from different types of gene (Smit *et al.*, 1993). Due to sharp contrast in lacking intron, having multiple gene copies and different expression sites in bombyxin genes, it was thought that different mechanisms underlie the evolution of ILPs in vertebrate and invertebrate (Kondo *et al.*, 1996; Iwami, 2000). But, presence of an intervening intron in the C peptide of insulin, IGF, and relaxin was led to suggest their evolution from a common ancestral gene (Crawford *et al.,* 1984). Still, more bombyxin genes remain undetected in the *B. mori* genome (Kondo *et al.*, 1996; Iwami, 2000) and therefore it is needed to identify all bombyxin genes by genomic analysis. If any bombyxin gene with the same location intron and similar expression site as vertebrate insulin gene could be found, an evolutionary relationship could be established.

To reveal the genomic evolution of bombyxin genes, I analyzed recently sequenced full genome of *B. mori* and identified five novel family bombyxin genes. I present here the characterization of these new genes. It was found that three of the genes have introns at almost same position as the human insulin gene. Furthermore, transcript expression of new bombyxin genes was investigated in different tissues and stages using RT-PCR and *in situ* hybridization. Furthermore, I performed developmental expression profile and observed effect of starvation on expression of these novel genes. While some of the new bombyxin genes expressed in neurosecretory cell, one of them solely expressed in non feeding states fat body and induced upon starvation. Elucidation of spatial localization of bombyxin mRNA in different tissues will give an indication of the physiological function(s)

in *B*. mori. Moreover, this characterization and expression pattern will provide important basis to elucidate evolutionary relationship and function of insulin family members in insect.

MATERIALS AND METHODS

Gene identification, structure, and alignment

Gene identification: Nucleotide sequences of new bombyxin genes were identified from the *B. mori* genome (The international silkworm genome consortium, 2008). The *B. mori* genome database KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase/) (Shimomura *et al*., 2009) was searched for additional genes encoding bombyxin and insulin-related peptide using nucleotide and peptide sequences of previously identified bombyxin genes. The deduced amino acid sequences of significant matches were examined for insulin-like characteristics, including B chain and C-peptides, dibasic processing sites flanking the C-peptide, a signal peptide, and a stop codon following the A chain. Signal peptides were predicted using a program SIG-pred (http://bmbpcu36.leeds.ac.uk/prot_analysis/Signal.html) (Zhang *et al*., 2009). Endoprotease cleavage sites for C-peptides were determined according to the procedures outlined earlier (Seidah and Chretien, 1997).

Exon-intron structure: Exon-intron boundaries were confirmed by comparing the genomic DNA and cDNA sequences (Appendix V). The exon-intron splice junctions adhere to the GT-AG consensus sequence of splice donor and acceptor sites in eukaryotic genes philosophy (Shapiro and Senapathy, 1987) was used to confirm the exon-intron position.

Alignment analysis: Nucleotide and amino acid sequence analysis was performed using a bioinformatic software Geneious Pro 4.8. (Biomatters, Auckland, New Zealand) (Drummond *et al*., 2009).

Phylogenetic analysis

A phylogenetic analysis of all previously identified bombyxin genes, novel bombyxin genes, and human insulin family members was performed. Entire amino acid sequences for the peptides were subjected to phylogenetic analysis using Geneious Pro 5.0.2 software.

Experimental insects

Eggs of a racial hybrid, Kinshu (Japanese race) X Showa (Chinese race), of *B. mori* were obtained from Ueda Sanshu (Ueda, Japan). Larvae were reared at 25±1°C under a photoperiodic cycle of 12h light and 12h dark on artificial diet (Silkmate, Nihon Nosan Kogyo, Yokohama, Japan) (Sakurai, 1984). The 24h period of the photophase following the scotophase during which the fourth instar larvae molted was designated day 0 of the fifth instar (V0). Similarly, the 24 h period of the photophase following the scotophase during which the fifth instar larva pupated was designated day 0 of pupal stage (P0). Adult emerging day was considered as day 0 (A0). The first day to tenth days after V0 denoted V1-10. Tissue samples were collected from different stages and the stages were termed as V2, V8, P2 and A2. 2 days after V0 was named as V2, for example.

RNA extraction, cDNA synthesis, and PCR amplification

RNA extraction: RNA was extracted from various tissues by the acid guanidinium thiocyanate phenol-chloroform method (Chomezynski and Sacchi, 1987) with minor modifications (Tsuzuki *et al,* 2001). After DNAse (Promega, Madison, WI, USA) treatment and repetition of phenol-chloroform treatment, RNA was precipitated using

absolute ethanol. RNA concentration and purity were estimated by UV absorbance at 260 and 280 nm. The 260:280 nm ratios were almost 2.0, which indicated high purity.

cDNA synthesis: One µg RNA was used as a template for cDNA synthesis in a 20 µl final volume reaction mixture using 100 U ReverTra Ace (Toyobo, Osaka, Japan) and 5 pmol oligo(dT)$_{12\text{-}18}$ primer at 42^0C for 60 min and reaction was stopped by heating the solution at 99^0C for 5 min. The reverse transcription products were diluted to 80 µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0). For control, cDNA samples were prepared without adding reverse transcriptase to demonstrate absence of genomic contamination.

PCR amplification: The resulting cDNA was used as a template to amplify DNA fragment by PCR in a 10 µl mixture using GoTaqGreen Master Mix (Promega) with gene-specific primers (Table 1 and 2). Primers were designed using Primer3 software at http://frodo.wi.mit.edu/primer3/ with default criteria (Rozen and Skaletsky, 2000). Two sets of primers were used for RT-PCR experiment. As bombyxin genes are the multi-gene family, the gene-specific primers were designed from signal peptide and C peptide site, which were least common region sites among these family genes, to avoid the mis-amplification (Table 1). Another set of bombyxin primers was picked from different locations of sequences and used for RT-PCR experiment only (Table 2).

Amplification was performed in a thermal cycler (Mastercycler ep384, Eppendorf, Humburg, Germany) with denaturation at 94^0C for 30s, annealing as mentioned in Table 1 and 2 for 30s and extension at 72^0C for 30s. PCR products were separated on a 1.5% (w / v) agarose gel, stained with ethidium bromide and visualized with UV light. RT-PCR reactions were performed at least three times.

Cloning, plasmid purification, DNA sequencing and sequence analysis

The PCR products of respective bombyxin gene primers were cloned into the pGEM-T vector (Promega). Plasmid DNA was purified using NucleoSpin Plasmid QuickPure kit (Macherey-Nagel GmbH & Co. KG, Germany). This kit has an alkaline lysis and silica absorption spin column to purify plasmid DNA.

The sequencing reactions were carried out in a total volume of 10 µl using ABI PRISM BigDye Terminator ver. 3.1 Cycle Sequencing kit (Applied Biosystems, Foster city, CA, USA) according to the manufacturer's protocol. The 10 µl sequence reaction mixture contained 0.75 µl 5X sequence buffer, 0.25 µl 10µM T7 primer (5'-TAATACGACTCACTATAGGG-3'), 0.5 µl BigDye terminator ver. 3.1 and 100-200 ng DNA. After mixing, the contents of the sequencing reaction were subjected for the following cycle profile; 96^{0}C for 1 min, 25 cycles at 96^{0}C for 30 s, 55^{0}C for 15 s and 60^{0}C for 4 min in the thermal cycler.

On completion, the reaction was stopped by adding 2.0 µl of 125 mM EDTA. PCR product was precipitated with 2.5 times absolute ethanol and 1/10th volume of 3 M sodium acetate (pH 5.5) by incubating at room temperature for 15 min. Pellets were washed with 70% ethanol and heat denatured at 85^{0}C for 5 min, the sequencing reaction was analyzed on sequencer.

The nucleotide sequences of the clones were analyzed for their homology by Geneious Pro 4.8. Comparison with open reading frame and sequenced bombyxin genes reveals perfect matching and confirmed that desired bombyxin genes have expressed. At least three individual clones were analyzed for sequence determination.

In situ hybridization by DNA probes

Wholemount *In situ* hybridization: In situ hybridization to whole brains was performed following the method illustrated earlier (Iwami *et al.,* 1996a). In brief, dissected brains were washed in 10 mm phosphate buffered saline (PBS, pH 7.4) with 0.1% Tween20 (PBT) and fixed in a solution of 85% ethanol, 4% formaldehyde and 5% acetic acid on ice for 45 min, as described (Hossain *et al.*, 2006, 2008). The brains were then incubated in a solution of PBT containing 15% (w / v) sucrose at 4^0C for about 16 h. After washing with PBT, the brains were treated with proteinase K (0.05 mg/ml) at 37^0C for 40 min, and fixed again with 3% (w / v) paraformaldehyde in PBT at room temperature for 20 min. Tissues were then washed three times with PBT and hybridized with 50 ng respective bombyxin probes - labeled with digoxigenin (Table 1 for sense and antisense sequences) using a Dig-labeled kit (Roche Diagnostics, Mannheim, Germany). Hybridization was carried out at 37^0C for 16 hrs in a 100 µl hybridization solution [50% (v / v) formamide, 5X standard sodium citrate (SSC; 0.15 M NaCl, 0.015 M sodiam citrate), 5% dextran sulphate and 100 µg/ml sonicated salmon sperm DNA]. After several times washing, the brains were incubated with a 1 : 500 diluted alkaline phosphatase conjugated anti-digoxigenin IgG (Roche) at room temperature. Color development was performed with 4-nitroblue-tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP) solution (Roche) in the presence of 1 mM levamisole (Sigma-Aldrich, Saint Louis, Missouri, USA). After dehydration with ethanol, the brains were clarified with methyl salicylate and observed with a microscope (BX-50F, Olympus, Tokyo, Japan). Negative control omitted the labeled probes as well as with sense dig-labeled probes, and no signals were detected.

Section *in situ* hybridization: For section in situ hybridization, freshly dissected tissues were embedded in Tissue-Tek O.C.T (Optimal Cutting Temperature) compound (Sakura Fintek, Torrance, CA, USA) and fixed in -80^{0}C. Fixed tissues were sectioned at 10 μm with a cryostat at -15^{0}C, mounted on slides, air dried and fixed for 12-16 hours at 4^{0}C in 4% paraformaldehyde in 0.1 M Na-phosphate buffer (PB, pH 7.4). Sections were then treated with proteinase K (5 μg/ml in 100 mM Tris-HCl pH 8, 50 mM EDTA) at 37^{0}C for 20 min, refixed for 20 min with 4% paraformaldehyde, washed with PB, treated with 0.2 N HCl, acetylated with 25 mM acetic anhydride in 0.1 M triethanolamine (pH 8) for 10 min at room temperature, washed with PB, dehydrated by grading of ethanol. The sections were hybridized with 75 ng probes labeled with digoxigenin (Table 1 for sense and antisense sequence) using a Dig-labeled kit (Roche) in the presence of hybridization buffer (50% formamide, 5% dextran sulfate, 6.3% 20XSSC, 100 μg/ml sonicated salmon sperm DNA,) and incubated 16 hours at 37^0 C in a humidified chamber. Post-hybridization washes were carried out with 10X, 2X and, 0.2X SSC at 37^{0}C. Tissue sections were then overlaid with blocking solution (1.5% blocking reagent, Roche) and DIG was visualized with the anti-DIG antibody coupled to alkaline phosphatase (AP) using NBT-BCIP solution (Roche).

In situ hybridization by RNA probes

As the wholemount *in situ* hybridization experiment by DNA probes was giving some mystifying and non-reproducible result, the experiment was performed again by using RNA probe which was very specific and accurate.

Wholemount *in situ* hybridization: After dissection, the brains were fixed as mentioned earlier for DNA probes. Tissues were deproteinized with proteinase K (50 μg/ml in PBS)

at 37^{0}C for 30 min, rinsed in 0.2% glycine (in PBT), washed in PBT and re-fixed with 4% (w / v) paraformaldehyde in PBT at room temperature for 20 min. After incubation in prehybridization solution [50% (v / v) formamide, 5X SSC, 1µg/ml yeast t-RNA, 50µg/ml Heparin, 0.1% TritonX, 0.1% CHAPS (3-{(3-cholamidopropyl)dimethyl ammonio}-propanesulfonate), 5% dextran sulphate, 5 mM EDTA, 2% blocking reagent (Roche)] at 50^{0}C for 90 min, the samples were hybridized with probes (each 1 ng/µl final concentration) in the presence of hybridization buffer and incubated at 50^{0}C overnight.

Probes for *in situ* hybridization were prepared for each new bombyxin from PCR products using gene specific primers (Table 1) with T7 and SP6 promoters. Digoxygenin (DIG)-labeled RNA probes were synthesized by means of in vitro transcription using DIG RNA Labeling Mix (Roche). Sense probes were used as negative control.

After washing several times with a series wash buffers: wash buffer 1 [50% formamide, 5X SSC, 1%SDS (sodium dodecyl sulfate)], wash buffer 2 (2X SSC, 0.1% CHAPS) wash buffer 3 (0.2XSSC, 0.1% CHAPS) and KTBT (50mM Tris-HCl pH 7.5, 150 mM NaCl, 10mM KCl, 0.1% Triton X-100) at 50^{0}C, the tissues were blocked in blocking solution (1.5% blocking reagent) for 90 min. The tissues were incubated with anti-DIG antibody coupled to alkaline phosphatase (Roche) at 1:2500 dilutions at 4^{0}C overnight followed by extensive wash by KTBT. Samples were incubated in staining buffer (0.1MTris-HCl pH 9.5, 0.1 M NaCl, 0.05M MgCl2 and 0.1% Tween 20). Staining was developed with NBT-BCIP solution (Roche). After extensive wash with PBT and dehydration with graded ethanol, brains were clarified with methyl salicylate. Clarified samples were observed under a microscope (BX-50F, Olympus, Tokyo, Japan).

Section *in situ* hybridization: Dissected tissues were washed and fixed as the same way mentioned earlier for section in situ hybridization by DNA probe. Fixed tissues were sectioned at 10 µm with a cryostat at -15^{0}C, mounted on coated slides (Matsunami,

Tokyo, Japan), air dried for at least 30 min and fixed for overnight at 4^0C in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Sections were then deproteinized with proteinase K (10 μg/ml in 100 mM Tris–HCl pH 8, 50 mM EDTA) at 37^0C for 20 min, refixed for 20 min with 4% paraformaldehyde, washed with PB, acetylated with 25 mM acetic anhydride in 0.1 M triethanolamine (pH 8) for 10 min at room temperature, washed with PB and dehydrated by grading series of ethanol.

After complete drying, the sections in the slides were hybridized with 2 ng/μl final concentration probes in the presence of hybridization buffer (50% formamide, 10 mM Tris HCl (pH 7.6), 200 μg/ml tRNA, 1X Denhardt's solution, 10% dextran sulfate, 600mM NaCl, 0.25% SDS and 1mM EDTA). The slides were covered with parafilm and incubated overnight at 50^0C in a humidified chamber with 50% formamide. The sections in slide were washed in 50% formamide in 2X SSC. Excess riboprobe was removed by treatment with 10 μg/ml ribonuclease A (Sigma-Aldrich, St. Louis, MO, USA) in TNE (10mM Tris-HCl pH 7.5, 0.5 M NaCl, 1mM EDTA) for 15 min at 37^0C. Post-hybridization washes were carried out with 2X SSC and with 0.2X SSC at 50^0C water bath. The slides were enclosed by Pap pen and blocked by 1.5% blocking reagent (Roche) for 30 min. The tissues were incubated with anti-DIG AP (Roche) at 1:1000 dilutions in blocking solution in the humidified chamber for 1 hr. After washing in PB and incubating in detection buffer for 3 min, staining was developed by NBT-BCIP solution in humid chamber. The humid chamber was kept continuously under slight rotation to reach the solution/reagents all over the slides.

Immunohistochemistry

To confirm the bombyxin expression site in the brain, immunohistochemistry was performed by the known bombyxin-A1 antibody. *B. mori* brains were fixed (in 85%

ethanol, 4% formaldehyde and 5% acetic acid on ice for 30 min), washed in 0.1% Triton X-100 in PBT and 2% BSA (bovine serum albumin) in PBT. These brains were incubated with of primary antibody - anti bombyxin A rabbit at a dilution of 1:100 in 2% BSA /PBT for 2 days at 4^0C. After washing four times in PBT, secondary antibody - FITC conjugated anti rabbit IgG from goat in 2% BSA /PBT at a concentration 1:400 was added and incubated in 4^0C for 12 hours. After several washes by PBT, samples were observed under fluorescence microscope (BX-50F, Olympus). Absence of detectable fluorescence in the control, demonstrated the specificity of the reaction.

Developmental expression profile

To check the developmental expression profile, tissues were collected from day 0 (V0), 2 (V2), 4 (V4) and 8 (V8) of fifth instar. RNA was extracted as mentioned earlier. Extracted RNA was used to prepare template cDNA. Tissues that were highly expressed in RT-PCR expression analysis was used for this experiment. Amplification was performed in a condition mentioned earlier using bombyxin primer (Table 1). RT-PCR reactions were performed with at least three samples and gave reproducible result.

Starvation effect on bombyxin expression

To study the effect of starvation on gene expression, starved larvae were reared under the same conditions (as mentioned earlier of this section) without food from fifth instar day 3. Tissues were collected from day 5 insects and RNA was extracted to prepare template cDNA. Control larvae were reared with food in same condition. RT-PCR reactions were performed as mentioned earlier.

RESULT

Identification of novel family bombyxin genes

Five new family bombyxin genes were identified by performing Blast searches on the *B. mori* genome database using all known bombyxin family genes and found those genes as novel family genes, which were named bombyxin-V to -Z. All of these new families consisted of a single gene except V which had two genes V1 and V2 (representative nos. fcaL21f12f and fcaL20e07f, respectively, in CYBERGATE database). Nucleotide, amino acid, and cDNA sequences of these novel family genes are appended in Appendix I-III. Nucleotide position no 14, 30, 50, 172 and 175 of V1 and V2 were found unmatched (Appendix IV) and thus V family was subdivided to 2 genes. The newly identified *bombyxin-Y1* gene was the same as the 8k-bombyxin-like peptide gene identified by Okamoto *et al.* (2009a).The deduced amino acid sequences of these novel bombyxin genes showed 21% (between *bombyxin-E1* and *-X1, bombyxin-X1* and *-Y1*) to 50% (between *bombyxin-W1* and *-A1*) identity among the prepropeptide of bombyxin family members and 21% (*bombyxin*-X1) to 26% (*bombyxin-V1* and *bombyxin*-Z1) identity with human preproinsulin (Table 3). These bombyxin gene groups were ascertained as new family because same family bombyxin genes have at least 85% sequence similarity (Kondo *et al*., 1996).

Sequence alignment and phylogenetic analysis

The amino acid sequences of preprobombyxin deduced from novel bombyxin genes were aligned (Fig. 1). Representative of previously identified preprobombyxins; human preporinsulin, relaxin, and insulin-like growth factor were also aligned along with. The open reading frame (ORF) of the newly identified bombyxin genes apparently encoded

preprobombyxin with four domains in the order of signal peptide, B chain (domain), C-peptide (domain), and A chain (domain) (Fig. 1). All the products had the same basic structure as that of the preprobombyxins previously characterized and preproinsulin. Multiple sequence alignment of the amino acid sequences of preprobombyxins revealed that the A and B chains of identified bombyxins have high similarity to those of previously identified bombyxins and other insulin members. On the other hand, the conservation of amino acid sequences was relatively low in the C peptide and even lower in the signal peptide throughout all bombyxin and insulin family genes.

To investigate the evolutionary relationships between the novel bombyxin genes, previously identified bombyxin genes, and human insulin family members, phylogenetic analysis was performed (Fig. 2). Phylogenetic analysis revealed that newly identified bombyxin genes did not belong to any known family of bombyxins and thus considered as novel families of bombyxin multigene family.

Gene characterization and structure

Genomic structure and chromosomal location of these genes were characterized. Fig. 3 shows exon-intron organizations of newly identified bombyxin genes. *Bombyxin-V1* and -*V2* were localized on chromosome 9. Chromosome 1 harbored *bombyxin-W1, -Y1*, and -*Z1, whereas X1* was localized on chromosome 11.

Comparison of the genomic and cDNA sequences (Appendix V) revealed that *bombyxin-V1* and -*V2* had one intervening intron of 754 and 833 nucleotides sized each at 5' untranslated region (UTR). *Bombyxin-W1*, -*X1,* and -*Y1* were lacking intron and thus considered as traditional bombyxin genes. *Bombyxin-Z1* consisted of four exons intervened by 3 introns. The 5' UTR includes the noncoding exon 1 and 2. The protein coding sequence consisted of part of exons 3 and 4. First (154 bp) and second (327 bp)

intron were at 5'-UTR, third one was in the C-peptide having 2905 bp. Fig. 4 shows comparative schematic gene structure of prepropeptides for novel bombyxin genes and human insulin gene.

The mature form of insulin is a monomer consisting of two chains (Fig. 5), an A chain of 21 amino acids and B chain of 30 amino acids (in human), linked by two disulfide bridges, A7–B7 and A20–B19. The A chain contains an intra-chain disulfide bridge between A7 and A11 (De Meyts, 2004). Insulin like structure also could be drawn for newly identified bombyxin genes (Fig. 5). Position of cysteine residues and disulfide bonds were as like as vertebrate insulin. This reveals that novel bombyxins also form insulin like structure.

Expression analysis of the bombyxin genes by RT-PCR

To identify the tissues that express bombyxin genes, RT-PCR expression analysis was performed. Fig. 6 shows tissue specific expression profile of novel bombyxin genes in all life stages studied - fifth instar day 2 (V2), fifth instar day 8 (V8), pupa day 2 (P2), and adult day 2 (A2).

Almost same *RpL3* expression among all stages examined indicated presence of similar amount of cDNAs in respective samples. No amplification was observed without reverse transcriptase indicating cDNAs were without genomic DNA contamination.

Bombyxin-V1 mRNA was highly expressed in brain of all four studied stages and slightly expressed in Malpighian tubule and ovary of P2 and A2. High expression of *bombyxin-W1* mRNA was observed in brain of all stages. Larval ovary and pupal fat body showed very low and moderate expression respectively.

High expression of *bombyxin-X1* was observed exclusively in fat bodies in post-feeding stages. X1 did not express in V2. The expression was appeared in the non-feeding stage - V8.

Bombyxin-Y1 showed high level of transcript in brain and ovary of larval stages. In P2, *Y1* was highly expressed in fat bodies, moderately expressed in testis and lowly in midgut, and brain. In A2, *Y1* showed the highest expression in ovary, whereas fat body and ovary showed low level expression.

Interestingly, *bombyxin-Z1* was found as a female biased gene, which was highly expressed in female fat body and ovary in V2 and V8. In P2, *Z1* transcript was high in ovary and fat body. A2 ovary showed a high expression. Female brain of adult showed comparatively high expression than male brain.

Gene sequencing

Bombyxin gene copies of the *B. mori* genome have at most 56% and 85% similarity between and within the families, respectively (Iwami, 2000; Kondo *et al.,* 1996). To verify the risk of amplifying closer non-target genes and to see whether targeted gene transcripts have expressed, cDNAs of the transcripts from the identified bombyxin genes were sequenced. Alignment analysis of ORF and sequenced bombyxin genes reveals perfect matching. This result confirms that desired bombyxin genes were expressed.

Localization of the bombyxin gene transcripts in brain revealed by *in situ* hybridization

Wholemount *in situ* hybridization by RNA probe was performed to specify the localization of bombyxin-expressing cells and to confirm the RT-PCR results. Fig. 7 (A, B) shows that *bombyxin-V1* mRNA was localized to medial large neurosecretory cells (MNCs) in V2 brains. In most of the brains four pairs MNCs were expressed (Fig. 7B). Section *in situ* hybridization showed the same expression pattern (Fig. 7C). Immunohistochemistry

result revealed that *bombyxin-A1* also expressed in medial neurosecretory cells like novel *bombyxin-V1*. *Bombyxin-W1* (Fig. 8A, B) and -*Y1* (Fig. 8C, D) transcripts were also localized to the MNCs of brain. No signal was detected in the sections hybridized with sense probes, indicating that the signals were specific to the transcripts of interest (data not shown).

Spatial localization of the bombyxin gene transcripts in the ovary and fat body

Expression of bombyxin mRNA was further confirmed by identifying the bombyxin transcript in the V8 and P2 ovaries by section *in situ* hybridization using frozen sections. This experiment was performed by both DNA and RNA probes separately. *Bombyxin-Y1* mRNA was dominantly and selectively detected in follicle cells surrounding the egg cells of the V8 ovary (Fig. 9A-D). *Bombyxin-Z1* transcripts were localized in the similar localization of V8 and P2 ovaries. (Fig. 9E-H, Fig. 10A-D). There was no signal in the space within the ovariole, between the ovarioles, and between ovariole and ovariole capsule. No signal was detected in the sections hybridized with sense probe, indicating that the signal was specific to the transcripts of interest (data not shown for RNA probe experiments).

Frozen section of V8 and P2 fat bodies were used to detect *bombyxin-X1* and -*Z1* transcriptional localization but could not find any detectable signal (Fig. 11A, B). This may be due to ubiquitous and very low bombyxin transcripts at the cellular level. The fat body in the *B. mori* is a very big tissue that covers whole body.

Stage specific expression of the bombyxin genes during development

To reveal the developmental expression profile, a comprehensive survey of transcription was conducted by semi-quantitative RT-PCR using gene specific primers and cDNA from the tissues that were highly expressed by respective genes in RT-PCR expression analysis experiment. Fig. 12 demonstrates developmental expression profile. All genes, except *bombyxin-X1* were expressed throughout the studied stages of fifth instar day 0 (V0), day 2 (V2), day 4 (V4) and day 8 (V8). *X1* expression was appeared and became very high at V8 stage; whereas it was very low or undetectable at V0, V2, and V4 stages. It may indicate its relation with ecdysteroid titer.

Effect of starvation on bombyxin gene expression

Bombyxin-X1 is strongly expressed when animals do not feed. This observation led to consider whether *bombyxin-X1* expression could also be induced upon starvation. Same starvation experiment was also performed on *bombyxin-Z1* to check its effect on fat body and ovary. Fig. 13 shows the effect of starvation on these genes expression. When fifth instar day 3 feeding larvae were started to starve, *bombyxin-X1* mRNA was increased in fifth instar day 5. There was no significant difference of *bombyxin-Z1* mRNA expression in fat body and ovary between starvation and fed controls.

DISCUSSION

Gene characterization and structure

From the nucleotide and amino acid sequence analysis, bombyxin-V to -Z was identified as novel bombyxin family genes. The *bombyxin-Y1* gene was partly characterized as the 8k-bombyxin-like peptide gene (Okamoto *et al.,* 2009a).Together with previously identified 32 genes (Iwami *et al*., 1989, 1990; Kawakami *et al*., 1989; Kondo *et al*., 1996; Tsuzuki *et al*., 1997; Yoshida *et al*., 1997, 1998; Iwami 2000), total number of bombyxin gene copy is revealed to be 38 in the haploid *B. mori* genome and are classified into 11 families, A to *G* and V to Z. The family A, B, C and V consists of 10, 12, 6 and 2 gene copies respectively. Other families comprise of single gene. The genomes of other insects revealed to contain multiple genes for ILPs; there are seven in the fruit fly *Drosophila melanogaster* (Brogiolo *et al.,* 2001), seven in malaria mosquito - *Anopheles gambiae* (Krieger *et al*., 2004) eight in yellow fever mosquito - *Aedes aegypti* (Riehle *et al*., 2006), two in honey bee – *Apis mellifera* (Wheeler et al., 2006); four in flour beetle *Tribolium castaneum* (Li *et al*., 2008), and at least six in *Samia cynthia ricinii* (Kimura-Kawakami *et al*., 1992).

Compared with the diverse structures of the insulin-related peptides in these invertebrates, vertebrate insulins are very limited in structural variation. There may be little room for mutational divergence to occur in vertebrate insulins because of the low copy number of their genes. Only point mutations resulting in abnormal human insulins that cause diabetes mellitus have been reported (Steiner *et al*., 1985). The presence of a large number of bombyxin gene copies in the *B. mori* genome might meet the demand for a large quantity of bombyxins for growth and development at a specific stage(s).

The encoded hormone precursors of novel bombyxin genes exhibit a very similar organization as observed for most other members of the insulin superfamily. All new bombyxin genes apparently encode preprobombyxin with four domain in the order of signal peptide, B chain, C-peptide and A chain, similar to other bombyxin genes (Iwami *et al.*, 1989, 1990; Kawakami *et al.* 1989; Kondo *et al.*, 1996; Tsuzuki *et al.*, 1997; Yoshida *et al.*, 1998), preprobombyxin-related peptides (Kimura-Kawakami *et al.*, 1992; Iwami *et al.*, 1996b; Brogiolo *et al.*, 2001; Kreiger *et al.*, 2004), and preproinsulins (Steiner *et al.*, 1985). Of the four domains of preprobombyxins, the highest similarities is seen in the A chain. The sequences of the C-peptide and the signal peptide are less conserved than those of the A chain and the B chain (Fig. 1). From these observations it is concluded that the degree of amino acid conservation is in the order A chain > B chain > C-peptide and signal peptide. The general features of vertebrate and non-vertebrate insulin-related peptides and their genes (novel bombyxin genes as well) are thus well conserved. The sequences of C peptides are considerably more divergent than the sequences found in the A and B chain of insulin and they also differ in size. C-peptide sequences do not appear to be well conserved during evolution (Claeys *et al.*, 2002). Because of the high amount of sequence divergence, it had been hypothesized that the main role of the C-peptide is to facilitate proper folding of A and B chains prior to cleavage. (Permutt *et al.*, 1981).

Insulin was found to be a polypeptide in 1928 with its amino acid sequence identified in 1952. It is a dipeptide, containing A and B chains respectively, linked by disulphide bridges, and containing 51 amino acids. The A and B chains comprise of 21 and amino acids respectively. The two chains are joined by 2 disulphide bonds. In proinsulin, a connecting peptide links the N-terminus of the A chain to the C-terminus of the B chain. (Wilcox, 2005; Dodson and Steiner, 1998). Paired basic amino acids (Lys-Arg) appear to be highly conserved proteolytic cleavage sites for C-peptides from lepidopteran ILP

precursors (Kimura-Kawakami *et al.*, 1992; Iwami *et al.*, 1996b) and even mammalian (Bell *et al.*, 1980) insulin precursors. In fact, this is also visible from present alignment comparison of novel bombyxin prepropeptides - V1 to -Z1 (Fig.1). The conserved six cysteine residue pattern, found throughout the insulin superfamily (Claeys *et al.*, 2002), is likewise present in prepropeptides of bombyxin-V1 to -Z1 (Fig. 1, 5). Multiple alignment of the newly identified bombyxin sequence with other known bombyxin sequence, human insulin and IGF show that the cystein pattern in both the A and B chains is extremely well conserved (Fig. 1). Phylogenetic analysis gave an interesting result. Human insulin, IGF I and II were found as subfamily of novel bombyxin-X. Relaxin was found as subfamily of bombyxin-B. This interesting relationship indicates close relations between bombyxin and human insulin family members.

The propeptide is processed into an active form by linkage of the A and B peptide by disulfide bridges followed by proteolytic cleavage of the C peptide, as with insulin and related peptides in vertebrates. An exception is the IGF, which possesses a short C peptide that is not proteolytically removed and an extension on the carboxy terminus termed the D and E peptide, the latter of which is removed by proteolytic cleavage (Lu *et al.*, 2005). Consensus cleavage sites (Seidah and Chretien, 1997) between B and A chains of all novel bombyxins suggest that these ILPs have multiple dibasic proteolytic cleavage sites like other bombyxins or ILPs, in contrast to only one in vertebrate IGFs (McMurtry *et al.*, 1997). Thus these active peptides consist of two separate polypeptide chains and resemble to insulin rather than IGFs, which have single polypeptide. The predicted peptide structure of *bombyxin-X1* and -*Y1* has a short C peptide. Moreover, the prepropeptide for *bombyxin-X1* has an extended A peptide. These criteria are more like to vertebrate IGFs. Extended A peptide of *X1* seems resemblance to IGF's extra D domain. On the basis of sequence features, some ILPs are predicted to be more similar to IGFs than to insulin. *B. mori* IGFLP, *D. melanogaster* ILP6, one ILP in *A. aegypti* and

one in *T. castaneum* have shorter C peptide and this feature was considered consistent with IGFs (Okamoto *et al.*, 2009a, b, Riehle *et al.*, 2006, Li *et al.*, 2008). The prepropeptide for bombyxin-X1 also has shorter C-peptide having only 14 amino acids (Fig. 1). Extended A chain of X1 seems resemblance to IGF's extra D domain. The prepropeptide for bombyxin-Y1 has sequence similarity with 8k-bombyxin-like peptide gene previously identified by Okamoto *et al.,* (2009a).

All previously identified bombyxin genes were lack of introns both in the 5' untranslated region and in the C-peptide region (Iwami, 1990, 1995; Iwami *et al.*, 1989, 1990; Kondo *et al.*, 1996; Tsuzuki *et al.*, 1997; Yoshida *et al.*, 1997, 1998, Iwami, 2000). Present study reveals for the first time that *Bombyxin-V1, -V2,* and *-Z1* have intervening introns, unlike long-established bombyxin genes. This is the first report of the *bombyxin* genes having intron.

A preliminary evaluation of the relatedness of homologues can be generated from the number and relative position of introns. Insulin gene intron sizes in different vertebrate species are shown in following chart (Docherty and Steiner, 1997; Perler *et al.*, 1980; Mansour *et al.*, 1998):

Organism	5' UTR intron	C-peptide intervening intron
Human	179	786
Chimpanzee	179	794
Pig	163	393
Rat 1	119	0
Rat 2	119	499
Mouse 1	119	0
Mouse 2	119	486
Cow	263	478
Chicken	719	3424
Zebrafish	99	2428
Tilapia fish	73	316

Although most vertebrates have single insulin, rats, mice, and three fish species (tuna, bonito and toadfish) have two insulins (Humbel *et al.*, 1972; Dayhoff, 1978; Cordell *et al.*,

1979; Lomedico *et al.*, 1979, Bell *et al.*, 1980; Steiner *et al.*, 1985). The insulin gene from all characterized vertebrate organisms contains two introns, one is within the region transcribed into the 5' UTR and the other interrupts the C-peptide encoding region; (Steiner 1985), murine insulin genes have only one intron in the C-peptide region. A 786 base pair intron was found within the DNA region encoding the C-peptide of human proinsulin. The rat insulin II gene contains a shorter intron, while the chicken insulin gene contains a longer (about 3500 base pair) intron in exactly the same position. In the human insulin gene, a second intron of 179 base pairs in the DNA segment encoding the 5' untranslated region of proinsulin mRNA is present while a 119 base pair intron is present in both rat and chicken insulin genes (Permutt *et al.*, 1981). Since both of these intron positions are highly evolutionary conserved, these might have a regulatory role (Lomedico *et al.*, 1979, Darmet *et al.,* 1996).

One of rat gene for preproinsulin contains a long intron interrupting the coding sequences; the other does not (Lomedico *et al.*, 1979). This difference poses a clearly formulated problem. The most straightforward interpretation is one of the genes has lost the large intron in such a precise way that the coding region of the gene was left intact (discussed by Gilbert, 1979). The older gene structure has more introns, as predicted by the hypothesis that the intons have a role in the assembly of genes (Gilbert, 1979).

Structurally, the insulin protein has been highly conserved throughout vertebrate evolution. *Amphioxus* occupies a key position in phylogeny because it is believed to be an extant representative of the ancestral invertebrate species that gave rise to vertebrates. Amphioxus ILP has same structure like mammalian insulin (Chan et al., 1990). Though the evolutionary divergence time between humans and hagfish is about 400 million years, their insulin sequences share 61% amino acid identity (Chan and Steiner 2000). Amphioxus ILP may be representative of the ancestral gene which

subsequently duplicated to form vertebrate and invertebrate ILPs including bombyxin (discussed by Chan and Steiner, 2000).

The gene for preproinsulin provides an interesting example of evolution (Perler et al., 1980). *Bombyxin-V1, -V2,* and *-Z1* have introns at almost the same positions in vertebrate insulin genes. Intron of C-peptide is much longer (2905 bp) than those present in 5'-UTR (154 and 327 bp) in case of *bombyxin-Z1,* like other vertebrate insulin genes. Due to existence of C-peptide intervening intron in insulin, IGFs, and relaxin genes (Crawford *et al.,* 1984) suggested that these genes have evolved from a common ancestral gene by gene duplication. This type of intronic position similarity is also apparent at the gene level of relaxin, insulin, and bombyxin-Z1. These resemblances indicate that the bombyxin and vertebrate insulin genes share a common ancestral gene. Existence of two introns at 5' UTR in Z1, one intron in V family, and no intron in other bombyxin genes may be due to their serial evolution from a common ancestral gene. The intronless bombyxin genes in *B. mori* might have been originated from an intron-containing ancestral gene.

Gene expression in brain

In insects, the nervous system appears to be the primary source of ILPs (Krieger *et al.*, 2004). Present study reveals that the newly identified bombyxin genes were expressed in a tissue specific manner suggesting its specific function. These genes were expressed in diversified tissues including brain. In the present study, three bombyxin genes were predominantly expressed in four pairs of medial neurosecretory cells (MNCs) of brain. In particular, previously identified bombyxin genes of all 7 families were expressed exclusively in same location of *B. mori* brain (Iwami, 1990, 1995; Tsuzuki *et al.*, 1997;

Yoshida *et al*., 1997, 1998; Iwami, 2000). The brain is reported as the main tissue that expresses the bombyxin gene throughout *B. mori* development from the embryonic to adult stages when analyzed by northern hybridization (Adachi *et al*., 1989; Iwami, 1990).

Analogous findings were reported for other lepidopterans such as *S. cynthia ricini* (Kimura-Kawakami *et al.,* 1992; Yagi *et al.,* 1995), *Agrius convolvuli* (Iwami *et al*., 1996b), *Manduca sexta* (Bollenbacher *et al*., 1997), and *Spodoptera littoralis* (Vand de Velde *et al.,* 2007). Moreover, the medial neurosecretory cells appear to be important sites for ILP gene expression in other insect orders as well (Sevala *et al*., 1993; Brogiolo *et al.,* 2001; Ikeya *et al.,* 2002; Krieger *et al.,* 2004). Small clusters of dorsomedial neurosecretory cells were immunostained by anti-ILP sera in different life stages and species of Diptera (Duve and Thorpe, 1979; Zitnan *et al*., 1993; Krieger *et al*., 2004), Orthoptera (Raabe, 1986; Hansen *et al*., 1990), Coleoptera (Sevala *et al*., 1993) and Hemiptera (Sevala *et al*., 1992).

Bombyxin is present in *B. mori* brain throughout the stages from larval hatching to adult eclosion, and the developmental fluctuation of bombyxin content in the brain suggests that it is secreted into the hemolymph to regulate the developmental and/or physiological function of *B. mori* (Mizoguchi *et al*., 1990). Neuropeptides secreted from MNCs of brain may regulate the developmental and /or physiological function. In mosquito, brain secreted neuropeptide stimulates the production of ecdysteroid hormones by the ovaries which inturn induce fat body to secret yolk proteins responsible for ovary development (Brown *et al*., 2008). Peptide gonadotropins that originate from MNCs regulate vitellogenesis and oogenesis in many insects (Brown *et al*., 2008; Bayazit, 2009). From the similarities in the distribution of neurosecretory cells in the brain of various insects, one would expect that the product of these cells should also be similar.

Previous study on bombyxin and insulin related genes reported that these genes expressed predominantly in the brain and at low levels in a number of other tissues (Iwami *et al.*, 1996a; Brogiolo *et al.*, 2001; Riehle *et al.*, 2006), in contrast to the insulin gene which is expressed in the gastroenteric organs and is almost silent in the brain. In vertebrates, insulin originates exclusively from endocrine cells in specialized gut regions (i.e. pancreas of higher vertebrates), whereas related peptides and growth factors are secreted from other tissues as well, including the reproductive tract. In addition to brain, very low amount of ILP transcripts have been found to localize in insect epidermis, fat body, gut, prothoracic glands, pterotheca, thoracic muscle, ovaries, testes, mature oocyte, silk gland, and Malpighian tubules (Kromer-Metzgen and Lagueux, 1994; Iwami *et al.*, 1996a; Broughton *et al.*, 2005). In the present study, three of the newly identified bombyxin genes were highly expressed in tissues other than brain.

Gene expression in ovary

The developing *B. mori* ovariole serves as a model system for studying differential gene expression (Papantonis, 2008). *Bombyxin-Z1* seems to be female-biased gene as it was expressed exclusively in female ovary, female fat body (in larval stages), and even in brain of female adult. Invertebrate insulin and IGFs exert effects on oocyte maturation (El-Etr *et al.*, 1979), embryogenesis (Froesch *et al.*, 1985), and ovarian development (Sim and Denligger, 2009). Bombyxin induces meiosis in ovary (Orikasa *et al.,* 1993) and induce morphological changes of BM-N4 cells (Tanaka *et al.*, 1995). In insects, fat body and ovary is site of vitellogenin synthesis (Isaac and Bownes, 1982). Due to presence of bombyxin in the developing embryos shortly after oviposition but before appearance of neurosecretory cells in the brain (Fugo *et al.*, 1987), it seems probable that bombyxin in ovariol is transferred to embryo.

In a review, ILP and ecdysteroid hemolymph titers were similar in life stages of *B. mori*, thus suggesting that the two hormones function cooperatively (Mizoguchi, 1994). In *A. aegypti,* insulin signaling cascade controls the production of ecdysteroids in the follicle cells surrounding the ovary (Riehle and Brown, 1999) and ILP has been reported to regulate egg maturation and metabolism in this insect (Brown *et al.*, 2008). Knocking down of insulin-like peptide-1 halted ovarian development in mosquito (Sim and Denlinger, 2009). Involvement of bombyxin in the regulation of ovarian development has been suggested by many researchers (Orikasa *et al.*, 1993; Tanaka *et al.*, 1995, Iwami *et al.*, 1996a, Fullbright *et al.*, 1997a, b). Mosquito insulin receptor was reported to localize in the cell membranes of follicle cells that produce ecdysteroids (Riehle and Brown, 2002). Fullbright et al. (1997a) observed putative insulin receptors in ovarian cells of three different lepidopteran species, suggesting a function in ovarian development that may be similar to that of IGFs and relaxins in vertebrates. Moreover, mutations of the insulin receptor, a receptor tyrosine kinase, produced fruit fly phenotypes displaying impaired ovarian ecdysteroidogenesis (Tu *et al.*, 2002), reduced folliculogenesis, and juvenile hormone deficiency (Tatar, 2004).

Ecdysteroids are synthesized in the ovarian follicle cells of female insects. The follicular epithelium develops alongside the oocyte have been reported as expression site of ecdysteroid-regulated genes in *D. melanogaster,* (Buszczak *et al.*, 1999; Carney and Bender, 2000) and honeybee (Paul *et al.*, 2005, 2006; Takeuchi *et al.*, 2007). In many insects, ecdysteroid and ecdysteroid-regulated genes are involved in oogenesis (Cho *et al.*, 1995; Kozlova and Thummel, 2000; Sun *et al.*, 2002). E75 and BR-C genes govern egg chamber development during oogenesis in *D. melanogaster* (Deng and Bownes, 1997; Buszczak *et al.*, 1999; Tzolovsky *et al.,* 1999) and involve in regulation of vitellogenesis in *A. aegypti* (Pierceall *et al.*, 1999; Sun *et al.*, 2002). Ecdysteroid-regulated gene are involved in reproduction in adult honey bees (Paul *et al.*, 2005, 2006).

Bombyxin-Z1 was localized in the follicular cells of ovary, the same expression site of the ecdysteroid-regulated gene. From the facts mentioned above, it is tempting to hypothesize that bombyxins at least that of a particular family, control oogenesis and embryogenesis of *B. mori* and thus have a role in reproduction. This role and expression of bombyxin is similar to those of the mammalian ovarian hormone relaxin (Wu and Brown 2006).

Gene expression in fat body and other tissues

Vertebrate insulin genes are expressed in the cells of gastroenteric organ. So far, this was a major criterion to differentiate vertebrate and invertebrate insulins or insulin-related peptides (Iwami, 2000). Present study shows the occurrence of a temporal fluctuation in relative *bombyxin-X1* transcript quantities in fat body. *Bombyxin-X1* was exclusively expressed in the fat body of molting period and post feeding stages. Furthermore, the expression was enhanced by starvation. This indicates its relationship with ecdysteriod titer. In most arthropods, the timing of molts and metamorphosis is coordinated by a rise in the titer of the steroid hormone 20-hydroxyecdysone (20E) (Henrich *et al*., 1999; Warren *et al*., 2006). During metamorphosis, DILP6, one of the ILPs of *D. melanogaster* was reported to be produced by fat body and required for growth. Its expression was controlled by the steroid hormone ecdysone in *D. melanogaster* (Slaidina, 2009). At the end of larval development, animals stop feeding and prepare for pupal development. The transition from larval to pupal development is controlled by the steroid hormone ecdysone (20E). 20E reduces food consumption and then induces starvation during molting and pupation in *B. mori* (Wang *et al*., 2010).

Saegusa *et al.* (1992) showed that the hemolymph titer of bombyxin was low during the fourth and fifth larval instars, and rose steeply one day after pupation to reach the maximal level, which lasted until adult emergence. The titers during pupal–adult development were more than ten times those during the larval stages. A large amount of bombyxin thus seems necessary for pupal-adult development and a large number of bombyxin gene copies are expected to be responsible, at least in part, for the production of the large amount of bombyxin at this stage.

These suggest that *bombyxin-X1* might involve in cell proliferation and differentiation at a time when the growth of many of the internal tissues and imaginal discs depends not on feeding but on hormone regulated events (Nijhout *et al.*, 2006). Considering these findings, it appraises that *bombyxin-X1* might have an exclusive growth function and may play an important role in metamorphosis. Whether *bombyxin-X1* really exhibits such activity requires further investigation.

The functional class of ILPs represented by bombyxin-X1 may be conserved in other insect species, as an ecdysone-induced, fat-body-specific ILP has recently been described in *B. mori* (Okamoto *et al.*, 2009a) and *D. melanogaster* (Okamoto *et al.*, 2009b and Slaidina *et al.*, 2009) as IGF-like peptide.

IGF-I mRNA is ubiquitously expressed in tissues including liver, uterus, lung, ovary, kidney, heart, testes, pancreas, stomach/intestine, skeletal muscle, mammary gland, brain, spleen, placenta, cartilage, and pituitary (Rechler and Nissley, 1986). The richest source of IGF-I is liver, which contains approximately 30 times more IGF-I mRNA than the highest level in other tissues (Murphy *et al.*, 1987). *Bombyxin*-X1 shares some specific features with vertebrate IGF-I that distinguish both of them from insulin. It's gene is expressed in the fat body, a tissue sharing common function with the vertebrate liver, where IGF-I is mainly produced. This study revealed a specific class of ILPs induced

upon metabolic stress that promotes growth following developmentally induced cessation of feeding and support the view of Slaidina *et al*., (2009). The insect fat body is known as the nutrient sensor organ (Edgar, 2006). Metabolic function of the fat body is similar to that of the vertebrate liver (Liu *et al*., 2009). Fat body development and function are largely regulated by insulin and ecdysteroid hormone (Liu *et al.,* 2009). It indicates a potential metabolic activity of bombyxin.

CONCLUDING REMARKS

In conclusion, this study presented novel family bombyxin genes with the first evidence for bombyxin gene having intron. Five novel bombyxin family genes were identified. The results clearly indicate that bombyxin genes are expressed in tissue specific and also in some cases developmental stage specific manner. They are dominantly expressed in brain, fat body, and ovary. *Bombyxin-X1* shares some specific features with vertebrate IGF-I gene that distinguish both of them from insulin. It has shorter C peptide, extended A peptide, and expression site is the fat body, analogous tissue to that of the vertebrate liver (Liu *et al.*, 2009), where IGF-I is mainly produced (Murphy *et al.*, 1989). Overall, nearly similar structure, location of intervening intron, and high gasteroenteric expression between bombyxin and insulin genes propose an evidence of bombyxin gene evolution. Bombyxin gene might have been originated from a common ancestral gene like vertebrate insulin, IGFs, and relaxin genes. The characterization of the structure and expression of these genes provides a basis for future studies, their functions, and mode of action throughout the life. Based on the sequence diversity and expression specificity, it is tempting to think that like the members of the insulin family in humans, each of the bombyxins also serve distinct functions. However, further detailed studies on functional analysis might provide clues to clarify bombyxin function.

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TABLES AND FIGURES

Table 1. Oligonucleotide sequences used for RT-PCR and *in situ* hybridization

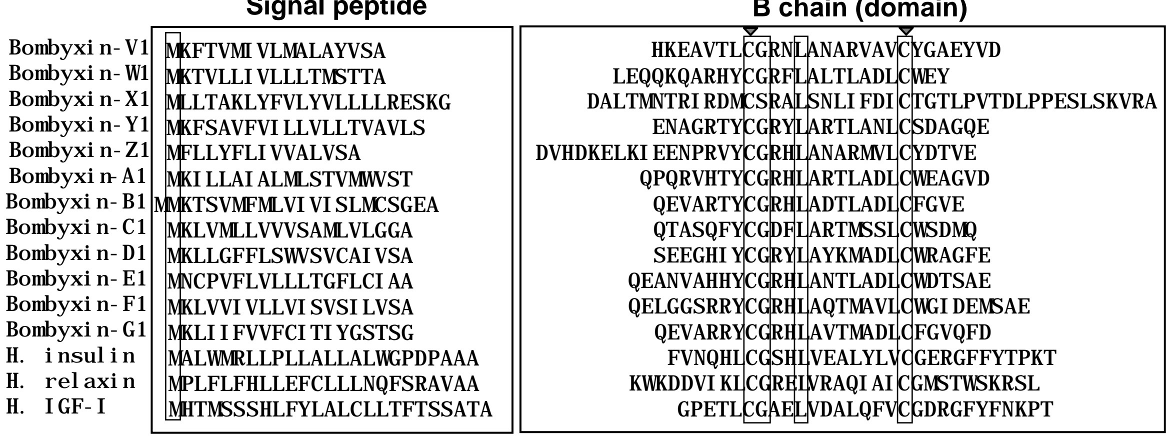
Gene	Forward primer	Reverse primer	Annealing temperature (^{0}C)
Bombyxin-V1	TTCACGGTGATGATAGTGCTG	CTTGTAGCGGGTCCAGTCAG	56
Bombyxin-W1	TCTACGACCGCGTTAGAACAA	TTTCTGGATGCGGTGCTC	55
Bombyxin-X1	TGACGGCAAAACTTTATTTCG	CCTGGATCTTTGCTTTGCAG	53
Bombyxin-Y1	TGAAATTTTCTGCGGTTTTTG	CGGACAGCCATGACCAGT	54
Bombyxin-Z1	TTCTGCTGTATTTCCTGATCGT	TCTTGGCGTACTGGGAAGAC	55
*RpL*3	AGCACCCCGTCATGGGTCTA	TGCGTCCAAGCTCATCCTGC	58

Table 2. Primer set used for RT-PCR

Gene	Forward primer	Reverse primer	Annealing temperature (^{0}C)
Bombyxin-V1	ACATCTTCAATCTTCCCATTG	ATTTCAAGAGTACGTCGGTG	56
Bombyxin-W1	TGATCGTTCTCCTGTTGACG	AAGCAGTACATCCAAAGTGCAG	54
Bombyxin-X1	TTGCTGACGGCAAAACTTTA	ATTCGACTAGCTGGGACACG	55
Bombyxin-Y1	CAAGAGCTCTCGAAAAGGC	AACACCGTAGAACATGTTGTC	56
Bombyxin-Z1	AACGCACGCATGATACTCTG	CAGGGCTTAAGGCAACATTC	60
*RpL*3	AGCACCCCGTCATGGGTCTA	TGCGTCCAAGCTCATCCTGC	58

Table 3. Amino acid sequence similarity (in percent) among preprobombyxin and human preproinsulin

Gene	Bombyxin													Human insulin
	A1	B1	C1	D1	E1	F1	G1	V1	V2	W1	X1	Y1	Z1	
Bombyxin-V1	39	44	35	36	34	31	39	100						26
Bombyxin-V2	39	44	35	36	34	31	39	100	100					26
Bombyxin-W1	50	47	40	46	45	37	41	28	28	100				25
Bombyxin-X1	25	32	26	28	21	27	28	24	24	34	100			21
Bombyxin-Y1	39	44	35	44	41	38	38	34	34	36	21	100		22
Bombyxin-Z1	41	46	33	47	44	35	33	34	34	37	29	38	100	26



	C peptide (domain)	A chain (domain)
Bombyxin-V1	KRTSGNTLFDDFEDVDLHGDWPWTGRRGALSADWT RYKR	QGIVNECCFKPCTTDVLLKYC
Bombyxin-W1	KRSDDSYYDGNNQDLTEPPSPQTAEHR IQKR	GVADDCCLRACTLDVLLLYC
Bombyxin-X1	KRASLFSAKQ RSRR	VADECCLRSCTVSQLVEYCPETW
Bombyxin-Y1	KRGEDWSWLS ASGRK	DGAVTENGVANECCLHPCTLEVLLSYC
Bombyxin-Z1	KRAQSYLDANIISAGDLSSWPGLSSQYAKTRAFALAEKSKR	GPGLVDECCLKPCYTYDLLNYC
Bombyxin-A1	KRSGAQFASYGSAWLMPYSEG RGKR	GIVDECCLRPCSVDVLLSYC
Bombyxin-B1	KRGGAQYAPYFWTRQYLGS RGKR	GVVDECCFRPCTLDVLLSYCG
Bombyxin-C1	KRSGSQYAGYGWPWLPPFSSS RGKR	GIVDECCYRPCTIDVLMSYCDN
Bombyxin-D1	KRSVAHYAGYGWPLLPS SEE RGKR	GIADECCLQPCTNDVLLSYC
Bombyxin-E1	KRSESSLASYSSRGWPWLPTPNFNKRA IKKR	GVVDECCIQPCTLDVLATYC
Bombyxin-F1	KRNSDMVYEDSGMPELLPADA RKKR	GIIDECCLQACTRDVLLSYC
Bombyxin-G1	KRNTQYEGYHWPLLAYSEE RIKR	QGIADECCLVPCTTNVLSSYC
H. insulin	RREAEDLQVGQVELGGGPGAGSLQPLALEGSLQ KR	GIVEQCCTSICSLYQLENYCN
H. relaxin	SQEDA------(95 amino acids)--------QKKR	RPYVALFELCCLICCTKRSLALYC
H. IGF-I	GYGSSSRRAPQT----	GIVDECCFRSCDLRRLEMYCA -(D domain)

Fig. 1. Amino acid sequence comparison of novel preprobombyxin, representative bombyxin (Iwami *et al.*, 1989, 1990; Kawakami *et al.*, 1989; Kondo *et al.*, 1996; Tsuzuki *et al.*, 1997; *Yoshida et al., 1997, 1998*), and human (H.) insulin , (Bell *et al*, 1980), human relaxin (Hudson *et al.*, 1983), and human preproinsulin-like growth factor-I (IGF-I) (Jansen *et al.,* 1983), as representatives of the vertebrate lineage. Gaps are introduced for maximum matching. Residues, which are completely conserved among all the insulin family prepropeptides are boxed. Inverted triangles indicate cysteine residues. The dibasic processing sites flanking the C peptide are underlined. Signal peptide was determined by using SIG-Pred: Signal peptide prediction software that uses weight-matrix choice.

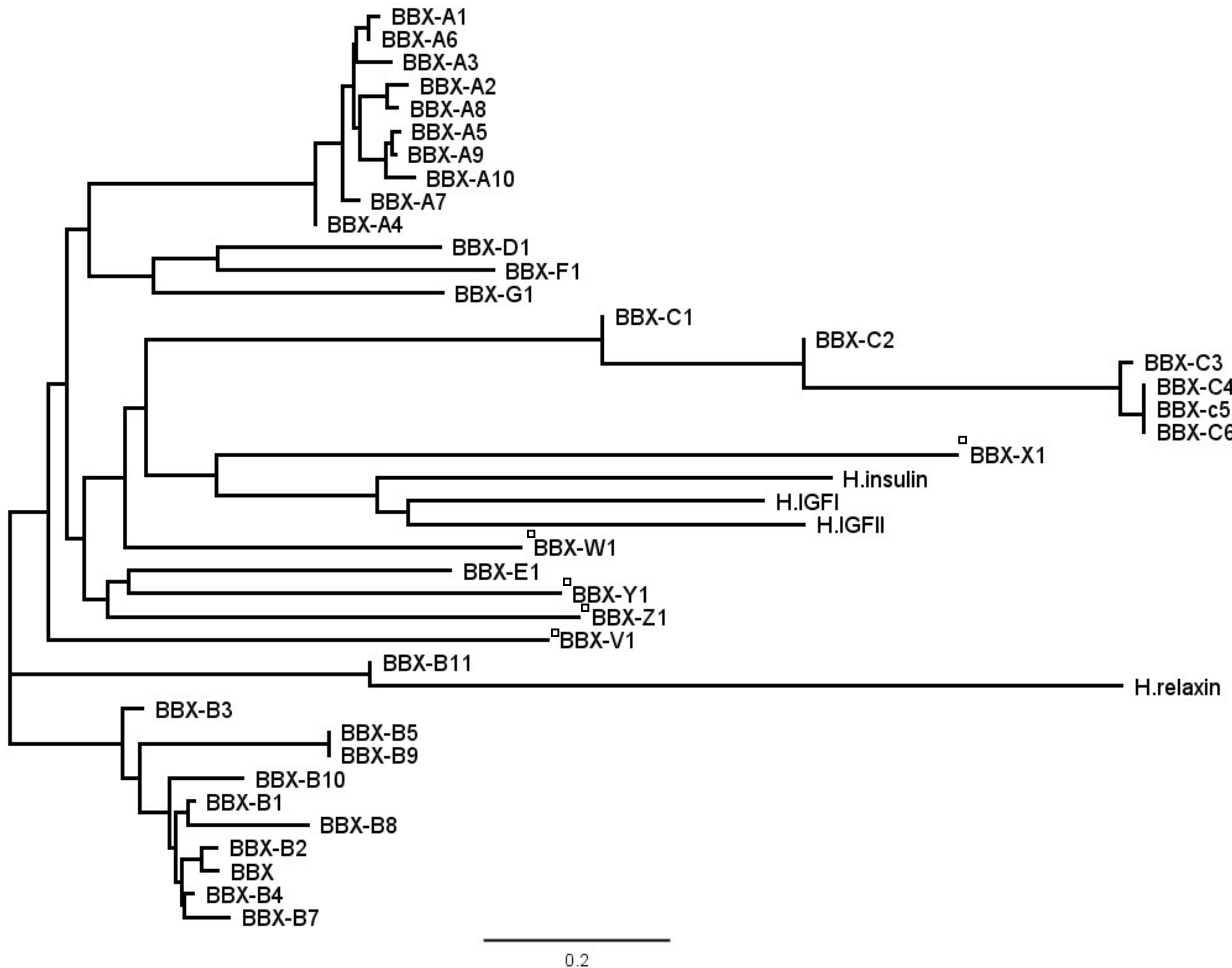


Fig. 2. A phylogenetic tree showing relationship between bombyxin family members, human insulin (Bell *et al.*, 1980), relaxin (Hudson *et al.*, 1984), IGFI (Rall *et al.*, 1987) and IGFII (Irminger *et al*, 1987). BBX = *bombyxin,* H = Human. The phylogenetic tree was generated on the basis of entire amino acid sequences using Geneious Pro 5.0.2 software. The scale bar indicates an evolutionary distance of 0.2 amino acid substitutions per position. Novel bombyxin members are highlighted by boxes.

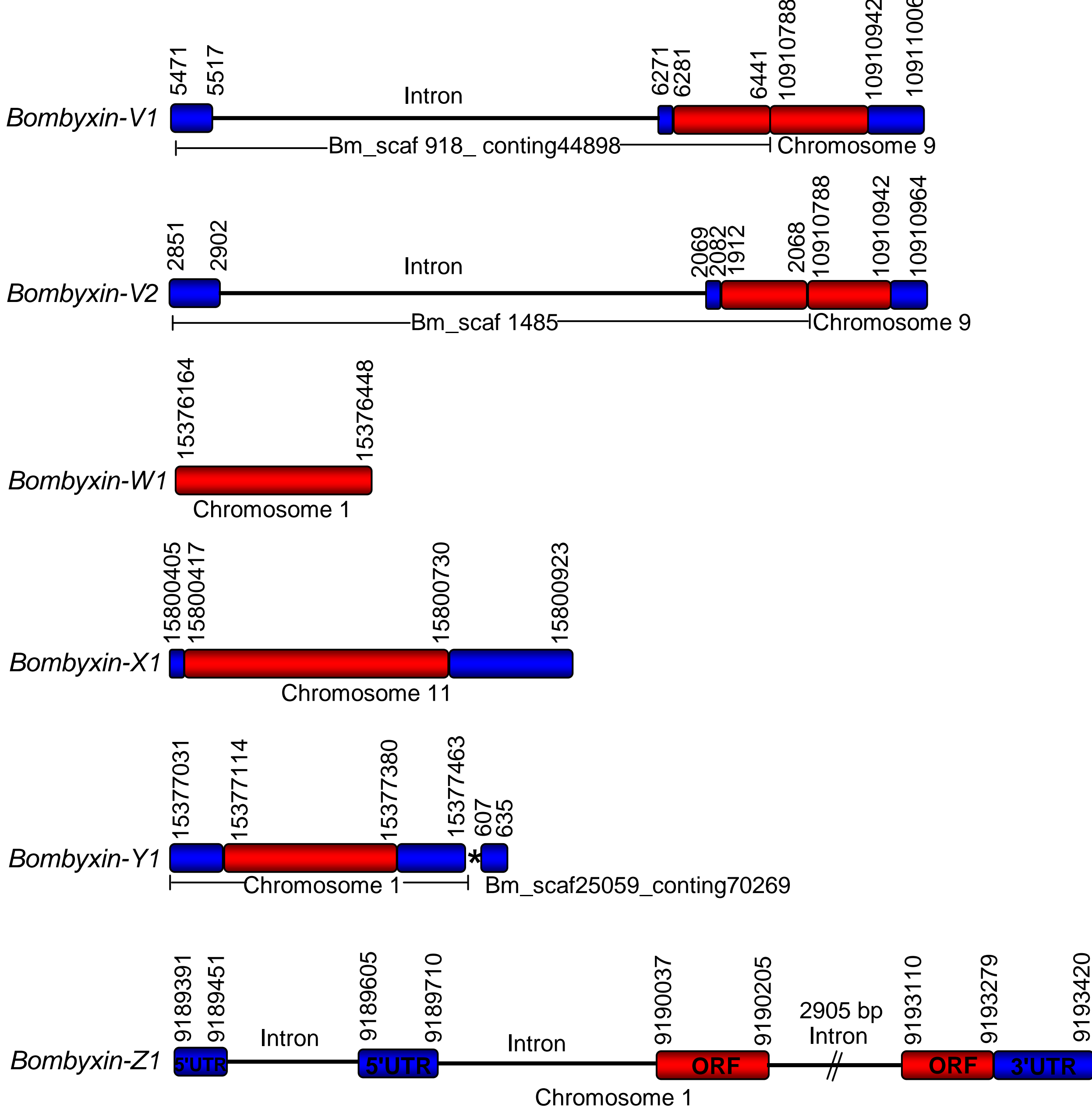


Fig. 3. Schematic representation of intron – exon organization of identified bombyxin genes. Comparison of genomic DNA and cDNA sequences confirmed intron-exon boundaries. Thick colored lines, exon; red thick lines, ORF; blue thick lines, UTR; thin lines, intron; "//", a break due to space limitation; "*", gap. Numbers above the gene structure indicate chromosome/scaffold position (based on KAIKObase version 2) in the gene.

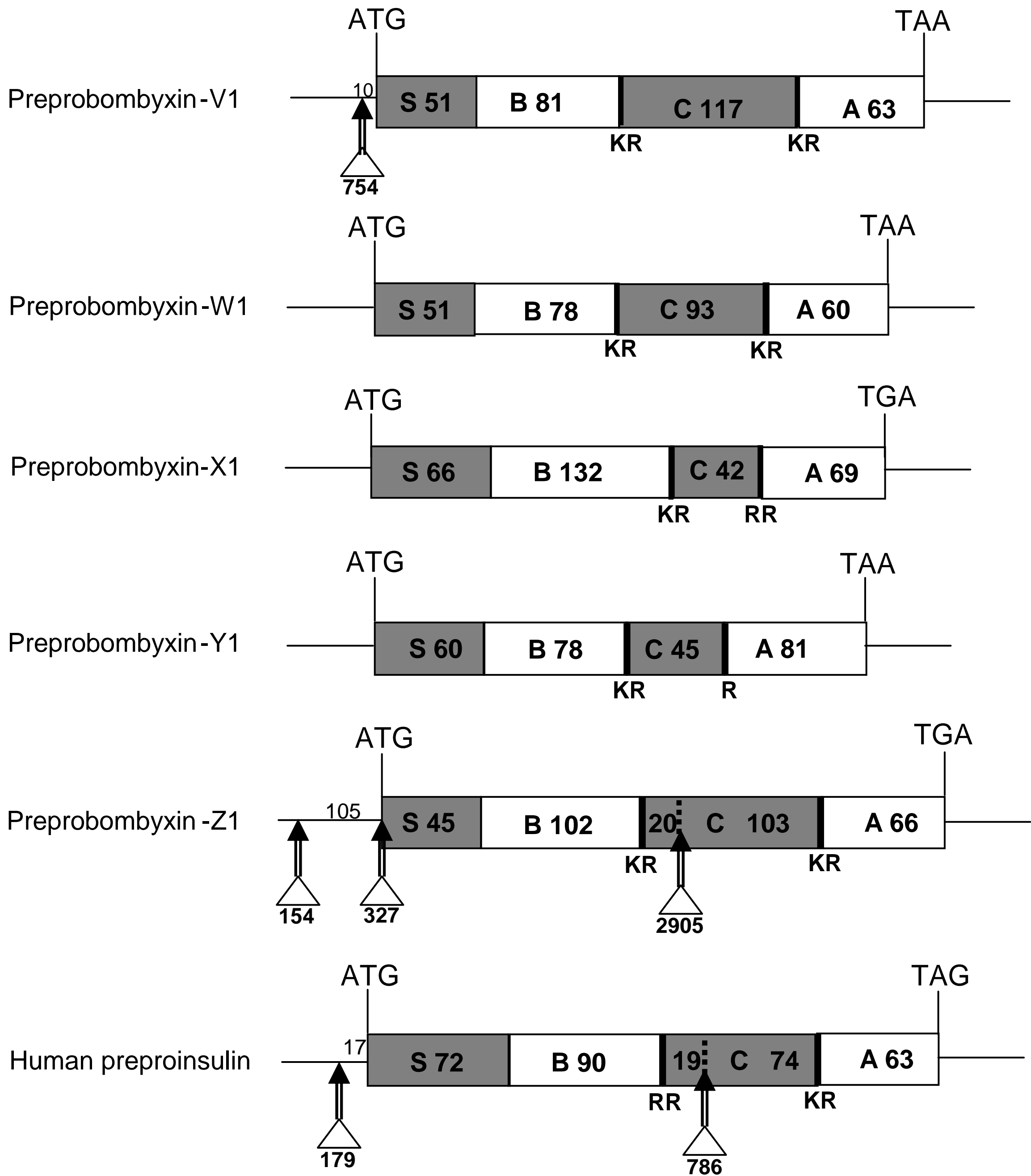


Fig. 4. Comparative schematic gene structure of prepropeptides for novel bombyxin genes and human insulin. ATG, initiation codon; TAA/TAG/TGA, stop codon. Exons are shown in boxes, open boxes designate the domains that form mature molecules and ash colored boxes those which are eliminated after translation. Dibasic amino acid residues, KR and RR, indicate the post-translational processing sites. S, signal peptide; B, B chain; C, C-peptide; A, A chain. Numbers indicate the nucleotide number in the respective coding segments and intronic position. Arrows indicate the position of introns.

Fig. 5. Comparison between insulin-related structure of novel bombyxin genes and human insulin (Bell *et al.*, 1980). Upper and lower lines are A chains and B chains, respectively. The identical residues between the peptides are colored and bridging disulfide bonds are indicated by bar lines.

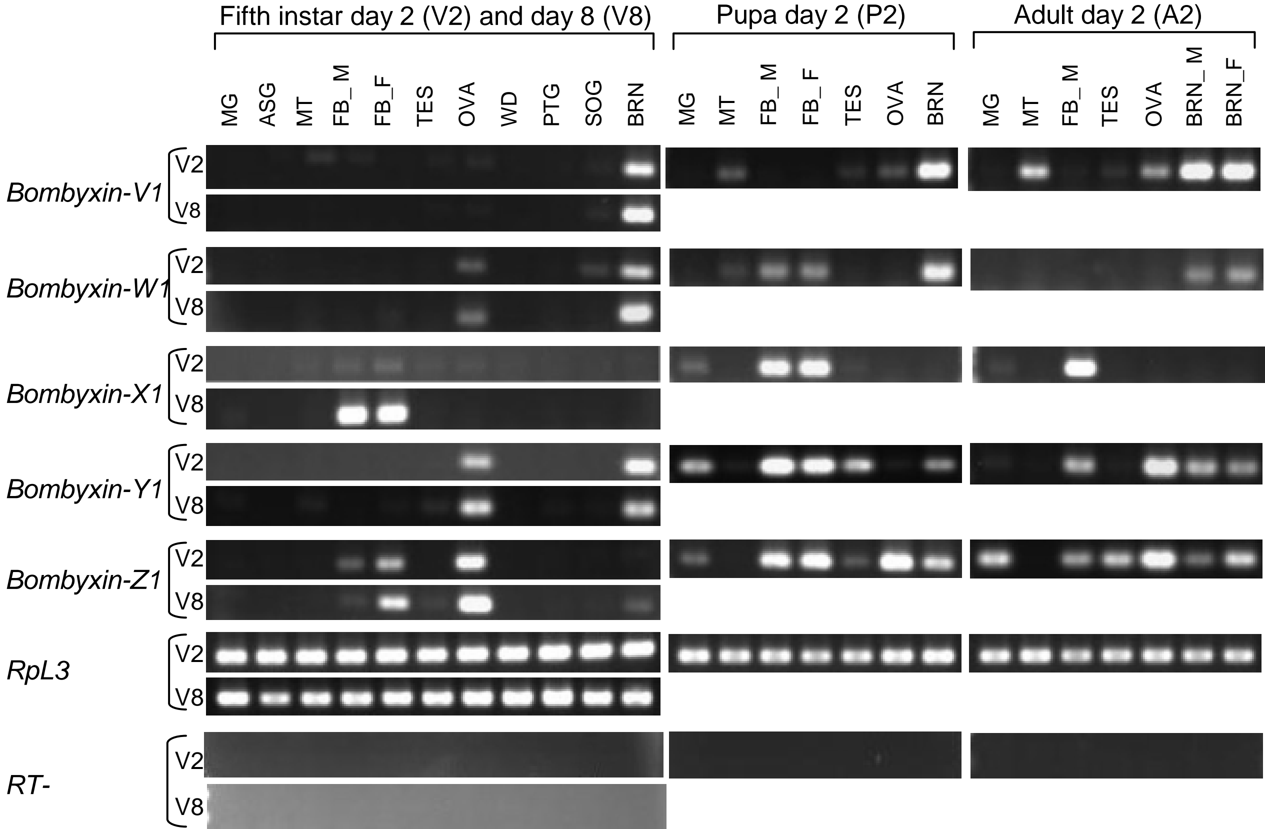


Fig. 6. Expression analysis of novel bombyxin genes in various tissues. Tissues of day 2 and day 8 of fifth instar larvae (V2, V8); day 2 of pupa (P2) and day 2 of adult (A2) were used. Abbreviations : MG, midgut; ASG, anterior silk gland; MT, Malpighian tubule; FB_M - male fat body; FB_F - female fat body; TES, testis; OVA, ovary; WD, wing disc; PTG - prothoracic gland; SOG, suboesophageal ganglion; BRN, brain of both male and female; BRN_M, male brain and BRN_F, female brain. *RpL3* was used as control. The number of PCR cycle was 27. There was no amplification without reverse transcriptase in the reaction (RT-), indicating no genomic cDNA contamination. These data are representative of at least 3 experiments.

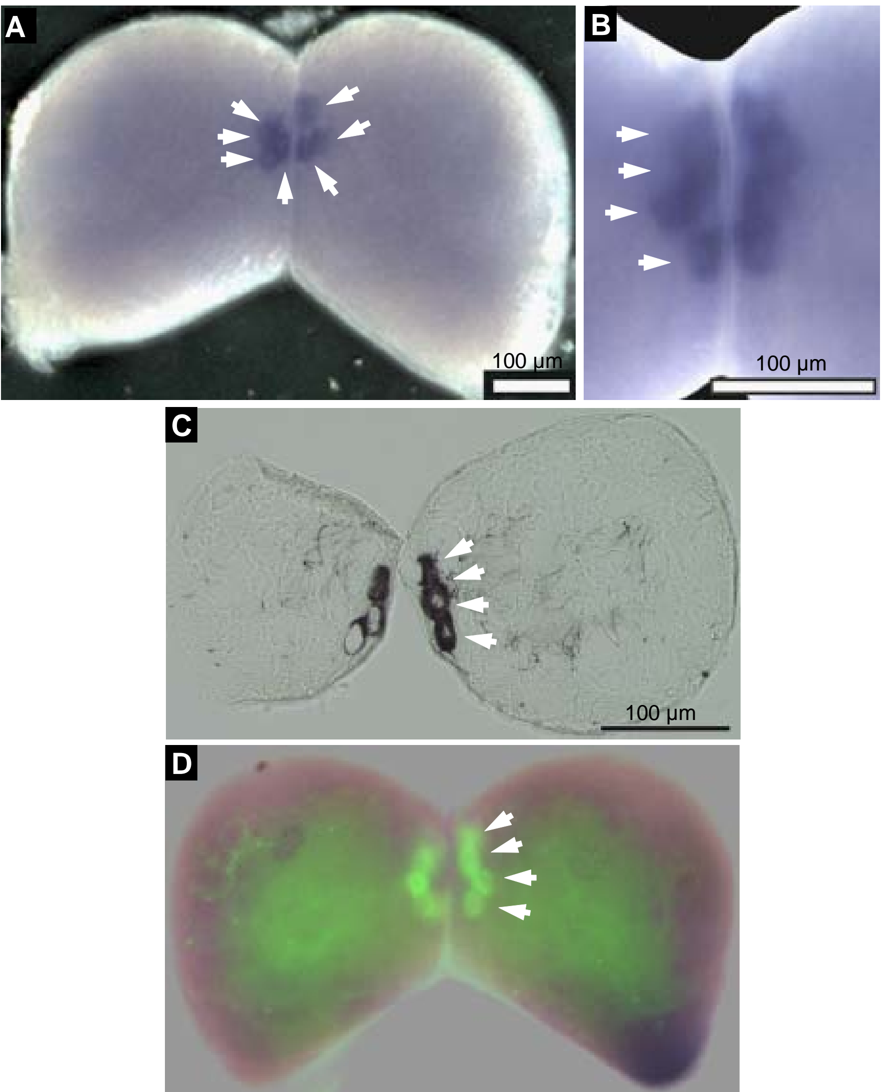


Fig. 7. Localization of *bombyxin-V1* mRNA in the medial neurosecretory cells (MNCs) of the fifth instar day 2 larval brain by *in situ* hybridization using DIG-labeled RNA antisense probe: (A) Expression in whole brain, (B) Magnified micrograph of *V1* expression, (C) Section *in situ* hybridization showing *bombyxin-V1* expression in MNCs. Arrows indicate mRNA localizations. These data are representative of at least 10 hybridizations. No signal was detected in hybridizations performed with sense probes (data not shown). Scale bars = 100 µm. (D) Immunohistochemistry with rabbit antiserum prepared against bombyxin A family was used as primary antibody and FITC-conjugated anti rabbit IgG from goat was used as secondary antibody.

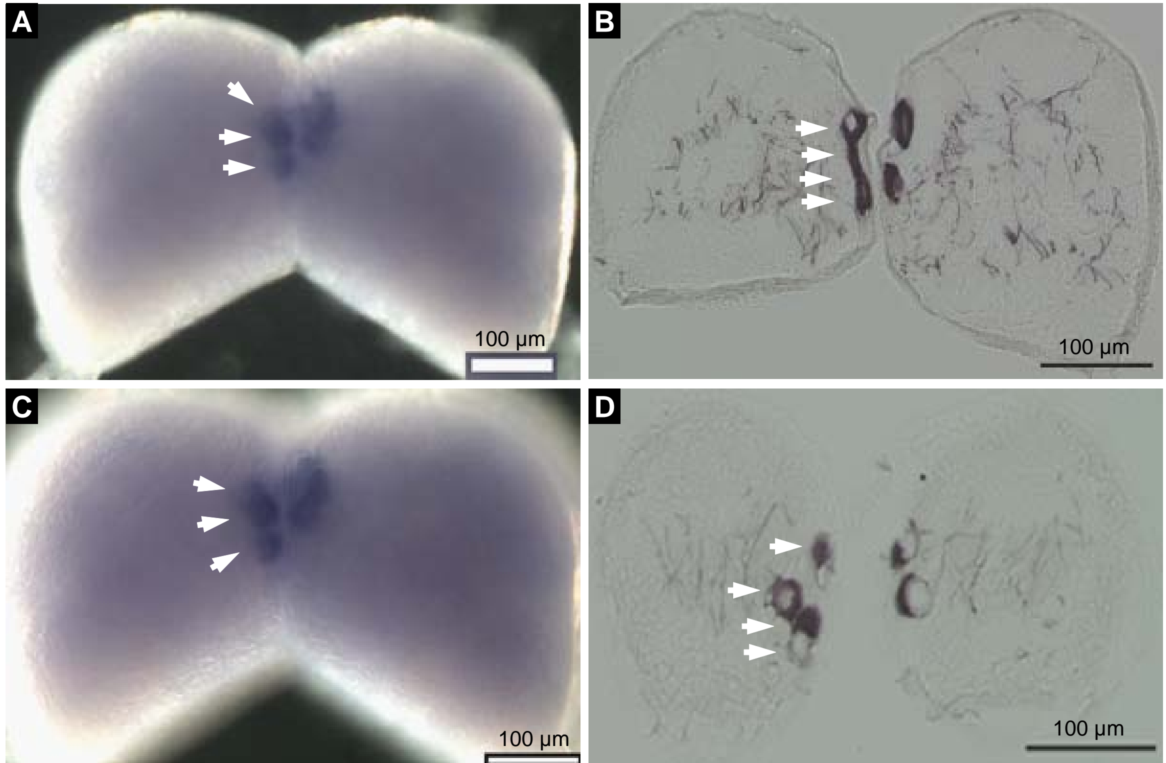


Fig. 8. *In situ* hybridization for localization of bombyxin transcripts using DIG-labeled RNA antisense probe. (A, B) *Bombyxin-W1* mRNA expression in the MNCs of the fifth instar day 2 larval brain, (C, D) *Bombyxin-Y1* mRNA expression in the MNCs of the fifth instar day 2 larval brain. Arrows indicate mRNA localizations. These data are representatives of at least 10 hybridizations. No signal was detected in hybridizations performed with sense probes (data not shown). Scale bars = 100 μm.

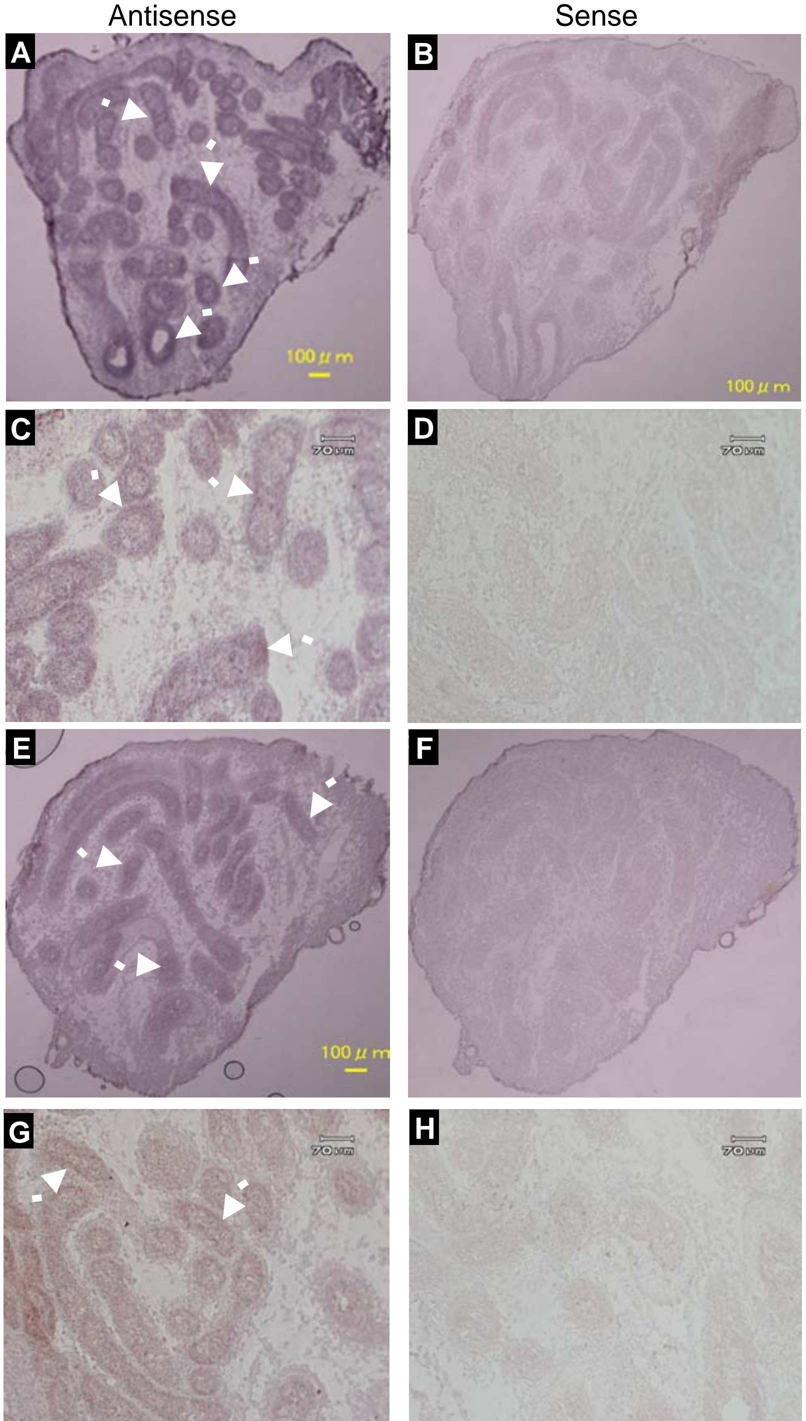


Fig. 9. Section *in situ* hybridization of *bombyxin-Y1* (A-D) and *bombyxin-Z1* (E-F) mRNAs in fifth instar day 8 ovary with the antisense (A,C,E,G) and sense (B,D,F,H) DIG-labeled DNA probes. Arrows indicate mRNA localizations. There was no signal in the samples hybridized with sense probes.

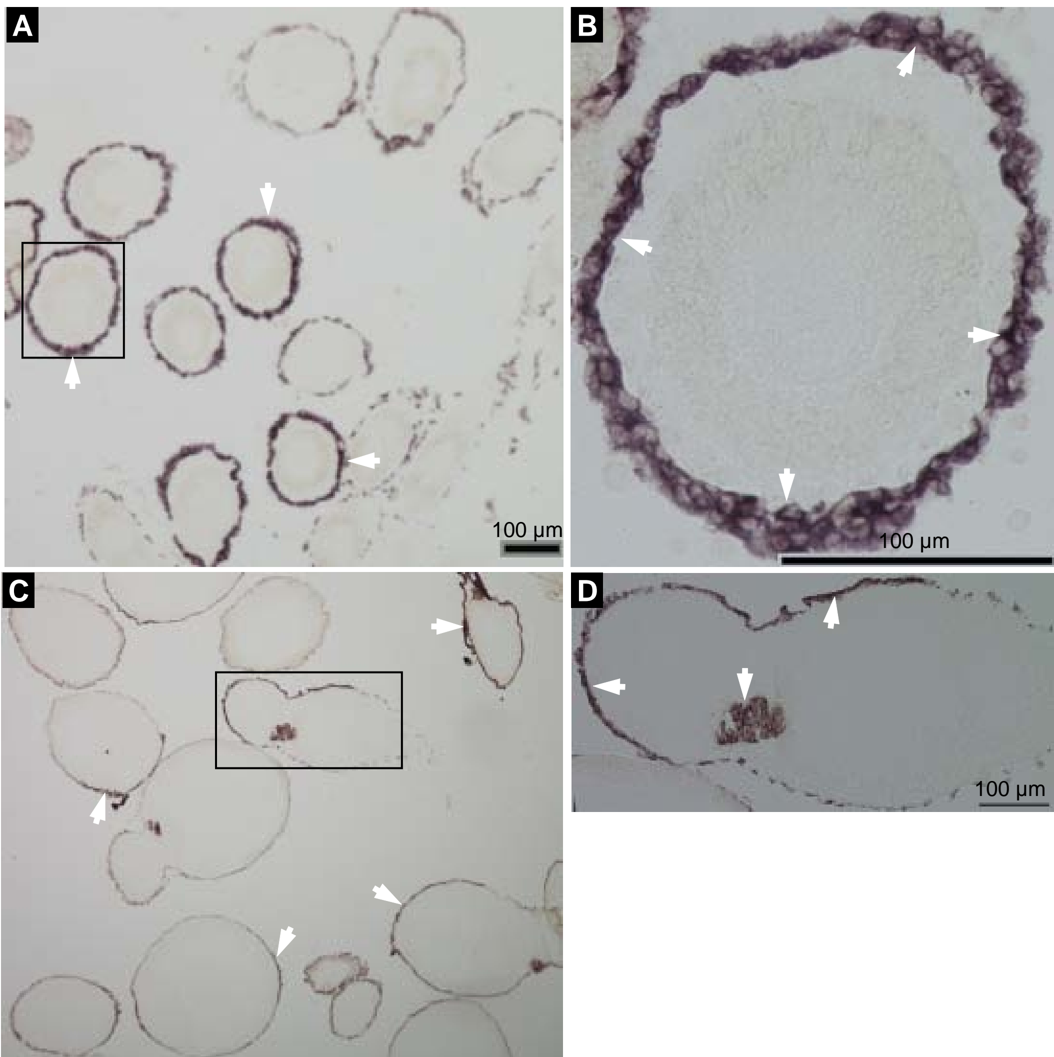


Fig. 10. Bombyxin mRNA localization in the ovary by *in situ* hybridization using DIG-labeled RNA antisense probe. (A, C) Expression of *bombyxin-Z1* in the fifth instar day 8 and pupa day 2 ovaries, respectively. (B, D) Magnified micrographs corresponding to the area shown in A and C, respectively. Staining is seen in the follicular cells of ovary. Arrows indicate mRNA localizations. These data are representative of at least 10 hybridizations. No signal was detected in hybridizations performed with sense probes (data not shown). Scale bars = 100 µm.

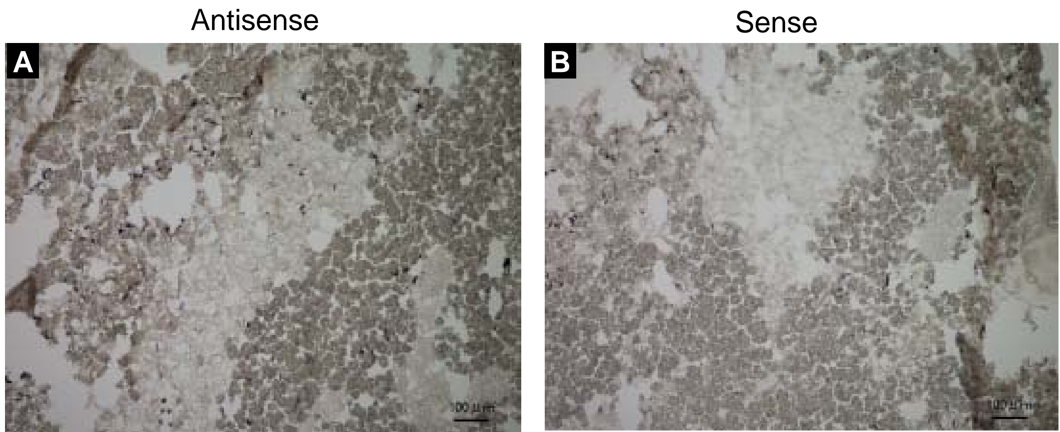


Fig. 11. Section *in situ* hybridization of pupa day 2 fat body by *bombyxin-Z1* antisense (A) and sense (B) probe. There was no detectable signal in the sections hybridized with antisense probe when compared to sense probe. Scale bars = 100 μm.

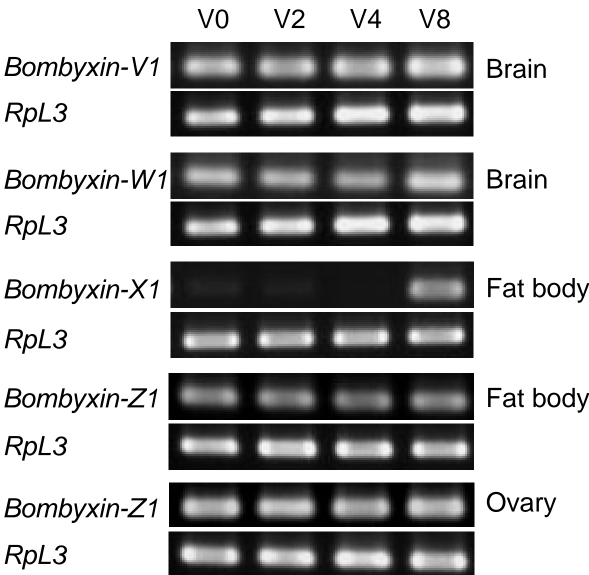


Fig. 12. Developmental changes of bombyxin gene expression. Transcript expression in tissues of fifth instar day 0 (V0), day 2 (V2), day 4 (V4) and day 8 (V8) stages was analyzed by RT-PCR. Vn represents n days after final larval ecdysis. Tissues exhibiting high expression of the appropriate bombyxin genes were used for this experiment. *RpL3* is shown to demonstrate equal amount of each sample. These data are representative of at least 3 experiments.

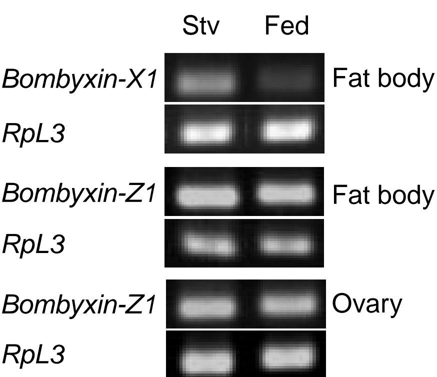


Fig. 13. Effect of starvation on expression of bombyxin genes. Larvae were starved from fifth instar day 3 and tissues were collected for starvation experiment on fifth instar day 5. Template cDNAs were analyzed by PCR. Stv and Fed indicate starved and control fed larvae, respectively. *RpL3* was used as internal standard. These data are representative of at least 3 experiments.

APPENDIX I

Nucleotide sequences of novel bombyxin genes

Following sequences start with the initiator methionine codon, ATG, and end with the terminator codon. Therefore, following sequences represent preprobombyxin coding region.

> Bombyxin-V1 (315)

ATGAAATTCACGGTGATGATAGTGCTGATGGCGTTAGCTTACGTCTCAGC

GCACAAGGAAGCAGTCACTCTGTGTGGACGGAACCTTGCAAACGCAAGGG

TTGCAGTTTGCTATGGAGCAGAATACGTTGATAAACGGACATCGGGAAAC

ACATTATTCGATGATTTCGAAGATGTAGACCTGCACGGCGATTGGCCGTG

GACTGGCCGTCGAGGCGCCCTCTCCGCTGACTGGACCCGCTACAAGCGCC

AAGGAATCGTCAACGAATGCTGTTTCAAACCATGCACCACCGACGTACTC

TTGAAATATTGTTAA

> Bombyxin-V2 (315)

ATGAAATTCACGGTGATGATAGTGCTGATGGCGTTAGCTTACGTCTCAGC

GCACAAGGAAGCAGTCACTCTGTGTGGACGGAACCTTGCAAACGCAAGGG

TTGCAGTTTGCTATGGAGCAGAATACGTTGATAAACGGACATCGGGAAAC

ACATTATTCGATGATTTCGAAGATGTAGACCTGCACGGCGATTGGCCGTG

GACTGGCCGTCGAGGCGCCCTCTCCGCTGACTGGACCCGCTACAAGCGCC

AAGGAATCGTCAACGAATGCTGTTTCAAACCATGCACCACCGACGTACTC

TTGAAATATTGTTAA

> Bombyxin-W1 (285)

ATGAAGACCGTCTTACTGATCGTTCTCCTGTTGACGATGTCTACGACCGC

GTTAGAACAACAAAAACAAGCCCGTCATTATTGCGGGCGATTCCTGGCGC

TAACGTTGGCTGACTTGTGCTGGGAGTATAAGCGCAGTGACGATTCGTAC

TACGATGGCAACAACCAGGACTTAACCGAGCCACCGAGCCCTCAGACGGC

GGAGCACCGCATCCAGAAACGAGGTGTTGCTGATGACTGCTGCCTCCGCG

CCTGCACTTTGGATGTACTGCTTTTATACTGTTAA

> Bombyxin-X1 (315)

ATGTTGCTGACGGCAAAACTTTATTTCGTCCTGTACGTTTTGCTTCTGTT

GAGAGAAAGCAAAGGCGACGCTCTCACCATGAACACAAGGATCAGAGATA

TGTGCAGCAGAGCGTTGTCGAACCTCATATTTGACATTTGCACAGGAACG

CTACCGGTTACGGATTTACCGCCCGAAAGTCTGTCGAAGGTACGTGCCAA

GAGAGCCTCTCTGTTTTCTGCAAAGCAAAGATCCAGGAGGCAGGTAGCCG

ACGAGTGCTGCCTTCGGTCTTGCACCGTGTCCCAGCTAGTCGAATACTGC

CCGGAGACTTGGTGA

> **Bombyxin**-Y1 (267)

ATGAAATTTTCTGCGGTTTTTGTGATCCTCCTCGTTCTACTGACGGTGGC

CGTGCTGAGTGAAAACGCGGGCCGTACCTACTGCGGTCGCTACCTGGCCC

GTACCTTGGCCAATCTCTGCAGCGACGCTGGTCAGGAAAAGCGCGGCGAA

GACTGGTCATGGCTGTCCGCATCCGGCCGTAAGGATGGCGCCGTGACTGA

GAATGGCGTCGCCAACGAGTGCTGCCTCCACCCCTGCACCCTAGAGGTTC

TGCTTTCCTACTGTTAA

> Bombyxin-Z1 (339)

ATGTTTCTGCTGTATTTCCTGATCGTTGTGGCGCTGGTGAGTGCTGACGT

CCACGACAAAGAGTTGAAAATTGAGGAAAATCCTCGGGTCTATTGCGGAC

GTCATTTGGCCAACGCACGCATGGTACTCTGCTATGACACTGTCGAGAAG

AGAGCCCAATCTTATCTCGACGCAAACATTATTTCGGCGGGAGATTTGAGC

TCCTGGCCTGGCCTGTCTTCCCAGTACGCCAAGACTCGCGCTTTTGCTCTC

GCCGAGAAATCTAAACGCGGCCCTGGCTTAGTCGACGAATGTTGCCTTAAG

CCCTGTTACACCTATGATCTACTTAATTACTGCTGA

APPENDIX II

Amino acid sequence of novel bombyxin genes

>Bombyxin-V1

MKFTVMIVLMALAYVSAHKEAVTLCGRNLANARVAVCYGAEYVDKRTSGN

TLFDDFEDVDLHGDWPWTGRRGALSADWTRYKRQGIVNECCFKPCTTDVL

LKYC

>Bombyxin-V2

MKFTVMIVLMALAYVSAHKEAVTLCGRNLANARVAVCYGAEYVDKRTSGN

TLFDDFEDVDLHGDWPWTGRRGALSADWTRYKRQGIVNECCFKPCTTDVL

LKYC

>Bombyxin-W1

MKTVLLIVLLLTMSTTALEQQKQARHYCGRFLALTLADLCWEYKRSDDSY

YDGNNQDLTEPPSPQTAEHRIQKRGVADDCCLRACTLDVLLLYC

>Bombyxin-X1

MLLTAKLYFVLYVLLLLRESKGDALTMNTRIRDMCSRALSNLIFDICTGT

LPVTDLPPESLSKVRAKRASLFSAKQRSRRQVADECCLRSCTVSQLVEYC

PETW

>Bombyxin-Y1

MKFSAVFVILLVLLTVAVLSENAGRTYCGRYLARTLANLCSDAGQEKRGE

DWSWLSASGRKDGAVTENGVANECCLHPCTLEVLLSYC

>Bombyxin-Z1

MFLLYFLIVVALVSADVHDKELKIEENPRVYCGRHLANARMVLCYDTVEK

RAQSYLDANIISAGDLSSWPGLSSQYAKTRAFALAEKSKRGPGLVDECCL

KPCYTYDLLNYC

APPENDIX III

cDNA sequences of novel *bombyxin* genes

>V1fcaL20e07f (Bombyxin-V1) (401)

GATCACACATCTTTAATCTTCCCATTGTTCCACATCGGAGATCATAATAC

AAGAGATTTTTAACATGAAATTCACGGTGATGATAGTGCTGATGGCGTTA

GCTTACGTCTCAGCGCACAAGGAAGCAGTCACTCTGTGTGGACGGAACCT

TGCAAACGCAAGGGTTGCAGTTTGCTATGGAGCAGAATACGTTGATAAAC

GGACATCGGGAAACACATTATTCGATGATTTCGAAGATGTAGACCTGCAC

GGCGATTGGCCGTGGACTGGCCGTCGAGGCGCCCTCTCCGCTGACTGGAC

CCGCTACAAGCGCCAAGGAATCGTCAACGAATGCTGTTTCAAACCATGCA

CCACCGACGTACTCTTGAAATATTGTTAAATATTAACCAAATTATTGTTG

A

>V2fcaL21f12f (Bombyxin-W1) (440)

ACATCTTCAATCTTCCCATTGTTTCACATCGGAGATCATAATAAAAGAGA

TTTTTAACATGAAATTCACGGTGATGATAGTGCTGATGGCGTTAGCTTAC

GTCTCAGCGCACAAGGAAGCAGTCACTCTGTGTGGACGGAACCTTGCAAA

CGCAAGGGTTGCAGTGTGTTATGGAGCAGAATACGTTGATAAACGGACAT

CGGGAAACACATTATTCGATGATTTCGAAGATGTAGACCTGCACGGCGAT

TGGCCGTGGACTGGCCGTCGAGGCGCCCTCTCCGCTGACTGGACCCGCTA

CAAGCGCCAAGGAATCGTCAACGAATGCTGTTTCAAACCATGCACCACCG

ACGTACTCTTGAAATATTGTTAAATATTAACCAAATTATTGTTGAAAAAA

AAAAGAAACTTAAAATATATTTCACGATGATTACCTANNA

>X1ovS329C05f (Bombyxin-X1) (521)

GAGCGTCCAAAATGTTGCTGACGGCAAAACTTTATTTCGTCCTGTACGTT

TTGCTTCTGTTGAGAGAAAGCAAAGGCGACGCTCTCACCATGAACACAAG

GATCAGAGATATGTGCAGCAGAGCGTTGTCGAACCTCATATTTGACATTT

GCACAGGAACGCTACCGGTTACGGATTTACCGCCCGAAAGTCTGTCGAAG

GTACGTGCCAAGAGAGCCTCTCTGTTTTCTGCAAAGCAAAGATCCAGGAG

GCAGGTAGCCGACGAGTGCTGCCTTCGGTCTTGCACCGTGTCCCAGCTAG

TCGAATACTGCCCGGAGACTTGGTGATGTGTGCAAATGAACTCGTGACGT

CTCCTGAATTCTGAAGTCGTGCTCTTGTTATGGCGAAAACTGTGTGTGTT

TGTGTGTGTGTGCTCCCGTAATGTGAATTTGAAAACATATCGTTAATTAA

GAAACACGTCGTGATTATTGTTTTGTTTCGAAAATTGGTATTAGTCTTTA

ACAAATCATATTAAGTAAANT

>Y1ovS326C11f (Bombyxin-Y1) (521)

ACAAGAGCTCTCGAAAAGGCAATTTATTTAATTGAATTAAATTCATCAAT
AGCCATCGACAGTCCGTCGTTTGACGAGCCACAATGAAATTTTCTGCGGT
TTTTGTGATCCTCCTCGTTCTACTGACGGTGGCCGTGCTGAGTGAAAACG
CGGGCCGTACCTACTGCGGTCGCTACCTGGCCCGTACCTTGGCCAATCTC
TGCAGCGACGCTGGTCAGGAAAAGCGCGGCGAAGACTGGTCATGGCTGTC
CGCATCCGGCCGTAAGGATGGCGCCGTGACTGAGAATGGCGTCGCCAACG
AGTGCTGCCTCCACCCCTGCACCCTAGAGGTTCTGCTTTCCTACTGTTAA
ATCCAGACAACATGTTCTACGGTGTTAAATAATTTGTACTAATTGATGTA
TCATTTTGGAAATAAAGATTTTTTTTAAATTCTAAAAAAAAAAAAAAAAA
AAAAAACTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGT
ATTACAATTCACTGGCCGTCG

>Z1fe100P26_F_N03 (Bombyxin-Z1) (651)

CGACGTACTCTTCGAATACGAACGCGAGCTGTCGTCTTCATCCCGACGTA

TTGAAATTTTAAACGGTTTCTAGGTGACGTCAAAGCAACCCTACATCGAG

GGTATAAAAGCTTGCTTAGTTCGATTTGAATGCACTTTGTCGCGTCTAGC

AGCCTAAGCACGAATACCACGATGTTTCTGCTGTATTTCCTGATCGTTGT

GGCGCTGGTGAGTGCTGACGTCCACGACAAAGAGTTGAAAATTGAGGAAA

ATCCTCGGGTCTATTGCGGACGTCATTTGGCCAACGCACGCATGGTACTC

TGCTATGACACTGTCGAGAAGAGAGCCCAATCTTATCTCGACGCAAACAT

TATTTCGGCGGGAGATTTGAGCTCCTGGCCTGGCCTGTCTTCCCAGTACG

CCAAGACTCGCGCTTTTGCTCTCGCCGAGAAATCTAAACGCGGCCCTGGC

TTAGTCGACGAATGTTGCCTTAAGCCCTGTTACACCTATGATCTACTTAA

TTACTGCTGAACAATTCAGTTTGCTTAACAGTAACCTACTACTGGTTAAT

ATTTATTCAAACCAGTAGTGATTGATCTTTTGTAGTGTTTGCATGTGTAA

TGTTTAATTTTCATTTAAAATTGTATCAACAAATAAAACTTTGAATTGCT

T

APPENDIX IV

Comparison between bombyxin-V1 and -V2 ORF

```
V1          ATGAAATTCACGGTGATGATAGTGCTGATGGCGTTAGCTTACGTCTCAGCGCACAAGGAA
V2          ATGAAATTCACGGTGATGATAGTGCTGATGGCGTTAGCTTACGTCTCAGCGCACAAGGAA
            ************************************************************

V1          GCAGTCACTCTGTGTGGACGGAACCTTGCAAACGCAAGGGTTGCAGTTTGCTATGGAGCA
V2          GCAGTCACTCTGTGTGGACGGAACCTTGCAAACGCAAGGGTTGCAGTTTGCTATGGAGCA
            ************************************************************

V1          GAATACGTTGATAAACGGACATCGGGAAACACATTATTCGATGATTTCGAAGATGTAGAC
V2          GAATACGTTGATAAACGGACATCGGGAAACACATTATTCGATGATTTCGAAGATGTAGAC
            ************************************************************

V1          CTGCACGGCGATTGGCCGTGGACTGGCCGTCGAGGCGCCCTCTCCGCTGACTGGACCCGC
V2          CTGCACGGCGATTGGCCGTGGACTGGCCGTCGAGGCGCCCTCTCCGCTGACTGGACCCGC
            ************************************************************

V1          TACAAGCGCCAAGGAATCGTCAACGAATGCTGTTTCAAACCATGCACCACCGACGTACTC
V2          TACAAGCGCCAAGGAATCGTCAACGAATGCTGTTTCAAACCATGCACCACCGACGTACTC
            ************************************************************

V1          TTGAAATATTGTTAA
V2          TTGAAATATTGTTAA
            ***************
```

Note: V1 and V2 ORF are completely similar.

Comparison between bombyxin-V1 and -V2 cDNA

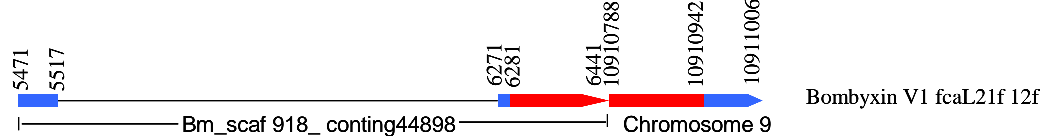
```
V1fcaL21f12f     ------ACATCTTCAATCTTCCCATTGTTTCACATCGGAGATCATAATAAAAGAGATTTT
V1fcaL20e07f     GATCACACATCTTTAATCTTCCCATTGTTCCACATCGGAGATCATAATACAAGAGATTTT
                       ******* *************** ******************* **********

V1fcaL21f12f     TAACATGAAATTCACGGTGATGATAGTGCTGATGGCGTTAGCTTACGTCTCAGCGCACAA
V1fcaL20e07f     TAACATGAAATTCACGGTGATGATAGTGCTGATGGCGTTAGCTTACGTCTCAGCGCACAA
                 ************************************************************

V1fcaL21f12f     GGAAGCAGTCACTCTGTGTGGACGGAACCTTGCAAACGCAAGGGTTGCAGTGTGTTATGG
V1fcaL20e07f     GGAAGCAGTCACTCTGTGTGGACGGAACCTTGCAAACGCAAGGGTTGCAGTTTGCTATGG
                 *************************************************** ** *****

V1fcaL21f12f     AGCAGAATACGTTGATAAACGGACATCGGGAAACACATTATTCGATGATTTCGAAGATGT
V1fcaL20e07f     AGCAGAATACGTTGATAAACGGACATCGGGAAACACATTATTCGATGATTTCGAAGATGT
                 ************************************************************

V1fcaL21f12f     AGACCTGCACGGCGATTGGCCGTGGACTGGCCGTCGAGGCGCCCTCTCCGCTGACTGGAC
V1fcaL20e07f     AGACCTGCACGGCGATTGGCCGTGGACTGGCCGTCGAGGCGCCCTCTCCGCTGACTGGAC
                 ************************************************************

V1fcaL21f12f     CCGCTACAAGCGCCAAGGAATCGTCAACGAATGCTGTTTCAAACCATGCACCACCGACGT
V1fcaL20e07f     CCGCTACAAGCGCCAAGGAATCGTCAACGAATGCTGTTTCAAACCATGCACCACCGACGT
                 ************************************************************

V1fcaL21f12f     ACTCTTGAAATATTGTTAAATATTAACCAAATTATTGTTGAAAAAAAAAAGAAACTTAAA
V1fcaL20e07f     ACTCTTGAAATATTGTTAAATATTAACCAAATTATTGTTGA-------------------
                 *****************************************

V1fcaL21f12f     ATATATTTCACGATGATTACCTANNA
V1fcaL20e07f     --------------------------
```

Note: V1 (V1fcaL21f12f) and V2 (V1fcaL20e07f) cDNAs are almost similar except few neuleotides marked by blue color letters.

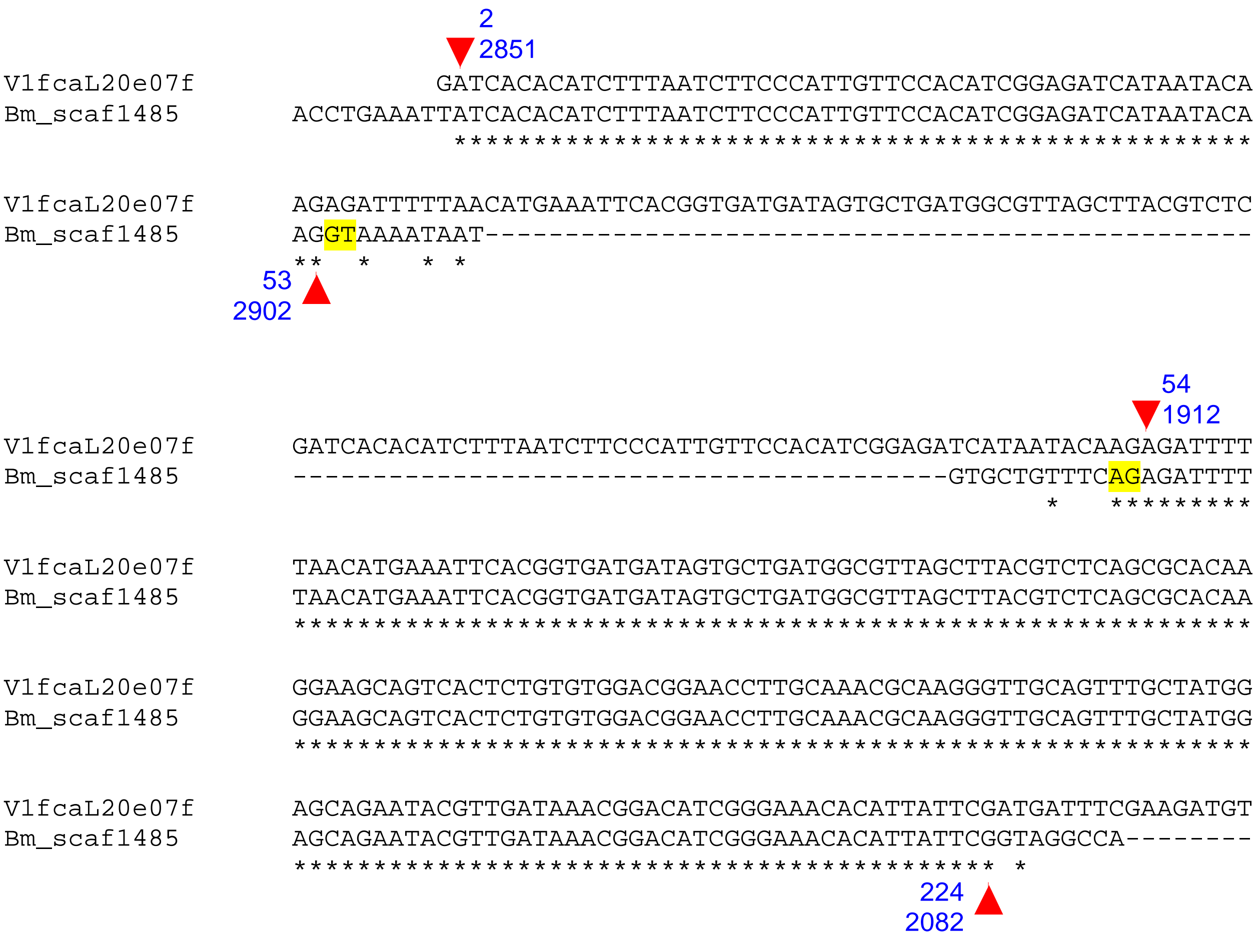
APPENDIX V

Comparison between cDNA and genomic sequence

Bombyxin-V1



Black narrow lines = intron, Thick lines = exon, Red thick lines = ORF, Blue thick lines = UTR

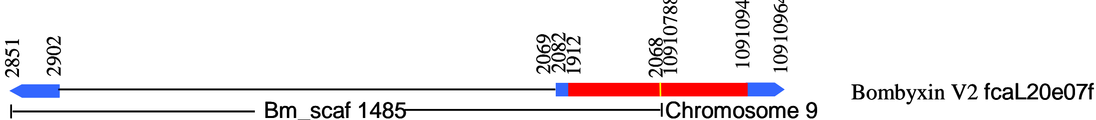


```
                                                                                225
                                                                               ▼10910788
V1fcaL20e07f       AGCAGAATACGTTGATAAACGGACATCGGGAAACACATTATTCGATGATTTCGAAGATGT
chr9               --------------------------------TATTTCAACAGATGATTTCGAAGATGT
                                                    *  * *   ****************

V1fcaL20e07f       AGACCTGCACGGCGATTGGCCGTGGACTGGCCGTCGAGGCGCCCTCTCCGCTGACTGGAC
chr9               AGACCTGCACGGCGATTGGCCGTGGACTGGCCGTCGAGGCGCCCTCTCCGCTGACTGGAC
                   ************************************************************

V1fcaL20e07f       CCGCTACAAGCGCCAAGGAATCGTCAACGAATGCTGTTTCAAACCATGCACCACCGACGT
chr9               CCGCTACAAGCGCCAAGGAATCGTCAACGAATGCTGTTTCAAACCATGCACCACCGACGT
                   ************************************************************

V1fcaL20e07f       ACTCTTGAAATATTGTTAAATATTAACCAAATTATTGTTGA
chr9               ACTCTTGAAATATTGTTAAATATTAACCAAATTATTGTTGAAAAAAAAAAAG
                   *****************************************
                                                          401 ▲
                                                     10910964
```

1st line cDNA sequence, 2nd line genomic sequence, red arrow area indicates matched area, V1fcaL20e07f represent cDNA of bombyxin-V1, chr9 indicates chromosome no. 9.

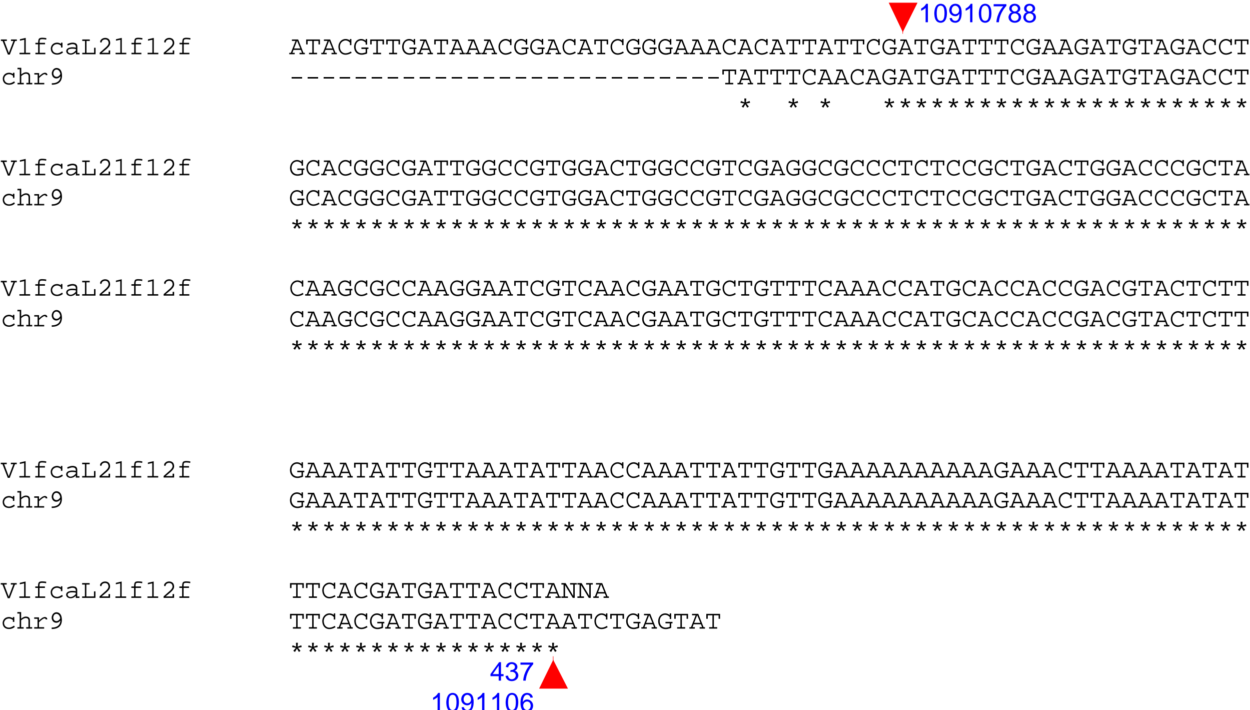
No homology of Bm_scaf1485 between 2902 and 1912 area with V1fcaL20e07f confirm this area as intron.

Bombyxin-V2



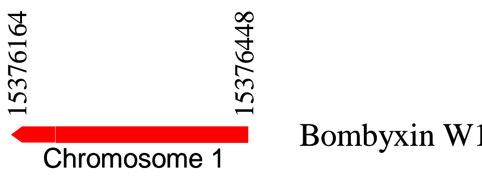
Black narrow lines = intron, Thick lines = exon, Red thick lines = ORF, Blue thick lines = UTR

```
                                       1
                                       5471
V1fcaL21f12f             ACATCTTCAATCTTCCCATTGTTTCACATCGGAGATCATA
Bm_scaf918_contig44898   AAATTATCACACATTTTCAATCTTCCCATTGTTCCACATCGGAGATCATA
                                   **** ****************** ****************

V1fcaL21f12f             ATAAAAGAGATTTTTAACATGAAATTCACGGTGATGATAGTGCTGATGGC
Bm_scaf918_contig44898   ATACAAGGTAAAA-------------------------------------
                         *** ***  *
                             47
                           5517

                                                                          48
                                                                          6271
V1fcaL21f12f             ACATCTTCAATCTTCCCATTGTTTCACATCGGAGATCATAATAAAAGAGA
Bm_scaf918_contig44898   -----------------------------------GTGCCGTTTCAGAGA
                                                                  *   *****

V1fcaL21f12f             TTTTTAACATGAAATTCACGGTGATGATAGTGCTGATGGCGTTAGCTTAC
Bm_scaf918_contig44898   TTTTTAACATGAAATTCACGGTGATGATAGTGCTGATGGCGTTAGCTTAC
                         **************************************************

V1fcaL21f12f             GTCTCAGCGCACAAGGAAGCAGTCACTCTGTGTGGACGGAACCTTGCAAA
Bm_scaf918_contig44898   GTCTCAGCGCACAAGGAAGCAGTCACTCTGTGTGGACGGAACCTTGCAAA
                         **************************************************

V1fcaL21f12f             CGCAAGGGTTGCAGTGTGTTATGGAGCAGAATACGTTGATAAACGGACAT
Bm_scaf918_contig44898   CGCAAGGGTTGCAGTGTGTTATGGAGCAGAATACGTTGATAAACGGACAT
                         **************************************************

V1fcaL21f12f             CGGGAAACACATTATTCGATGATTTCGAAGATGTAGACCTGCACGGCGAT
Bm_scaf918_contig44898   CGGGAAACACATTATTCGGTAGGCAAAA----------------------
                         ****************** *       *
                                      218
                                     6441
```



No homology of Bm_scaf918_contig44898 between 5517 and 6271 area with V1fcaL21f12f confirm this area as intron. V1fcaL21f12f represent cDNA of bombyxin-V1, chr9 indicates chromosome no. 9.

Bombyxin-W1



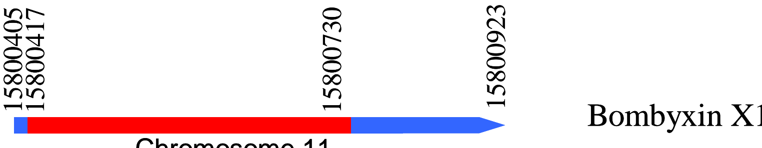
Red thick line = ORF

```
                                  1
                                  15376164
BBX-W1                            ATGAAGACCGTCTTACTGATCGTTCTCCTGTTGACGATGTCTACGACCGC
chr1            CAGCGTAAAAATGAAGACCGTCTTACTGATCGTTCTCCTGTTGACGATGTCTACGACCGC
                          **************************************************

BBX-W1          GTTAGAACAACAAAAACAAGCCCGTCATTATTGCGGGCGATTCCTGGCGCTAACGTTGGC
chr1            GTTAGAACAACAAAAACAAGCCCGTCATTATTGCGGGCGATTCCTGGCGCTAACGTTGGC
                ************************************************************

BBX-W1          TGACTTGTGCTGGGAGTATAAGCGCAGTGACGATTCGTACTACGATGGCAACAACCAGGA
chr1            TGACTTGTGCTGGGAGTATAAGCGCAGTGACGATTCGTACTACGATGGCAACAACCAGGA
                ************************************************************

BBX-W1          CTTAACCGAGCCACCGAGCCCTCAGACGGCGGAGCACCGCATCCAGAAACGAGGTGTTGC
chr1            CTTAACCGAGCCACCGAGCCCTCAGACGGCGGAGCACCGCATCCAGAAACGAGGTGTTGC
                ************************************************************

BBX-W1          TGATGACTGCTGCCTCCGCGCCTGCACTTTGGATGTACTGCTTTTATACTGTTAA
chr1            TGATGACTGCTGCCTCCGCGCCTGCACTTTGGATGTACTGCTTTTATACTGTTAAAGTGA
                *******************************************************
                                                                   285
BBX-W1                                                        15376448
chr1            TCTGA
```

1st line cDNA sequence, 2nd line genomic sequence, red arrow area indicates matched area.

BBX-W1 represent bombyxin-W1, chr1 indicates chromosome no. 1.

Bombyxin-X1



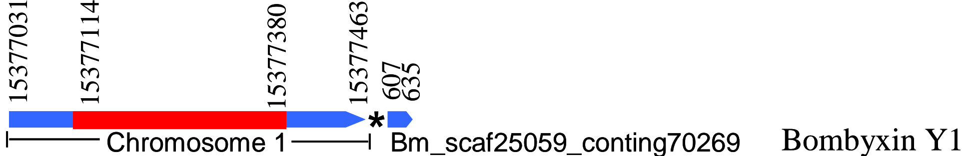
Thick lines = exon, Red thick line = ORF, Blue thick lines = UTR.

```
                          1
                          15800405
X1ovS329C05f                    GAGCGTCCAAAATGTTGCTGACGGCAAAACTTTATTTCGTCCTGTACGTT
chr11               CAGCAGACGAGAGCGTCCAAAATGTTGCTGACGGCAAAACTTTATTTCGTCCTGTACGTT
                                **************************************************

X1ovS329C05f        TTGCTTCTGTTGAGAGAAAGCAAAGGCGACGCTCTCACCATGAACACAAGGATCAGAGAT
chr11               TTGCTTCTGTTGAGAGAAAGCAAAGGCGACGCTCTCACCATGAACACAAGGATCAGAGAT
                    ************************************************************

X1ovS329C05f        ATGTGCAGCAGAGCGTTGTCGAACCTCATATTTGACATTTGCACAGGAACGCTACCGGTT
chr11               ATGTGCAGCAGAGCGTTGTCGAACCTCATATTTGACATTTGCACAGGAACGCTACCGGTT
                    ************************************************************

X1ovS329C05f        ACGGATTTACCGCCCGAAAGTCTGTCGAAGGTACGTGCCAAGAGAGCCTCTCTGTTTTCT
chr11               ACGGATTTACCGCCCGAAAGTCTGTCGAAGGTACGTGCCAAGAGAGCCTCTCTGTTTTCT
                    ************************************************************

X1ovS329C05f        GCAAAGCAAAGATCCAGGAGGCAGGTAGCCGACGAGTGCTGCCTTCGGTCTTGCACCGTG
chr11               GCAAAGCAAAGATCCAGGAGGCAGGTAGCCGACGAGTGCTGCCTTCGGTCTTGCACCGTG
                    ************************************************************

X1ovS329C05f        TCCCAGCTAGTCGAATACTGCCCGGAGACTTGGTGATGTGTGCAAATGAACTCGTGACGT
chr11               TCCCAGCTAGTCGAATACTGCCCGGAGACTTGGTGATGTGTGCAAATGAACTCGTGACGT
                    ************************************************************

X1ovS329C05f        CTCCTGAATTCTGAAGTCGTGCTCTTGTTATGGCGAAAACTGTGTGTGTTTGTGTGTGTG
chr11               CTCCTGAATTCTGAAGTCGTGCTCTTGTTATGGCGAAAACTGTGTGTGTTTGTGTGTGTG
                    ************************************************************

X1ovS329C05f        TGCTCCCGTAATGTGAATTTGAAAACATATCGTTAATTAAGAAACACGTCGTGATTATTG
chr11               TGCTCCCGTAATGTGAATTTGAAAACATATCGTTAATTAAGAAACACGTCGTGATTATTG
                    ************************************************************

X1ovS329C05f        TTTTGTTTCGAAAATTGGTATTAGTCTTTAACAAATCATATTAAGTAAANT--------
chr11               TTTTGTTTCGAAAATTGGTATTAGTCTTTAACAAATCATATTAAGTAAATTGTGGCCGT
                    *************************************************  *
                                                                 519
                                                            15800923
```

1st line cDNA sequence, 2nd line genomic sequence, red arrow area indicates matched area

X1ovS329C05f represent cDNA of bombyxin-X1, chr11 indicates chromosome no. 11.

Bombyxin-Y1



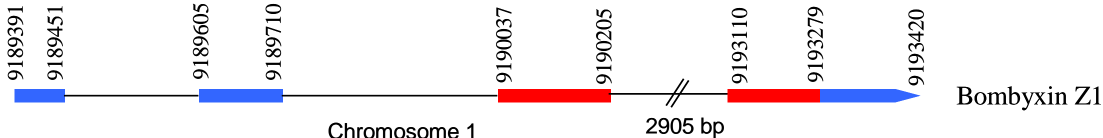
Thick lines = exon, Red thick lines = ORF, Blue thick lines = UTR, *=gap

```
Y1ovS326C11f                           ACAAGAGCTCTCGAAAAGGCAATTTATTTAATTGAATTAAATTCATCAAT
Chromosome 1
TCTCGGGAAGACAAGAGCTCTCGAAAAGGCAATTTATTTAATTGAATTAAATTCATCAAT
                                       **************************************************

Y1ovS326C11f       AGCCATCGACAGTCCGTCGTTTGACGAGCCACAATGAAATTTTCTGCGGTTTTTGTGATC
Chromosome 1       AGCCGTCGACAGTCCGTCGTTTGACGAGCCACAATGAAATTTTCTGCGGTTTTTGTGATC
                   **** *******************************************************

Y1ovS326C11f       CTCCTCGTTCTACTGACGGTGGCCGTGCTGAGTGAAAACGCGGGCCGTACCTACTGCGGT
Chromosome 1       CTCCTCGTTCTACTGACGGTGGCCGTGCTGAGTGAAAACGCGGGCCGTACCTACTGCGGT
                   ************************************************************

Y1ovS326C11f       CGCTACCTGGCCCGTACCTTGGCCAATCTCTGCAGCGACGCTGGTCAGGAAAAGCGCGGC
Chromosome 1       CGCTACCTGGCCCGTACCTTGGCCAATCTCTGCAGCGACGCTGGTCAGGAAAAGCGCGGC
                   ************************************************************

Y1ovS326C11f       GAAGACTGGTCATGGCTGTCCGCATCCGGCCGTAAGGATGGCGCCGTGACTGAGAATGGC
Chromosome 1       GAAGACTGGTCATGGCTGTCCGCATCCGGCCGTAAGGATGGCGCCGTGACTGAGAATGGC
                   ************************************************************

Y1ovS326C11f       GTCGCCAACGAGTGCTGCCTCCACCCCTGCACCCTAGAGGTTCTGCTTTCCTACTGTTAA
Chromosome 1       GTCGCCAACGAGTGCTGCCTCCACCCCTGCACCCTAGAGGTTCTGCTTTCCTACTGTTAA
                   ************************************************************

Y1ovS326C11f       ATCCAGACAACATGTTCTACGGTGTTAAATAATTTGTACTAATTGATGTATCATTTTGGA
Chromosome 1       ATCCAGACAACATGTTCTACGGTGTTAAATAATTTGTACTAATTGATGTATCATTTTGGA
                   ************************************************************

Y1ovS326C11f       AATAAAGATTTTTTTTAAATTCTAAAAAAAAAAAAAAAAAAAAAAAAACTCGAGGGGGGGCC
Chromosome 1       AATAAAGATTTTTTTTAAATTCTATGTTTCGCCTTTTTTATTTTATTTTTAATTAAGTCC
                   ************************        *     *  *  *      * **
                                 433
                           15377463
Y1ovS326C11f       CGGTACCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCG
Chromosome 1       ---TACCTGTTTTTGT-----------------------------------
                      ****   **
```

```
Y1ovS326C11f               TCATTTTGGAAATAAAGATTTTTTTTAAATTCTAAAAAAAAAAAAAAAAA
Bm_scaf25059contig70269    ------------------------TCGAATTCCCGCGGCCGCCATGGCGG
                                                   *  476               *
                                                    ▼607
Y1ovS326C11f               AAAAAACTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGT
Bm_scaf25059contig70269    CCGGGAGC--ATGCGACGTCGGG--CCCAATTCGCCCTATAGTGAGTCGT
                                *    * * *  * * **  *************************

Y1ovS326C11f               ATTACAATTCACTGGCCGTCG
Bm_scaf25059contig70269    ATTA-----------------
                           ****
                           504▲
                           635
```

Note: *= No homology between downstream of chr 1: 15377463 and upstream Bm_scaf25059contig70269: 607 indicates there is a gap between 433 and 476. Bm_scaf25059contig70269 is not mapped so it is assumed that this scaffold is a part of the chromosome 11.

Bombyxin-Z1



Black narrow lines = intron, Thick lines = exon, Red thick lines = ORF, Blue thick lines = UTR

```
                                        5
                                        9189391
Z1fe100P26_F_N03           CGACGTACTCTTCGAATACGAACGCGAGCTGTCGTCTTCATCCC
Chromosome 1         AATTCTTCCAGTACTCTTCGAATACGAACGCGAGCTGTCGTCTTCATCCC
                               ****************************************

Z1fe100P26_F_N03     GACGTATTGAAATTTTAAACGGTTTCTAGGTGACGTCAAAGCAACCCTAC
Chromosome 1         GACGTATTGAAATTTTAAA-GGTATTTAAA--------------------
                     ******************* *** * **
                                      65
                                 9189451


Z1fe100P26_F_N03     CGACGTACTCTTCGAATACGAACGCGAGCTGTCGTCTTCATCCCGACGTA
Chromosome 1         --------------------------------------------------

                                     66
                                     9189605
Z1fe100P26_F_N03     TTGAAATTTTAAACGGTTTCTAGGTGACGTCAAAGCAACCCTACATCGAG
Chromosome 1         -TATAATTCTA---GGTTTCTAGGTGACGTCAAAGCAACCCTACATCGAG
                      *  **** **   ************************************

Z1fe100P26_F_N03     GGTATAAAAGCTTGCTTAGTTCGATTTGAATGCACTTTGTCGCGTCTAGC
Chromosome 1         GGTATAAAAGCTTGCTTAGTTCGATTTGAATGCACTTTGTCGCGTCTAGC
                     **************************************************

Z1fe100P26_F_N03     AGCCTAAGCACGAATACCACGATGTTTC-TGCTGTATTTCCTGATCGTTG
Chromosome 1         AGCCTAAGCACGAATACCACGGTACGGCATG-------------------
                     ********************* *    * **
                                       171
                                  9189710

                                           172
                                           9190037
Z1fe100P26_F_N03     AGCCTAAGCACGAATACCACGATGTTTCTGCTGTATTTCCTGATCGTTGT
Chromosome 1         ----------TTTATTTGCAGATGTTTCTGCTGTATTTCCTGATCGTTGT
                                  **     ******************************

Z1fe100P26_F_N03     GGCGCTGGTGAGTGCTGACGTCCACGACAAAGAGTTGAAAATTGAGGAAA
Chromosome 1         GGCGCTGGTGAGTGCTGACGTCCACGACAAAGAGTTGAAAATTGAGGAAA
                     **************************************************
```

```
Z1fe100P26_F_N03          ATCCTCGGGTCTATTGCGGACGTCATTTGGCCAACGCACGCATGGTACTC
Chromosome 1              ATCCTCGGGTCTATTGCGGACGTCATTTGGCCAACGCACGCATGGTACTC
                          **************************************************

Z1fe100P26_F_N03          TGCTATGACACTGTCGAGAAGAGAGCCCAATCTTATCTCGACGCAAACAT
Chromosome 1              TGCTATGACACTGTCGAGAAGAGAGCCCAATCTTATCTCGGTGAGTTTAA
                          ****************************************   *     *
                                                             340
                                                         9190205

                                                                    341
                                                                    9193110
Z1fe100P26_F_N03          TGCTATGACACTGTCGAGAAGAGAGCCCAATCTTATCTC-GACGCAAACA
Chromosome 1              -------------------------------TTTATTTCAGACGCAAACA
                                                          **** ** **********

Z1fe100P26_F_N03          TTATTTCGGCGGGAGATTTGAGCTCCTGGCCTGGCCTGTCTTCCCAGTAC
Chromosome 1              TTATTTCGGCGGGAGATTTGAGCTCCTGGCCTGGCCTGTCTTCCCAGTAC
                          **************************************************

Z1fe100P26_F_N03          GCCAAGACTCGCGCTTTTGCTCTCGCCGAGAAATCTAAACGCGGCCCTGG
Chromosome 1              GCCAAGACTCGCGCTTTTGCTCTCGCCGAGAAATCTAAACGCGGCCCTGG
                          **************************************************

Z1fe100P26_F_N03          CTTAGTCGACGAATGTTGCCTTAAGCCCTGTTACACCTATGATCTACTTA
Chromosome 1              CTTAGTCGACGAATGTTGCCTTAAGCCCTGTTACACCTATGATCTACTTA
                          **************************************************

Z1fe100P26_F_N03          ATTACTGCTGAACAATTCAGTTTGCTTAACAGTAACCTACTACTGGTTAA
Chromosome 1              ATTACTGCTGAACAATTCAGTTTGCTTAACAGTAACCTACTACTGGTTAA
                          **************************************************

Z1fe100P26_F_N03          TATTTATTCAAACCAGTAGTGATTGATCTTTTGTAGTGTTTGCATGTGTA
Chromosome 1              TATTTATTCAAACCAGTAGTGATTGATCTTTTGTAGTGTTTGCATGTGTA
                          **************************************************

Z1fe100P26_F_N03          ATGTTTAATTTTCATTTAAAATTGTATCAACAAATAAAACTTTGAATTGC
Chromosome 1              ATGTTTAATTTTCATTTAAAATTGTATCAACAAATAAAACTTTGAATTGC
                          **************************************************

Z1fe100P26_F_N03          TT
Chromosome 1              TTTTCTTCATTT
                          **
                     651
                 9193420
```

1st line cDNA sequence, 2nd line genomic sequence, red arrow area indicates matched area, Z1fe100P26_F_N03 represent cDNA of bombyxin-Z1.

No homology of chromosome 1 between 9189451 and 9189605, 9189710 and 9190037, 919205 and 9193110 area with Z1fe100P26_F_N03 confirm this area as intron.

Accompanying Published Paper

Zoological Science (in press)

Identification of novel bombyxin genes from the genome of the silkmoth *Bombyx mori* and analysis of their expression

Abu F M Aslam, Taketoshi Kiya, Kazuei Mita, and Masafumi Iwami

Zoological Science (in press)

Identification of novel bombyxin genes from the genome of the silkmoth *Bombyx mori* and analysis of their expression

Running Title: Bombyxin V – Z genes

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[3]National Institute of Agrobiological Sciences, Tsukuba, 305-8634, Japan

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E-mail address: masafumi@kenroku.kanazawa-u.ac.jp

Discipline: Molecular Biology

Abstract

Insulin family peptide members play key roles in regulating growth, metabolism, and reproduction. Bombyxin is an insulin-related peptide of the silkmoth *Bombyx mori*. We analyzed the full genome of *B. mori* and identified five novel bombyxin families, V to Z. We characterized the genomic organization and chromosomal location of the novel bombyxin family genes. In contrast to the previously identified bombyxin genes, bombyxin-V and -Z genes had intervening introns at almost the same positions as vertebrate insulin genes. We performed reverse transcription-polymerase chain reaction and in situ hybridization in different tissues and developmental stages to observe their temporal and spatial expression patterns. The newly identified bombyxin genes were expressed in diverse tissues: bombyxin-V, -W, and -Y mRNAs were expressed in the brain and bombyxin-X mRNA in fat bodies. Bombyxin-Y gene was expressed in both brain and ovary of larval stages. High level of bombyxin-Z gene expression in the follicular cells may suggest its function in reproduction. The presence of a short C-peptide domain and an extended A chain domain, and high expression of bombyxin-X gene in the fat body cells during non-feeding stages suggest its insulin-like growth factor-like function. These results suggest that the bombyxin genes have originated from a common ancestral gene, similar to the vertebrate insulin gene, and evolved into a diverse gene family with multiple functions.

Key words: bombyxin, brain, fat body, ovary, gene expression, insulin, insulin-like growth factor

INTRODUCTION

The insulin family consists of insulin, insulin-like growth factor (IGF), and relaxin, which exert a wide variety of physiological effects on growth, metabolism, and development of vertebrates. Insulin family members have also been identified in invertebrates, including insect species of different orders (Nagasawa et al., 1984, 1986; Iwami et al., 1989; Lagueux et al., 1990; Brogiolo et al., 2001; Riehle and Brown, 2002; Krieger et al., 2004; Wheeler et al., 2006; de Azevedo and Hartfelder, 2008; Li et al., 2008). Among these, bombyxin, a family of peptides produced in the brain of the silkmoth *Bombyx mori*, was the first insulin-related peptide isolated from insects (Nagasawa et al., 1984, 1986). Bombyxin has been considered as a critical factor regulating insect molting and metamorphosis (Nagasawa et al., 1984), tissue growth (Nijhout and Grunert, 2002), carbohydrate metabolism (Satake, 1997), and ovarian development (Fullbright et al., 1997). The exact physiological function of bombyxin in *B. mori* is still unclear, however, and further study is necessary for the elucidation of its function (Iwami, 2000; Garde and Hoffmann, 2005).

Bombyxin genes are expressed predominantly in the brain (Kawakami et al., 1989; Iwami, 2000) in contrast to the insulin gene, which is expressed in the pancreas. Recently, insulin-like peptides of *B. mori* and *Drosophila melanogaster* have been identified and found to be highly produced in the fat body cells, suggesting to be insect IGFs (Okamoto et al., 2009a, b; Slaidina et al., 2009).

Thus far, thirty-two bombyxin genes belonging to seven families have been isolated from the *B. mori* genome (Kondo et al., 1996; Iwami, 2000). These results suggests that a high degree of diversification in the structure and genomic organization of bombyxin genes has occurred throughout the long course of evolution (Kondo et al., 1996; Iwami, 2000; Bayazit, 2009).

It is however still unknown whether the genes encoding insulin and its structurally related peptides have emerged from a single ancestral (insulin-type) gene (Smit et al., 1993). Due to sharp contrasts in the number of introns, multiple gene copies, and different expression sites in bombyxin genes, it has been thought that different mechanisms underlie the evolution of insulin family genes between vertebrates and invertebrates (Kondo et al*.,* 1996; Iwami, 2000). Previous reports indicated that more bombyxin genes remain to be found in the *B. mori* genome (Kondo et al., 1996; Iwami, 2000) and thus we aimed to identify all bombyxin genes using genomic analysis

We analyzed bombyxin genes in the *B. mori* genome database and identified five novel family genes. The present paper is the first report of a comprehensive survey of insulin-related genes in *B. mori* genome after the completion of the genome project. We present here the characterization of these genes and show that two family genes have introns at almost the same positions as human insulin gene. We found the novel bombyxin genes are expressed in various tissues and developmental stages using reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization. In addition, characterization of structure and genomic organization of bombyxin genes will provide an important basis to elucidate evolutionary relationships of insulin family members in insects.

MATERIALS AND METHODS

Gene identification, structure, and alignment analysis

Nucleotide sequences of new bombyxin genes were identified from the *B. mori* genome (The International Silkworm Genome Consortium, 2008). The *B. mori* genome database KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase/) (Shimomura et al., 2009) was searched for additional genes encoding bombyxin and insulin-related peptide using nucleotide and peptide sequences of previously identified bombyxin genes. The deduced amino acid sequences of significant matches were examined for insulin-like characteristics, including the presence of signal peptide, B chain, C-peptide, and A chain and dibasic processing sites flanking the C-peptide. Signal peptides were predicted using SIG-pred (http://bmbpcu36.leeds.ac.uk/prot_analysis/Signal.html) (Zhang et al., 2009), and endoprotease cleavage sites for C-peptides were determined according to the procedures outlined by Seidah and Chretien (1997). Intron-exon boundaries were confirmed by comparison between the genomic and cDNA sequences. The cDNA sequences used are sequences of representative nos. in the CYBERGATE database (http://150.26.71.213/cgi-bin/main_MX): *V1*, fcaL21f12f; *V2*, fcaL20e07f; *X1*, ovS329C05f; *Y1*, ovS326C11f; *Z1*, fe100P26_F_N03. The cDNA sequence for *W1* was identified in this study. Geneious Pro 4.8 (Biomatters, Auckland, New Zealand) was used for nucleotide and amino acid sequence analysis and for phylogenetic analysis with default parameters.

Experimental animals

Eggs of a racial hybrid of *B. mori*, Kinshu × Showa, were obtained from Ueda Sanshu (Ueda, Japan), and larvae were reared on an artificial diet (Silkmate II, Nihon Nosan Kogyo, Yokohama, Japan) at 25±1°C under a 12-h light/12-h dark photoperiodic cycle (Sakurai et al., 1998). Newly molted fifth instar larvae were fed from the beginning of the photophase following the scotophase during which they molted to fifth instars. The 24 h period of the photophase following the scotophase during which the fourth instar larva molted was designated day 0 of the fifth instar (V0). Similarly, the 24 h period of the photophase following the scotophase during which the fifth instar larva pupated was designated day 0 of the pupal stage (P0), and that of pupa eclosed was designated day 0 of adult stage (A0). Days 2, 4, and 8 after V0 and days 2 after P0 were thus denoted as V2, V4 V8, and P2, respectively. Day 2 after A0 was denoted as A2.

RNA isolation, cDNA synthesis, and PCR amplification

Total RNA was extracted from various tissues using the acid guanidinium thiocyanate phenol-chloroform method with minor modifications (Tsuzuki et al., 2001). RNase-free DNase I-treated (Promega, Madison, WI, USA) RNA (1 µg) was used as a template for cDNA synthesis using ReverTra Ace (Toyobo, Osaka, Japan) and an oligo(dT) primer in a 20 µl reaction mixture. The reverse transcription products were diluted with 80 µl TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH

8.0). The resulting cDNA was used as a template to amplify DNA fragments using PCR in a 10 µl mixture containing GoTaq Green Master Mix (Promega) and gene-specific primers (Table 1). As control, cDNA samples were prepared without adding reverse transcriptase to demonstrate the absence of genomic contamination. Primers were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/), with default criteria (Rozen and Skaletsky, 2000). Because bombyxin genes belong to a multi-gene family, the gene-specific primers were designed from the signal peptide and C-peptide sites, which are the least common sites among these genes, to avoid non-specific amplification. Amplification was performed in a thermal cycler (Mastercycler ep384, Eppendorf, Hamburg, Germany), with denaturation at 94°C for 30 s, annealing as mentioned in Table 1 for 30 s, and extension at 72°C for 30 s. RT-PCR reactions were performed at least three times and gave reproducible results.

Cloning, plasmid purification, DNA sequencing, and sequence analysis

The *B. mori* genome contains multiple bombyxin gene copies having at most 56% and 85% similarity between and within families, redspectively (Iwami, 2000; Kondo et al., 1996). To evaluate the risk of amplifying highly similar non-target genes and to confirm whether targeted gene transcripts were expressed, cDNAs of the transcripts from the identified bombyxin genes were sequenced. The PCR products of respective bombyxin gene primers were cloned into the pGEM-T vector (Promega) and sequenced. For sequence determination, at least three individual clones were analyzed.

In situ hybridization

Whole-mount in situ hybridization*:* Dissected brains were washed in 10 mM phosphate-buffered saline (PBS, pH 7.4) with 0.1% Tween20 (PBT) and fixed in a solution of 85% ethanol, 4% formaldehyde, and 5% acetic acid on ice for 45 min, as described previously (Hossain et al., 2006, 2008). Tissues were deproteinized with proteinase K (50 µg/ml in PBS) at 37°C for 30 min and re-fixed in 4% (w/v) paraformaldehyde in PBT at room temperature for 20 min. After incubation in prehybridization solution (50% (v/v) formamide, 5X sodium and sodium citrate (SSC), 1 µg/ml yeast t-RNA, 50 µg/ml heparin, 0.1% TritonX, 0.1% CHAPS (3-{(3-cholamidopropyl)dimethyl ammonio}-propanesulfonate), 5% dextran sulfate, 5 mM EDTA, and 2% blocking reagent (Roche Diagnostics GmbH, Mannheim, Germany)) at 50°C for 90 min, the samples were hybridized with riboprobes (1 ng/µl each) at 50°C overnight. Probes for each bombyxin gene were prepared using the plasmid where the RT-PCR products were cloned. Digoxygenin (DIG)-labeled RNA probes were synthesized using a DIG RNA Labeling Mix (Roche). Sense probes were used as negative controls. After being washed several times with a series of wash buffers (50% formamide, 5X SSC, and 1% SDS; 2X SSC and 0.1% CHAPS, and 0.2X SSC and 0.1% CHAPS) at 50°C and KTBT (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM KCl, and 0.1% Triton X-100) at room temperature, the tissues were blocked in 1.5% blocking solution for 90 min. The tissues were incubated with an anti-DIG antibody conjugated with alkaline phosphatase (Roche) at a 1:2500 dilution at 4°C overnight followed by extensive washing in

KTBT. Samples were incubated in staining buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 0.05 M $MgCl_2$ and 0.1% Tween 20). Staining was developed using NBT/BCIP solution (nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt) (Roche). After dehydration in ethanol, brains were clarified with methyl salicylate and observed under a microscope (BX-50F, Olympus, Japan).

Section in situ hybridization*:* Freshly dissected tissues were embedded in Tissue-Tek O.C.T compound (Sakura Fintek, Torrance, CA, USA) and frozen on dry ice. Frozen tissues were sectioned at 10 μm using a cryostat at -15°C, mounted on slides, air dried, and fixed for 12 - 16 h at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). Sections were then treated with proteinase K (10 μg/ml in 100 mM Tris–HCl, pH 8, and 50 mM EDTA) at 37°C for 20 min, re-fixed for 20 min, washed with PB, acetylated with 0.25 % acetic anhydride in 0.1 M triethanolamine (pH 8) for 10 min at room temperature, washed with PB, and dehydrated using a graded series of ethanol. The sections were hybridized with probes (2 ng/μl) in the presence of hybridization buffer (50% formamide, 10 mM Tris HCl, pH 7.6, 200 μg/ml yeast tRNA, 1X Denhardt's solution, 10% dextran sulfate, 600 mM NaCl, 0.25% SDS, and 1 mM EDTA) and incubated overnight at 37°C in a humidified chamber with 50% formamide. Slides were washed in 50% formamide and 2X SSC at 37°C for 30 min, treated with 10 μg/ml ribonuclease A (Sigma-Aldrich, St. Louis, MO, USA) in TNE (10 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 1 mM EDTA) for 15 min, and washed with 2X SSC and 0.2X SSC at 50°C. Staining was developed as previously mentioned.

RESULTS

Identification and analysis of novel families of bombyxin genes

We identified novel bombyxin genes, which were grouped into five families named bombyxin-V to -Z, by performing BLAST searches on the *B. mori* genome database using all known bombyxin family genes. The deduced amino acid sequences of these novel bombyxin genes showed 21% to 50% identity among the prepropeptide of bombyxin family members and 21% to 26% identity with human preproinsulin. Phylogenetic analysis revealed that the newly identified bombyxin genes did not belong to any known family of bombyxins and were thus considered as novel genes (Fig. 1A). Our newly identified bombyxin-Y1 gene was the same as the 8k-bombyxin-like peptide (8K-BLP) gene identified by Okamoto et al. (2009a). The open reading frames (ORFs) of the newly identified bombyxin genes encoded preprobombyxins with four domains in the order of signal peptide, B chain, C-peptide (domain), and A chain. The A and B chains of deduced bombyxins showed high similarity to those of previously identified bombyxins and other insulin members, while the C-peptide (domain) and signal peptide showed less similarity (Fig. 1B).

Organization of bombyxin genes: *bombyxin-V* and *-Z* have introns

We characterized the genomic structure and chromosomal location of the novel bombyxin genes. Figure 2 shows the exon–intron organization of the newly identified bombyxin genes.

Bombyxin-W1, -Y1 and *-Z1* were localized on chromosome 1, while chromosomes 9 and 11 harbored family V and X genes, respectively. The comparison of the genomic and cDNA sequences (data not shown) revealed that *bombyxin-V1* and *V2* had one intervening intron of 754 and 833 nucleotides, respectively, in the 5'-untranslated region (UTR), but could not be assigned on exact positions on chromosome 9. *Bombyxin-W1*, *-X1,* and *-Y1* lacked introns at the conserved intron sites of insulin-family genes and were thus considered traditional bombyxin genes. *Bombyxin-Z1* consisted of four exons and three introns. The first (154 bp) and second (327 bp) introns were in the 5'-UTR, and the third one was in the C-peptide region (2905 bp).

Expression of bombyxin genes in diverse tissues

To identify the tissues that express bombyxin genes, we performed RT-PCR expression analysis. Figure 3A shows tissue-specific expression profiles of the identified bombyxin genes in the life stages studied: V2, V8, P2, and A2.

Bombyxin-V1 (or *V2*) and *-W1* mRNAs were highly expressed in the brain at all four studied stages. High expression of *bombyxin-X1* was observed exclusively in the fat bodies in V8, P2, and A2. Analysis of *bombyxin-Y1* expression showed high levels of the transcript in the brain and ovary of larval stages. In P2, *Y1* was highly expressed in fat bodies and in A2, it showed the highest expression in the ovary. The expression pattern of *bombyxin-Y1* (identical to previously identified 8K-BLP gene) was partly presented by Okamoto et al. (2009a). Interestingly, *bombyxin-Z1* was mostly expressed in the female fat body and ovary in V2 and V8 and highly expressed in the ovary and fat body of P2 as well as in the ovary of A2.

Expression of bombyxin genes in different developmental stages

To profile the developmental expression of bombyxin genes, a survey of transcription was conducted semi-quantitatively using RT-PCR on the tissues in which the genes were highly expressed. Figure 3B shows developmental expression profiles. All genes except *X1* were expressed throughout V0, V2, V4, and V8. *X1* expression was low or undetectable at V0, V2, and V4 and began at V8 in the male fat body.

Spatial distribution of bombyxin gene transcripts

To specify the location of *bombyxin*-expressing cells, we performed in situ hybridization. *Bombyxin-V1* (Fig. 4A, B), *-W1* (Fig. 4D), and *-Y1* (Fig. 4F) mRNAs were localized to four pairs of the large medial neurosecretory cells in V2 brains. Section in situ hybridization showed the same expression pattern (Fig 4. C, E, G). We further confirmed the expression of bombyxin genes by identifying transcripts in V8 and P2 ovaries using in situ hybridization on frozen sections (Fig. 4H - K). *Z1* transcripts were predominantly and selectively detected surrounding the egg cells of the ovariole, which we assumed as follicle cells. *Y1* mRNA was also detected in a similar location in the ovary (data

not shown). We then used frozen sections of V8 and P2 fat bodies to detect *bombyxin-X1* and *-Z1* transcriptional location, but we could not find any signal, possibly due to ubiquitous and low level expression at the cellular level.

DISCUSSION

In the present study, we identified six novel bombyxin genes. Together with the previously identified 32 genes (Kawakami et al., 1989; Kondo et al., 1996; Tsuzuki et al., 1997; Yoshida et al., 1998; Iwami, 2000), 38 bombyxin genes have been identified in the haploid *B. mori* genome and classified into 11 families, A to G and V to Z. Analyses of the genomes of other insects have revealed the presence of multiple genes for insulin-related peptides: seven in *D. melanogaster* (Brogiolo et al., 2001), seven in *Anopheles gambiae* (Krieger et al., 2004), eight in *Aedes aegypti* (Riehle et al., 2006), two in *Apis mellifera* (Wheeler et al., 2006), four in *Tribolium castaneum* (Li et al., 2008), and at least six in *Samia cynthia ricini* (Kimura-Kawakami et al., 1992). The previously identified 25 bombyxin genes are clustered in a 50 kb range on chromosome 11 and 6 genes in a 15 kb range on an anonymous chromosome with unique distribution patterns: gene pairs, gene triplets, and single genes (Iwami, 2000). Newly identified 3 genes are located on chromosome 1, 2 on 9, and 1 on 11, without forming gene triplet nor gene pair. Gene clustering is usual for bombyxin genes that have highest multiple gene copies. The *Drosophila* insulin-like peptide (DILP) 1-5 genes occur in a gene cluster within 26 kb on chromosome 3 and DILP6-7 on X chromosome (Brogiolo et al., 2001). *Anopheles gambiae* (Ag) ILP1-4 clustered within 12 kb of DNA and separated from 6-7 by 23 kb on chromosome 3, AgILP5 is located on chromosome 2 (Krieger et al., 2004).

All of the newly identified bombyxin genes apparently encode preprobombyxin, having a signal peptide, B chain, C-peptide (domain), and A chain, similar to other bombyxin genes (Iwami et al., 1989, 1990; Kawakami et al., 1989; Kondo et al., 1996; Tsuzuki et al., 1997; Yoshida et al., 1998), preprobombyxin-related peptides of *S. cynthia ricini* (Kimura-Kawakami et al., 1992; Brogiolo et al., 2001; Krieger et al., 2004), and preproinsulins (Steiner et al., 1985). Of the four domains of preprobombyxins, the highest similarities were seen in the A-chain, and the degree of amino acid conservation was A chain > B chain > C-peptide (domain) and signal peptide. The general structural features of vertebrate and non-vertebrate insulin-related genes (including novel bombyxin genes) are thus well conserved. Although two dibasic amino acids are present in the C-peptide (domain), there is possibility that bombyxin-X1 and -Y1 remain as a single polypeptide chain, which is more like vertebrate IGFs in view of the previous analysis (Okamoto et al., 2009a).

All the previously identified bombyxin genes were lack of introns both in the 5' untranslated region and in the C-peptide region (Kondo et al., 1996; Iwami, 2000). Present study reveals for the first time that *bombyxin-V1, -V2*, and *-Z1* have intervening introns, unlike previously identified bombyxin genes. The most straightforward interpretation of not having introns in all bombyxin genes might be due to lose of intron from intron-containing bombyxin genes. We do not exclude other possibilities, for example, *bombyxin-Z1* might have acquired novel introns after they branched from other bombyxin genes. Presence of introns has already been reported in some insulin-like genes of

Drosophila (reviewed in Wu and Brown 200), *A. gambiae* (Krieger et al., 2004) and *A. aegypti* (Riehle et al, 2006). Structurally, the insulin protein has been highly conserved throughout vertebrate evolution. Vertebrate insulin genes, including human insulin genes, contain two introns: one within the 5'-UTR and the other in the C-peptide region (Steiner et al., 1985). Due to the presence of intron in the C-peptide region in insulin, IGFs, and relaxin genes, Crawford et al. (1984) suggested that these genes had evolved from a common ancestral gene by gene duplication. From our findings, the presence of intron at almost the same position in *bombyxin-Z1* indicates that bombyxin and vertebrate insulin genes share a common ancestral gene.

The newly identified bombyxin genes were expressed in a tissue-specific manner, suggesting a specific function. We found that three genes were predominantly expressed in four pairs of MNCs in the brain. Previously identified bombyxin genes of all 7 families were exclusively expressed in the same cells (Iwami, 2000). Analogous findings have also been reported for other lepidopterans (Van de Velde et al., 2007) and other insects (Brogiolo et al., 2001; Ikeya et al., 2002; Krieger et al., 2004).

The expression of bombyxin and insulin-related genes is low in a number of tissues other than brain (Iwami et al., 1996; Brogiolo et al., 2001; Riehle et al., 2006). In the present study, we found that three of the newly identified genes were highly expressed in tissues other than brain. *Bombyxin-Z1* was expressed in various tissues in P2 and A2 including the brain, fat body, and midgut. Interestingly, it was highly expressed in the ovary throughout the stages examined. Invertebrate insulin and IGFs assumed to have effects on oocyte maturation (El-Etr et al., 1979), embryogenesis (Froesch et al., 1985), and ovarian development (Sim and Denlinger, 2009). Bombyxin induces meiosis in the ovary (Orikasa et al., 1993). Brown et al., (2008) found that an insulin-like peptide in *A. aegypti* regulates egg maturation and metabolism. In many insects, insulin-related genes are involved in oogenesis (Kozlova and Thummel, 2000; Sun et al., 2002). The mosquito insulin receptor has been reported to localize in cell membrane of the follicle cells that produce ecdysteroids (Riehle and Brown, 2002). The follicular cells of the ovary have been reported as an expression site of ecdysteroid-regulated genes in insects (Carney and Bender, 2000; Paul et al., 2006; Takeuchi et al., 2007). The transcript of *bombyxin-Z1* was localized to the follicular cells of the ovary, the same site of transcripts of the ecdysteroid-regulated genes. From these facts, it is tempting to hypothesize that bombyxins, at least those of a particular family and possibly bombyxin-Z1, control the oogenesis and embryogenesis of *B. mori*, similar to the mammalian hormone relaxin, another member of the insulin family (Wu and Brown, 2006).

Vertebrate insulin genes are expressed in the pancreatic cells, and this has been a major criterion to differentiate vertebrate and invertebrate insulins or insulin-related peptides (Iwami, 2000). Our study revealed that *bombyxin-X1* was predominantly expressed in the fat body during the molting period. Ecdysone reduces food consumption and then induces starvation during molting and pupation in *B. mori* (Wang et al., 2010). DILP6, one of the insulin-like peptides of *D. melanogaster,* is specifically produced and required for growth during this period (Slaidina et al., 2009). Growth of the isolated wing imaginal disks of *M. sexta in vitro* requires both bombyxin and ecdysone to achieve normal growth (Nijhout et al., 2007). The functional class of IGF has recently been described in *B.*

mori (Okamoto et al., 2009a) and *D. melanogaster* (Okamoto et al., 2009b; Slaidina et al., 2009). Considering these findings, we hypothesize that bombyxin-X1 might have an IGF-like growth function and may play an important role in metamorphosis. Whether *bombyxin-X1* actually exhibits such activity requires further investigation.

In conclusion, five novel bombyxin family genes were identified. Our results clearly indicate that the identified bombyxin genes are expressed in a tissue-specific and, in some cases, developmental stage-specific manner. They are predominantly expressed in the brain, fat body, and ovary. Similarities in the structure, location of intervening introns, and high gasteroenteric expression between bombyxin and insulin genes suggest that the bombyxin genes might have originated from a common ancestral gene similar to the vertebrate insulin gene. Based on the sequence diversity and expression specificity, it is tempting to think that, similar to members of the insulin family in humans, each of the bombyxins also serves a distinct function. Further detailed studies might provide clues to clarify the functions of bombyxin families.

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FIGURE LEGENDS AND TABLE

Fig. 1. (A) A phylogenetic tree showing the relationships among bombyxin family members. BBX = bombyxin. The scale bar indicates an evolutionary distance of 0.3 amino acid substitutions per position. Novel bombyxins are boxed. (B) Amino acid sequence comparison of novel preprobombyxins, preprobombyxin-A1 (Nagasawa et al., 1986), human preproinsulin (Bell et al., 1980), and prepro-IGF-I (Jansen et al., 1983) as representatives of the vertebrate lineage. *Bombyxin-V2* is not shown in the figure as this ORF sequence is similar to that of V1. Gaps are introduced for maximum matching. Residues that are completely conserved among the prepropeptides are boxed. Inverted triangles indicate cysteine residues for disulfide bonds. The dibasic processing sites flanking the C peptide are underlined.

Fig. 2. Schematic representation of the intron-exon organization of newly identified bombyxin genes. Thick lines, exon; dark gray thick lines, ORF; light gray thick lines, UTR; thin lines, intron; "//", a break due to space limitation; "*", gap. Numbers above the gene structure indicate the chromosome/scaffold position (based on KAIKObase version 2) in the gene. Although bombyxin *V1* and *V2* have been assigned on the different positions on chromosome 9, the exact positions of the 5'-regions of them could not be assigned, while 3′-halves of *bomyxin-V1* and -*V2* are located at positions 10910788 - 10911006 and 10909184 - 10909360 on chromosome 9, respectively. Non-mapped portion of the chromosome is marked by scaffold and contig.

Fig. 3. (A) Expression analysis of newly identified bombyxin genes in various tissues and developmental stages. Tissues of V2 and V8 larvae, P2 pupa, and A2 adult were used. Abbreviations : MG, midgut; ASG, anterior silk gland; MT, Malpighian tubule; FB_M, male fat body; FB_F, female fat body; TES, testis; OVA, ovary; WD, wing disc; PTG, prothoracic gland; SOG, suboesophageal ganglion; BRN, brain of both male and female; BRN_M, male brain, and BRN_F, female brain. *RpL3* was used as a control. The number of PCR cycles was 27. There was no amplification without reverse transcriptase in the reaction, indicating no genomic DNA contamination. These data are representative of at least 3 experiments. (B) Developmental changes in bombyxin gene expression. Expressions in tissues of V0, V2, V4, and V8 stages were analyzed using RT-PCR. Tissues exhibiting high expression of the bombyxin genes were used for this experiment. Abbreviations used are the same as in (A). *RpL3* is shown to demonstrate the equal amount of cDNA in each sample. These data are representative of at least 3 experiments.

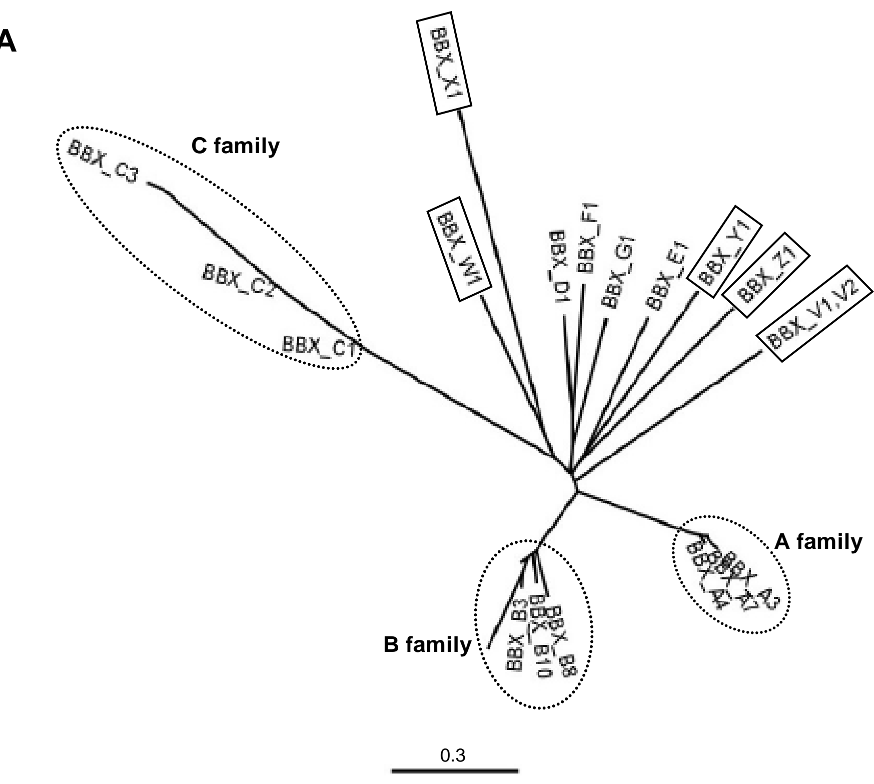
Fig. 4. Localization of bombyxin mRNAs in different tissues using in situ hybridization. (A, C) *Bombyxin-V1* expression in the medial neurosecretory cells (MNCs) of V2 brain. (B) Magnified micrograph of *V1* expression. (D, E) *Bombyxin-W1* expression in MNCs. (F, G) *Bombyxin-Y1* expression in MNCs. (H, J) *Bombyxin-Z1* expression in V8 and P2 ovaries, respectively. (I, K)

Magnified micrograph corresponding to the areas shown in H and J, respectively. Staining is seen in the follicular cells of the ovary. Arrows indicate mRNA localizations. These data are representative of at least 10 hybridizations. No signal was detected in hybridizations performed with sense probes (data not shown). Scale bars = 100 μm.

Table 1. Primers for PCR and in situ hybridization.

Gene	Forward	Reverse	Annealing temperature (。C)
bombyxin-V1	TTCACGGTGATGATAGTGCTG	CTTGTAGCGGGTCCAGTCAG	56
bombyxin-W1	TCTACGACCGCGTTAGAACAA	TTTCTGGATGCGGTGCTC	55
bombyxin-X1	TGACGGCAAAACTTTATTTCG	CCTGGATCTTTGCTTTGCAG	53
bombyxin-Y1	TGAAATTTTCTGCGGTTTTTG	CGGACAGCCATGACCAGT	54
bombyxin-Z1	TTCTGCTGTATTTCCTGATCGT	TCTTGGCGTACTGGGAAGAC	55
RpL3	AGCACCCCGTCATGGGTCTA	TGCGTCCAAGCTCATCCTGC	58

Figure 1



B

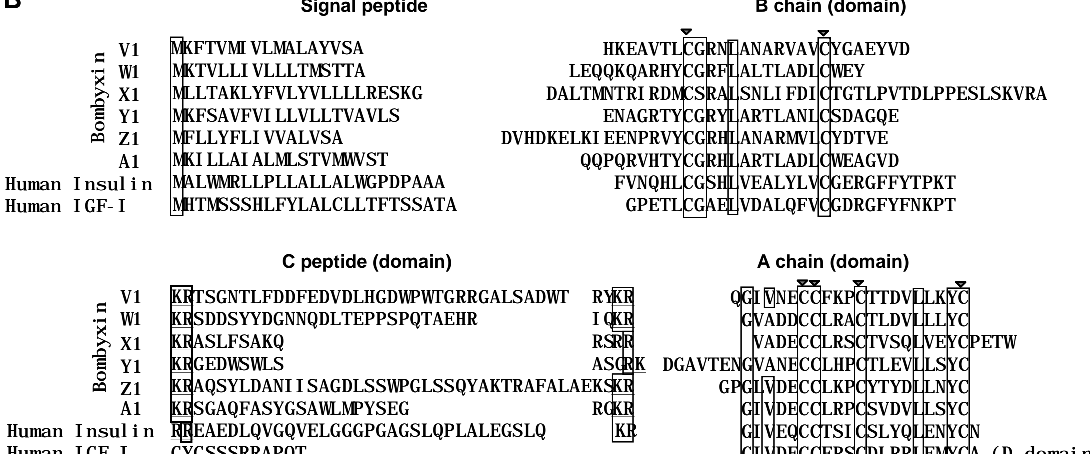


Figure 2

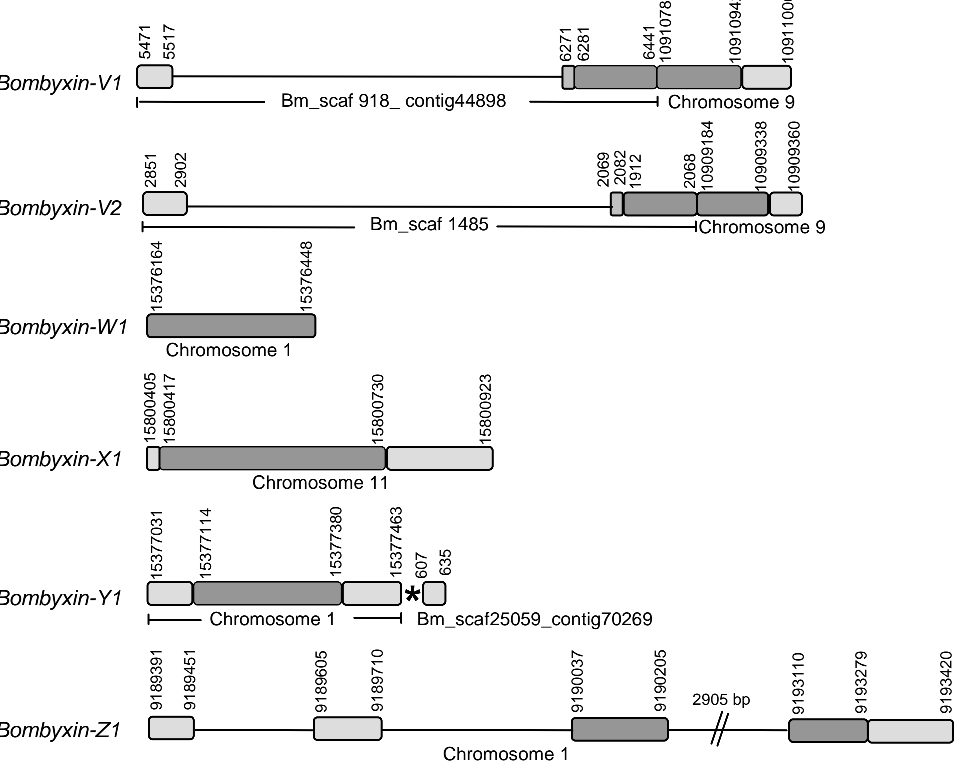
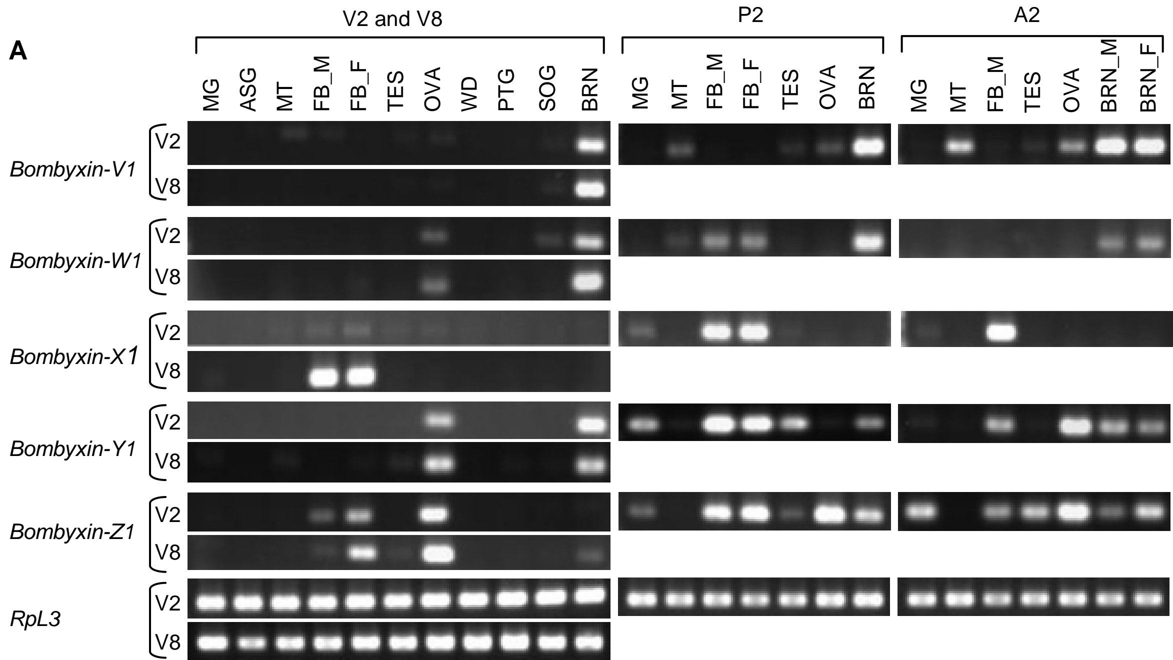


Figure 3



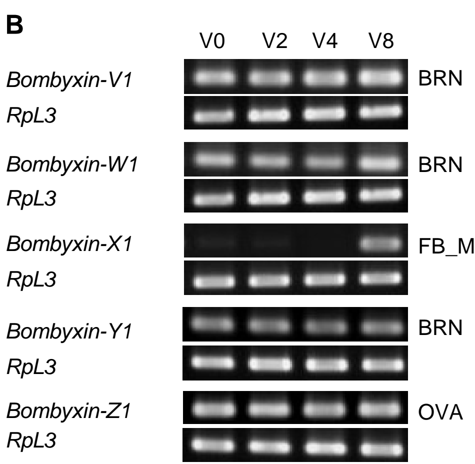


Figure 4