

Full Length Research Paper

Isolation and characterization of a PUF-domain of pumilio gene from silkworm *Bombyx mori*

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Pumilio is a sequence-specific RNA-binding protein that binds to target mRNA to repress its translation. The PUF-domain, the RNA-binding motif of pumilio, is highly conserved across species. In the present study, a partial pumilio gene with complete PUF-domain in *Bombyx mori* has been cloned using 3' and 5' RACE for the first time, designated as *BmPUM*. The sequence of *BmPUM* has been registered in GenBank under the accession number FJ461590. Comparative sequence analysis revealed that the deduced protein *BmPUM* contains a PUF-domain and shares 83% identity with *Drosophila pumilio*, hence belongs to pumilio family. One encoding sequence of *BmPUM* fragment was successfully expressed in *Escherichia coli*. Western blotting indicated that the anti-BmPUM antibody and anti-*Drosophila pumilio* antibody all could specifically detect *BmPUM* expressed in prokaryotic cells. The tissue expression pattern performed by real-time PCR and western blotting demonstrated that *BmPUM* expressed in various tissues, especially in testis and ovary. Those data collectively indicated that *BmPUM* belongs to the extremely high conserved RNA-binding domain. Conserved function of pumilio protein in invertebrates and vertebrates suggested that *BmPUM* could also play an important role in the proliferation of silkworm germline stem cell.

Key words: *Bombyx mori*, pumilio, PUF-domain, RACE, germline stem cell.

INTRODUCTION

Pumilio gene encodes a protein that binds to specific sequences in the 3' untranslated region (3'UTR) of relevant mRNA to act as a translation repressor. Pumilio proteins are characterized by their RNA binding motif, which is also called PUF-domain. The typical character of the PUF-domain is the presence of the eight tandem repeats, called Puf repeats, and each repeat is approximately 36 amino acids (Macdonald, 1992; Zamore et al., 1997; Zhang et al., 1997; Wharton et al., 1998). The entire cluster of Puf repeats is essential for RNA binding (Zamore et al., 1997; Zhang et al., 1997; Wharton et al., 1998) and protein-protein interaction (Edwards et al., 2000). To a large extent, expression of PUF domain alone is sufficient to rescue the defects of a PUF protein mutant (Wharton et al., 1998). The PUF protein family with its members is found conserved in many organisms,

including plants, animals, and microbes (Barker et al., 1992; Zamore et al., 1997; Zhang et al., 1997).

PUF proteins involve in diverse biological processes. In *Drosophila*, the posterior patterning of the early embryo is governed by Pumilio co-working with nanos in the repression of hunchback mRNA (Barker et al., 1992; Murata and Wharton, 1995). In Pumilio mutant embryo, hunchback mRNA is expressed uniformly throughout the posterior of the embryo and can not form anterior-to-posterior concentration gradient, as a result, the embryo develops no abdominal segment (Barker et al., 1992). Cyclin B is another target mRNA of Pumilio, by repressing translation of cyclin B messenger RNA, Pumilio can inhibit primordial germ cells enter into mitosis (Asaoka-Taguchi et al., 1999). Other studies demonstrate that *Drosophila pumilio* is also required for maintenance of germline stem cells and germline migration. In *Caenorhabditis elegans*, PUF protein also has multiple functions. For example, *C. elegans* FBF participates in hermaphrodite switch from spermatogenesis to oogenesis through its repression of *fem-3* mRNA and physical

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interaction with nanos (Kraemer et al., 1999). FBF also controls germline stem cells by repressing *gld-1* mRNA. In an FBF mutant, germline proliferation is initially normal, but stem cells are not maintained (Luitjens et al., 2000). Besides the function we discussed above, *C. elegans* PUF protein also required for germline survival, germline migration and germline mitotic arrest during embryogenesis (Kraemer et al., 1999; Subramaniam and Seydoux, 1999). Studies on *S. cerevisiae* find that PUF proteins are related with aging, mating-type switching and mitochondrial function. Thus, the conserved biochemical features and genetic function of PUF protein family members have identified from studies of model organisms.

Whereas the biological function of pumilio proteins have been gradually revealed in other organisms, there are scarcely related reports about pumilio gene in *Bombyx mori*. In the present study, we have therefore cloned and expressed a fragment of *BmPUM* for the first time and produced the antiserum to verify this gene in *B. mori*. In addition, bioinformatics tools were also used to analyze this gene, including conserved domain analysis and phylogenetic analysis. Through these analyses, we obtained primary information about this gene, which will provide a new insight into the further studies.

MATERIALS AND METHODS

Animal materials

The 306 strain of *B. mori* was inbred in our lab. All used tissues were separated from feeding fifth instar larva at the 5th day of the silkworm and used in RNA extraction and protein extraction immediately.

RNA extraction and cDNA synthesis

Total RNAs of different tissues including ovary, testis, fat body, silk gland, midgut, Malpighian tubules and blood-lymph were extracted using TRIzol Reagent (Invitrogen™ Co). Every step was performed according to the manufacturer's instruction. cDNAs were synthesized by using M-Mlv Reverse Transcriptase. The quality and concentration of RNA sample was examined by ethidium bromide (EB)-stained agarose gel electrophoresis and Gene spec III (Naka Instruments Co., Ltd.).

Pumilio gene cloning by RACE

Total RNA was extracted from the silkworm testis with TRIzol reagent (Invitrogen™ Co). Every step was performed following the instruction manual. Finally, the total RNA inspected with Gene spec III (Naka Instruments Co., Ltd.). The first-strand cDNA was synthesized by superscript III (Invitrogen™ Co). An expression sequence tag (GenBank no AU002534) was obtained by using *Drosophila pumilio* protein blast in NCBI (<http://www.ncbi.nlm.nih.gov/>) EST database. Then PCR was performed using the primers were designed to amplify this EST. The PCR product was purified and sub-cloned into pMD18-T vector followed by transformed *Escherichia coli* (TG1 strain). Plasmid was purified, the sequencing was performed using an automatic sequencer: CEQ8000 (Beckman Company).

The 3' end of the pumilio cDNA from testis was performed with 3' RACE. The first-strand cDNA was synthesized from 2 ug of total RNA using a cDNA synthesis primer AP (5'-GGCCACGCGTCTGACTAGTAC (T)₁₆-3'). The first round of PCR was performed with gene specific primer S1 (5'-gtcaagtgtacgctgtcc-3') and AP primer, then nested PCR was performed using 2 ul of the first PCR product with gene specific primer S2 (5'-atgccacacagaccaactc-3') and the abridged universal amplification primer (AUAP, 5'-GGCCACGCGTCTGACTAGTAC-3'). The PCR product was purified and sub-cloned into pMD18-T vector followed by sequencing.

5' RACE method was introduced to obtain the 5' end of the pumilio gene. Based on the only homology EST (expression sequence tag), two gene specific primers A1 (5'-cgtgtccgtcgagtttctg-3') and A2 (5'-atagactcgagcgccttctg-3') were synthesized and subsequently used to amplify the 5' end of pumilio gene. The first-strand cDNA for 5' RACE was synthesized followed by tailing cDNA with oligo (C). The first round of PCR was performed with A1 primer and Abridged Anchor Primer (AAP, 5'-GGCCACGCGTCTGACTA GTACGGIIGGGIIGGGIIG-3'). The first round PCR product was diluted 50-fold for nested PCR with the second round of amplification with A2 and AUAP. Under the same condition performed in the first round of PCR, the 5' RACE product was purified and sub-cloned into pMD18-T vector followed by sequencing.

Sequence comparison, protein prediction and phylogenetic analysis

Sequence assembling was carried out with DNASTar. Protein deduction, blasting, and domain prediction were performed at NCBI (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) and ExPASy (<http://expasy.org/tools/dna.html>), respectively. The protein sequence similarity analysis was dealt with ClustalW and BOXSHADE. Phylogenetic tree was made by ClustalW software.

Protein expression, purification and polyclonal antiserum production

One encoding fragment (603bp) of pumilio protein was amplified from testis cDNA using a sense primer 5-GCGGATCCGAGTCTATCCCGCCTGAACA -3 (containing the BamHI site) and an antisense primer 5-GGGAAGCITTCGGTCCGATCTTGTGCAT -3 (containing the HindIII site). The PCR product was cloned to pMD18-T vector and then transformed into *E. coli* (TG1). The positive clones were verified by digesting with BamHI and HindIII restriction enzymes. Then the digested fragment was subcloned into the pET30a expression vector and transformed into *E. coli* (BL21). Positive clones were confirmed by restriction digestion. For expression of recombinant protein, a positive clone was cultured in LB medium supplied with Kanamycin (50 µg/ mL) at 37°C with shaking to A₆₀₀ about 0.6. The culture was then induced with IPTG (final concentration of 0.2 mmol/ L) and further cultured at 37°C. At last, 12% SDS polyacrylamide gel was used to analyze the recombinant protein. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R250 to visualize the protein bands.

To purify pumilio-6His fusion protein, the induced bacteria was collected, lysed by sonication, and the products were purified using the Ni resin (QIAGEN, USA), and verified by SDS-PAGE. At last, the purified recombinant protein was used to immunize New Zealand white rabbits to raise polyclonal antibodies. Antisera were tested by western blot using recombinant proteins and tissue homogenates.

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were prepared from silkworm tissues (ovary, testis, fat body, silk gland, midgut, Malpighian tubules and blood-lymph). Two microgram of total RNA was reverse-transcribed with Oligo (dT) 12-18 (500µg/ml) and M-MLV reverse transcriptase (Takara) in a total volume of 20µl at 42°C for 60 min. Real-time quantitative PCR was performed with SYBR[®] Premix Ex Taq[™] kit (Takara) and the amplification was detected using the STRATAGENE Mx 3000P[™] (USA) according to the manufacturer's protocol. Pumilio-F (CTCGCCAACCACATCGTC) and Pumilio-R (TGTTGTTTCAGGCGGGATAG) was used as primers. The RT-PCR reaction for the housekeeping gene *Bm-Actin* (GenBank Acc. No NM_001126254) was also performed as an internal control.

Western blotting

Silkworm tissues were homogenized in lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM TRIS-HCl pH 7.4) containing four kinds of protease inhibitors: PMSF (35 µg/ml); EDTA (0.3 mg/ml); Pepstatin (0.7 µg/ml); Leupeptin (0.5 µg/ml). The supernatant was saved after tissue lysate being centrifugated at 12,000 g for 10 min, and the protein concentration was determined with the Bradford' method (Bradford, 1976). Protein samples (60 µg protein) were subjected to SDS-polyacrylamide gel electrophoresis (10% gel). After the protein samples were transferred onto PVDF membrane, the membrane was incubated with polyclonal antibody (1:1,000 dilution) which raised against the PUF-domain of *Drosophila Pumilio* (Macdonald, 1992) and BmPUM. Since the amino acid identities of the PUF-domain between *Drosophila Pumilio* and BmPUM had exceeded 80%, we strongly expected that the antibody would recognize BmPUM with similar sensitivity. Antibody detection was accomplished by incubation membrane with horseradish-peroxidase-conjugated secondary antibody (1:1,000). The peroxidase activity was developed with 0.1% H₂O₂ and 3, 3'-diaminobenzidine (DAB) as a chromogenic substrate.

RESULTS

Isolation of *Bombyx mori* pumilio cDNA

Since PUF-domain was strictly conserved, the protein sequence of *Drosophila Pumilio* was used as the probe to blast in NCBI, and the only cDNA fragment (GenBank no AU002534) of *B. mori* with high homology was obtained and initially amplified as the core sequence. Sequence alignment showed that this core sequence possesses 84% homology with *Drosophila Pumilio* gene implying that it was probably a partial sequence of pumilio gene. Then a 1,543 bp cDNA fragment was obtained by 3' RACE. We cloned this fragment to pMD18-T vector and then sequenced it. The obtained sequence was found overlap 50 bp with the core sequence, following the analysis by DNAstar software. Meanwhile, 5' RACE produced a 377 bp fragment with 75 bp overlap with the core sequence. Assembled by DNAstar software, the core sequence, 3' RACE and 5' RACE fragments were formed into a new cDNA sequence with 2,174 bp containing a complete conserved PUF-domain. This transcript was designated as BmPUM. After being confirmed

by amplifying and sequencing, this sequence was submitted to GenBank (Accession number. FJ461590).

Sequence analysis

Blasting in NCBI, transcript of BmPUM was found to contain a partial open reading frame (ORF) of 1095 bp encoding a peptide of 365 amino acids (Figure 1). The deduced protein sequence, designated BmPUM, was predicted to have a strictly conserved domain of pumilio possessing 84% homology with *Drosophila pumilio* protein and 85% with *Apis mellifera*.

Analysis with SMART software has revealed that the deduced amino acid sequence of the BmPUM shares the following features: a conserved Pumilio homology domain (PUM-HD) of 359-amino-acid long, which includes eight tandem imperfect repeats of 36 amino acids (Figure 1). With the exception of repeat 8, which is separated by a spacer of seven amino acids from repeat 7, the remaining Puf repeats are immediately adjacent to the previous one (Figure 1).

ClustalW alignment of the *B. mori* and *Drosophila Pumilio* homology domain showed that they share very high degree of homology and evolutionary conservation (82% identity; 89% similarity), with highly conserved protein sequence and structure of the Pumilio RNA-binding domain (Figure 2). Like *Drosophila*, *B. mori* PUM-HD also consisted of an N-terminal conserved part of 20 amino acids, eight imperfect repeats of 36 amino acids each, and a C-terminal conserved region. The only difference between *Drosophila pumilio* and BmPUM is the C-terminal part which is 44 aa long in *B. mori* while *Drosophila pumilio* is 131 aa long (Figure 2).

Based on the characteristics possessed by PUF family (Barker et al., 1992; Zamore et al., 1997; Zhang et al., 1997), the isolated BmPUM cDNA was predicted to encoded a PUF protein, which was also supported by the result of western blot (Figure 5B, 7).

Molecular evolution analysis of BmPUM protein

Database search was performed in the NCBI and fourteen proteins that contain Puf repeats and thus belong to pumilio protein family were picked. Proteins from animals [*Aedes aegypti* (XP_001656036), *Apis mellifera* (XP_391849), *Drosophila melanogaster* (NP_731316), *Strongylocentrotus purpuratus* (XP_794621), *Homo sapiens* (NP_056132), *Mus musculus* (BAE24797), *Oncorhynchus mykiss* (NP_001117710), *Xenopus laevis* (BAB20864), *Dugesia japonica* (CAG25892), *C. elegans* (NP_508980)] plants [*Arabidopsis thaliana* (NP_180483), *Oryza sativa* (NP_001044782)] and microbes [*D. discoideum* (AAD39751), *S. cerevisiae* (NP_013088)] were compared with BmPUM and analyzed by ClustalW and BOXSHADE software. The comparative analysis of the set of 15

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gacaagccggcggcggggcgctegcgctctgctegaggacttccggaacaaccgcttcccg
D K P A A G R S R L L E D F R N N R F P
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S R F V Q Q K L E R A T V Q E K Q M V F
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Q Y G N Y V V Q H V L E H G A G E D R S
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R L V A A V R G K V L Q L S Q H K F A S
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G A V L stop
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cattaatataaaataagcattctcgtataataatttacaatgataaaaaaaaaaaaaaaaaaaaa
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Figure 1. Complementary DNA sequence and deduced amino acid sequence for the BmPUM. The partial BmPUM transcript encodes a 365 aa protein. The eight Puf repeats are shaded by dark gray and light gray. The nucleotide sequence reported here appears in the GenBank databases under Accession No.FJ461590.

proteins revealed that the PUM-HD demonstrates a high level of evolutionary conservation (Figure 3). Interestingly, 9 out of 15 proteins compared (*B. mori*, *A. aegypti*, *A. mellifera*, *D. melanogaster*, *S. purpuratus*, *H. sapiens*, *M. musculus*, *O. mykiss*, *X. laevis*) showed high degree of evolutionary conservation (identity is exceed 75%) of the amino acid sequence and structure of the PUM-HD, with similar spacing between Puf repeats and conserved

core consensus sequence of each repeat (Figure 3). In contrast, the 6 remaining proteins (*D. japonica*, *C. elegans*, *A. thaliana*, *O. sativa*, *D. discoideum*, *S. cerevisiae*) differed from the other nine (Figure 3).

The evolutionary relationships among BmPUM and other PUF proteins have been figured out as the form of a phylogenetic tree based on the deduced amino acid sequences (Figure 4). The result showed that pumilio

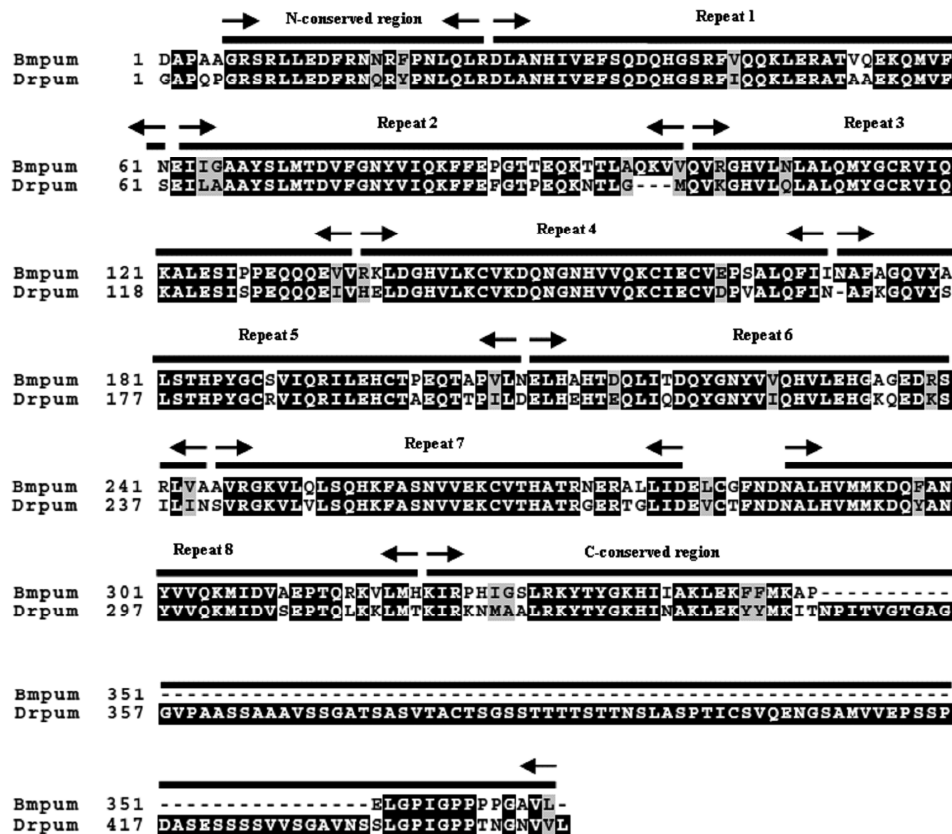


Figure 2. Comparative sequence analysis of BmPUM and *Drosophila pumilio* protein by ClustalW and BOXSHADE software. The amino acid sequence shows very high degree of homology and evolutionary conservation (82% identity, 89% similarity).

proteins from animals, plants, microbes can be classified into three clusters that probably represent divergent subfamilies. The first cluster contains *S. purpuratus* only. The second cluster is further divided into two subgroups; the first subgroup is comprised of *D. japonica*, *C. elegans*, *A. thaliana*, *O. sativa*, *D. discoideum*, *S. cerevisiae*. The second subgroup consists entirely of insect pumilio proteins (*B. mori*, *A. aegypti*, *A. mellifera*, *D. melanogaster*). The third cluster consists of vertebrate: amphibians (*X. laevis*), fishes (*O. mykiss*), and mammals (*H. sapiens*, *M. musculus*).

Protein expression, purification and polyclonal antiserum production

The partial coding sequence (603 bp) for BmPUM was cloned into the pET30a expression vector and the resulting construct was used to transform *E. coli* strain BL21 (DE3), a high level expression fusion protein of about 24 kD was obtained (Figure 5A), which was consistent with the expected molecular mass of the fusion protein of pET30a/BmPUM. The recombinant protein was purified using Ni resin according to the protocol (Figure 5A), and the purified protein was then

provided for antisera production.

The specificity of the antiserum for BmPUM was tested by western blotting analysis. The result showed that the antiserum can specifically detect the recombinant protein (Figure 5B), and also can specifically identify BmPUM from the tissue proteins (Figure 7).

Tissue expression pattern of BmPUM

To investigate expression pattern of BmPUM in different tissues, total RNAs (2 µg/sample) of different tissue samples from fifth instar larva at the 5th day of the silkworm were used as templates to detected BmPUM mRNA transcript levels by Real-time PCR. The result showed that BmPUM mRNA was detected in all tissues examined (Figure 6). Interestingly, BmPUM was expressed much more intensively in ovary and testis than in other tissues (Figure 6).

Western blotting analysis was then performed to examine the tissue distribution and expression level of BmPUM. Anti-BmPUM polyclonal antibody raised against the PUF-domain gave a specific signal in all the tissues examined (Figure 7). In addition, signals in ovary and testis were stronger than that in the other tissues indicat-

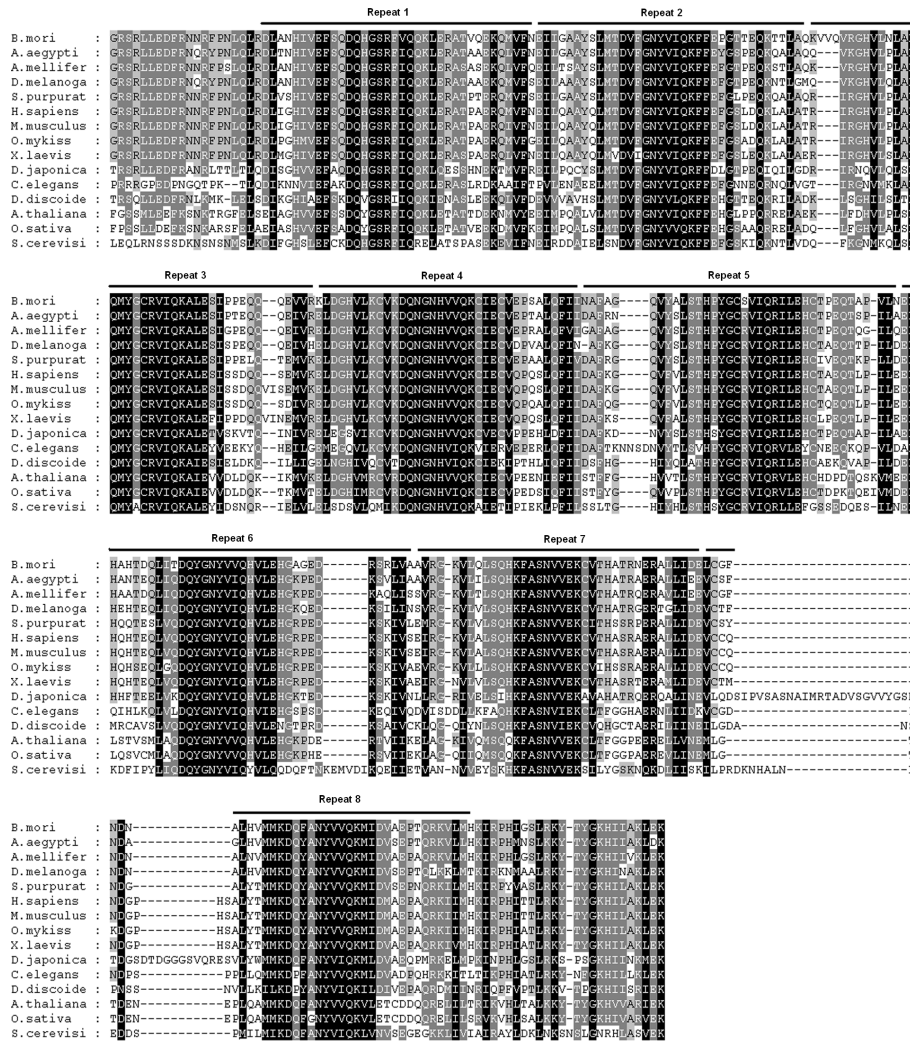


Figure 3. Multiple amino acid sequence alignment of PUM-HD located in pumilio with the other homologous deposited in NCBI databases with ClustalW and BOXSHADE software. The Puf repeats are indicated with dark straight line. The conserved residues are shaded in thick and in grey. The conserved Puf repeats are marked above the alignment.

ing its higher expression levels in ovary and testis (Figure 7), which is consistent with the levels of BmPUM mRNAs.

DISCUSSION

Members of Pumilio family of RNA-binding proteins repress translation through recognition of the target mRNAs by the PUF domain. Studies of pumilio in *D. melanogaster*, *C. elegans* and *S. cerevisiae* have shown that pumilio proteins are sequence specific RNA-binding proteins capable of recognizing specific sequence in the 3' UTR of the target mRNAs and acting as translational repressors during development and differentiation (Murata and Wharton, 1995; Zhang et al., 1997; Asaoka-Taguchi et al., 1999; Kraemer et al., 1999; Olivas, and Parker, 2000; Sonoda and Wharton, 2001; Tadauchi et

al., 2001; Crittenden et al., 2002).

We have isolated a cDNA clone for *BmPUM* for the first time. Partial nucleotide sequence of *BmPUM* was obtained by 3'RACE and 5'RACE for the complex hairpin structure which was probably caused by rich GC content in the 5' region. The results indicated that, like other species, the product of the *BmPUM* mRNA possesses a typical PUF-domain and is expected to function as a translational repressor. Wickens et al. (2002) found that PUM-HD is the only conserved region among invertebrate and vertebrate Pumilio family members (Wickens et al., 2002). Before Wickens et al. (2002) had also pointed out PUM-HD alone is sufficient for the function of Pumilio in *Drosophila* (Wharton et al., 1998).

Comparison of *BmPUM* and *Drosophila pumilio* showed that their PUF-domain part has 83% homology demonstrating a high degree of evolutionary conservation

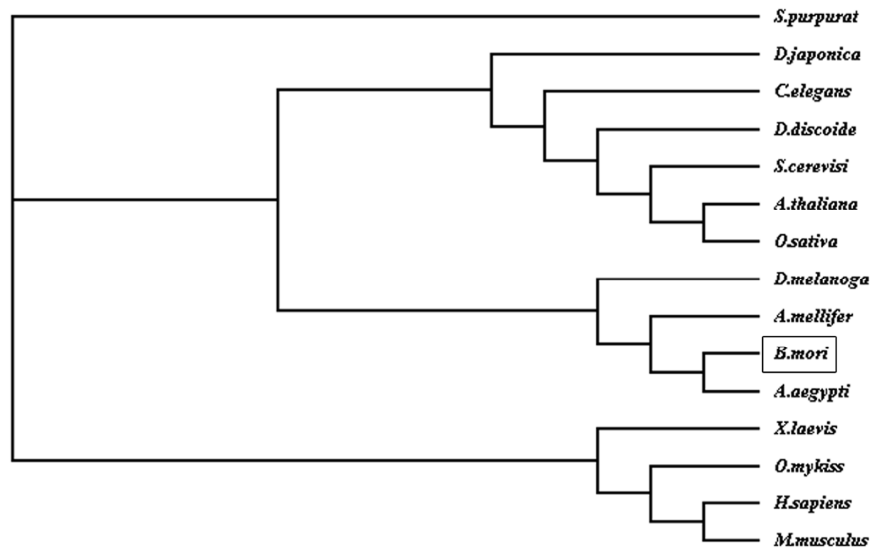


Figure 4. Phylogenetic relationships of PUM-HD in plants, animals and microbes. The analysis is performed with ClustalW software. BmPUM is marked with a blank box.

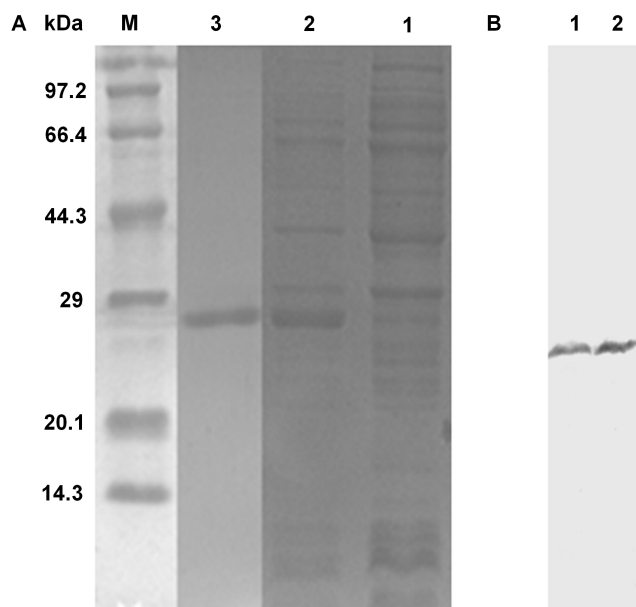


Figure 5. (A) SDS-PAGE of fusion protein. M. Protein marker; lane 1, Protein of *E. coli* BL21 carrying pET30a induced by IPTG; lane 2, Protein of *E. coli* BL21 carrying pET30a/BmPUM induced by IPTG; lane 3, purified His/ BmPUM fusion protein. (B) Western blot analysis of *E. coli* expressed protein using the anti-BmPUM antibody and anti-Drosophila pumilio antibody. Lane 1, *E. coli* expressed BmPUM fusion protein detected by anti-BmPUM antibody; lane 2, *E. coli* expressed BmPUM fusion protein detected by anti-Drosophila pumilio antibody.

of the pumilio RNA-binding domain structure. In addition, comparative analysis of a set of pumilio proteins from animals, plants and microbes also showed a high level of evolutionary conservation, suggesting that PUM-HD is an ancient protein domain. On the other hand, the comparison also revealed there are at least three evolutionarily

divergent groups within the pumilio family, and BmPUM belongs to a group that is represented by prototypical *Drosophila Pumilio*, which encompasses other invertebrate PUF proteins.

Our real-time PCR result and Western blotting analysis indicated that BmPUM is expressed in all tissues examined. The most significant difference was its relatively higher expression levels in the ovary and testis. This observation may well be attributed to the suggested roles of the pumilio protein in the development of germline stem cell (Moore et al., 2003; Bachorik and Kimble, 2005; Fox et al., 2005; Szakmary et al., 2005; Urano et al., 2005). In addition, widespread tissue expression of the BmPUM was also similar to *Drosophila Pumilio*. Recent reports demonstrated that besides germline, *pumilio* mutations in *Drosophila* affect other tissues as well (Forbes and Lehmann, 1998; Schweers et al., 2002). This is consistent with the fact that in *Drosophila* expression of *pumilio* is not restricted to the germline. Based on those research results, we inferred that BmPUM was probably involved in the control of the asymmetric division and cell fate specification in a variety of tissues.

In conclusion, BmPUM belongs to evolutionarily conserved ancient family of RNA-binding proteins. Studies of invertebrate and vertebrate species had shown that pumilio proteins are structurally and functionally conserved suggesting BmPUM could also play an important role in translational regulation of embryogenesis as well as cell development and differentiation (Crittenden et al., 2002; Wickens et al., 2002). On the basis of series observations, it was recently suggested that an ancestral function of Pumilio proteins is to support mitotic proliferation of stem cells (Wickens et al., 2002). Silkworm is an important economic insect and is regard-

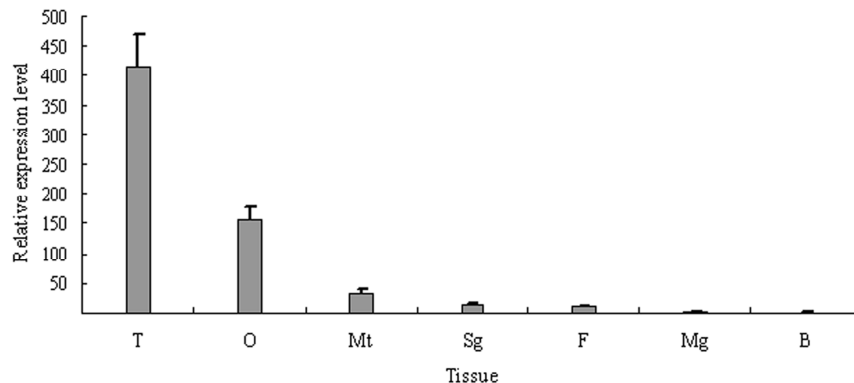


Figure 6. Expression profiles of BmPUM in *B. mori* day 3 fifth instar larvae. Real-time PCR analysis were performed using total RNA from testis (T), ovary (O), fat body (F), Malpighian tubules (Mt), silk gland (Sg), midgut (Mg), blood-lymph (B). *BmActin* was used as an internal control. The data show means \pm standard errors (N = 3).

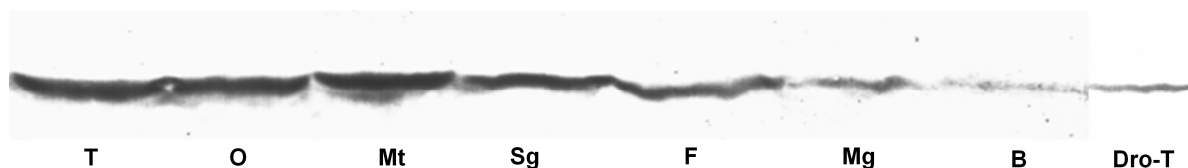


Figure 7. Western blot analysis of tissue expression of *Bombyx mori*. Lysates prepared from the indicated *Bombyx mori* tissues were electrophoresed on 10% acrylamide gel and then blotted onto PVDF membrane. The membrane was probed with anti-BmPUM antibody and anti-Drosophila pumilio antibody. O (Ovary); T (testis); Mt (Malpighian tubules); Sg (silk gland); F (fat body); Mg (midgut); B (blood-lymph); Dro-T (testis protein was detected by anti-Drosophila pumilio antibody)

ed as a model insect of Lepidoptera. The presence of BmPUM in the germ cell must have relationship with the development and economic trait of silkworm. Thus, functional characterization of BmPUM should be elucidated to further understand conserved molecular mechanisms that regulate asymmetric division and self-renewal of stem cells.

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