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Cloning and Alternative Splicing Analysis of *Bombyx mori* Transformer-2 Gene using Silkworm EST Database

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Abstract We have identified *Bombyx mori* transformer-2 gene (*Bmtra-2*) cDNA by blasting the EST database of *B. mori*. It was expressed in the whole life of the male and female silkworm and was observed as a band of 1.3 kb by Northern blot analysis. By comparing corresponding ESTs to the *Bmtra-2* DNA sequence, it was revealed that there were eight exons and seven introns, and all splice sites of exons/introns conformed to the GT/AG rule. *Bmtra-2* pre-mRNA can produce multiple mRNAs encoding six distinct isoforms of BmTRA-2 protein using an alternative splicing pathway during processing. Six types of *Bmtra-2* cDNA clones were identified by reverse transcription-polymerase chain reaction. All isoforms of BmTRA-2 protein contain two arginine/serine-rich domains and one RNA recognition motif, showing striking organizational similarity to *Drosophila* TRA-2 proteins.

Key words EST database; transformer-2 gene; *Bmtra-2*; alternative splicing; gene clone; *Bombyx mori*

The transformer-2 gene (*tra-2*) that encoded a pre-mRNA splicing protein for sex differentiation was first cloned in *Drosophila* [1–3]. Genetic studies have shown that the *tra-2* gene plays a key role in the metazoan sexual differentiation regulatory cascade in *Drosophila*. The female-specific transformer protein (TRA) functions in combination with TRA-2 proteins to direct female-specific doublesex gene (*dsx*) pre-mRNA splicing [4–6]. The *tra-2* gene has also been discovered in mammals, chickens and insects as an mRNA splicing factor [7–9]. It encodes a pre-mRNA splicing protein that consists of two arginine/serine-rich domains (RS domains) and one ribonucleoprotein (RNP) type RNA binding domain, also identified as the RNA recognition motif (RRM) [10–12].

Here we cloned the *Bombyx mori* transformer-2 gene (*Bmtra-2*) by blasting its expressed sequence tag (EST) database and using the DNA sequencing approach [13, 14], and found it could produce six alternatively spliced mRNAs encoding six isoforms homologous to *Drosophila* TRA-2.

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Materials and Methods

Silkworm strain

Silkworm strain p50 was donated by the Sericultural Research Institute, Zhejiang Academy of Agricultural Sciences (Hangzhou, China). Their sexes were distinguished by detecting the W chromosome-specific retrotransposable element (W-Samurai; GenBank accession number AB012905) with primers Samurai-1B and Samurai-2B [15].

B. mori EST database blasting with RRM

The cDNA sequence of *B. mori* that encoded an amino acid peptide containing the RRM was selected as a probe to blast the *B. mori* EST database (http://www.ncbi.nlm.nih.gov/dbEST/) for homologous clones, using the BLAST 2.1 program (http://www.ncbi.nlm.nih.gov/blast/). This approach led to the identification of an EST clone, CK496349. The deduced amino acid sequence of CK496349 has an RRM domain. This EST clone was used for further analysis by selecting the corresponding ESTs

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from the blast output and extending these ESTs to a new contig for further cycles of EST blasting. We continued to recycle the contig and blast the ESTs until no new ESTs were identified to the extended sequence [16]. In the relative identified ESTs, there were many alternatively spliced isoforms. The primer pairs listed in **Table 1** were designed according to these EST sequences.

Cloning of *Bmtra-2* DNA sequence

The genomic DNA was extracted from silkworm posterior silk glands with the phenol-chloroform extraction method and was used as the template for polymerase chain reaction (PCR) with the primer pairs listed in **Table 1**. PCR products were cloned into a T-A cloning site of pMD-T vector (TaKaRa, Dalian, China) and sequenced. These sequenced fragments were joined without any intervening sequence. The *Bmtra-2* DNA sequence was obtained.

The *B. mori* genomic database (http://www.ncbi.nlm.nih.gov/BLAST/Genome/Insects.html) was blasted with the cloned *Bmtra-2* gene. The genomic clones that were aligned with the *Bmtra-2* DNA sequence were identified and joined. The sequence upstream of the *Bmtra-2* gene was obtained and put into the promoter website (http://thr.cit.nih.gov/molbio/proscan/) to search the promoter regions and putative transcription factor-binding (TFB) sites to investigate its transcriptional regulation.

Analysis of alternative splicing of *Bmtra-2* pre-mRNA by reverse transcription (RT)-PCR

Total RNA was separately extracted from five kinds of tissues of 3-d fifth instar male and female larvae using EASYPrep RNA (TaKaRa): the fat body, Malpighian tubule, silk gland, testis or ovary. Poly(A)$^+$ RNA was isolated from the fat body using a Micro-FastTrack 2.0 mRNA isolation kit (Invitrogen Corp., California, USA). The first-strand cDNA synthesis was performed with an oligo(dT) primer (5′-TTTTTTTTTTTTTTTTTTXX-3′). PCR reactions were done for testing the alternatively spliced mRNAs with the primer pairs listed in **Table 1** and the LA RNA PCR kit (TaKaRa). PCR products were purified on a 1.5% agarose gel, and cloned into a T-A cloning site of pMD-T vector and sequenced. All procedures were carried out according to the protocol provided by the manufacturer.

Northern blot hybridization

Total RNA was isolated separately from the male and female silkworms at different stages by EASYPrep RNA: egg, larval, pupal and adult. For Northern blot hybridization, total RNAs were subjected to electrophoresis on a 1.2% agarose gel in the presence of 2.2 mM formaldehyde and transblotted onto a nylon membrane. The membrane was prehybridized and hybridized with the *Bmtra*-2 cDNA probe labeled with digoxigenin (Roche Corp., Mannheim, Germany) and detected with Ap-anti digoxigenin according to the manufacturer's instructions.

Results

In silico cloning of *Bmtra-2* cDNA by blasting silkworm EST database

It has been reported previously that TRA-2 is an RNA binding protein containing an RRM. In the *B. mori* EST

Table 1 Primers used in this study

Purpose	Primer	Sequence
Cloning *Bmtra-2* gene genomic DNA	TF1	CGTGCGGTCGCGTCTGTAGATTG
	TR1	TGCGACGACCGTCAATTTCCAT
	TF2	GCAAAGAATGAATGCACCGGAATG
	TR2	CTATTCGGTTGAGGTCAGCGTCG
	TF3	GTTATTAGTAAGTTCGCGACGC
	TR3	TAGTCGCTCACAAGTTACCACTAC
Producing the probes for Northern blot hybridization	F	ACTACGAACATCGCTATGTCTG
	R	GGTAGTAGTAGTCGTCTCTGTC
Testing the alternatively spliced *Bmtra-2* mRNA	L	GCAGAAAATATAGAAGCCCCATG
	M	GCAGAAAATATAGAAGCCCGACA
	S	GCAGAAAATATAGAAGCCCATC
	R1	CTAAGTTTACATTCTAGAGGCGTG
	R2	GTCGGAAGATAAAGAATATGATC
Distinguishing the sex of silkworms	Samurai-1B	CCAACTAATATCTTCACCCTCG
	Samurai-2B	CCTGGTCGGAAGCCAGACTGGTAA

The primers were designed according to the sequences of putative *Bombyx mori* transformer-2 gene (*Bmtra-2*) ESTs.

database, there was a clone, CK496349, with strong similarity to RRM in its deduced amino acid sequence. It was used as the probe for further analysis by electing the corresponding ESTs from the blast output and extending to a new contig for further cycles of EST blasting. A total of 27 ESTs were obtained, as shown in **Table 2**. These ESTs can be assembled to many contigs with a complete open reading frame encoding proteins that contain two RS domains at each end and the same RRM found in CK496349. The seven-glycine (G) region was located between the RRM and the C-terminal RS domain. One isoform was PA, the nucleotide and deduced amino acid sequences of which are shown in **Fig. 1**. The overall organization of these proteins was similar to the *Drosophila* TRA-2[264] and

Table 2 GenBank accession number, score (a match between two sequences) and E value (the random background noise that exists for matches between sequences) of *Bombyx mori* ESTs identified to putative *B. mori* transformer-2 gene (*Bmtra-2*) cDNA sequence

Bombyx mori EST	Score (bits)	E value
CK500353	1199	0.0
BP180739	1025	0.0
BP180462	1013	0.0
CK496349	916	0.0
AV405806	886	0.0
BP183601	882	0.0
CK519526	864	0.0
CK517820	862	0.0
CK560418	781	0.0
CK547947	728	0.0
CK549959	722	0.0
CK548460	718	0.0
CK549244	716	0.0
CK550574	712	0.0
CK492807	712	0.0
CK543632	702	0.0
AV399252	702	0.0
CK492266	688	0.0
CK547249	656	0.0
AU006051	642	0.0
CK512485	622	e-177
CK562230	613	e-174
CK490326	601	e-170
CK548747	515	e-145
CK540533	507	e-142
CK509207	412	e-114
CK549760	357	3e-97

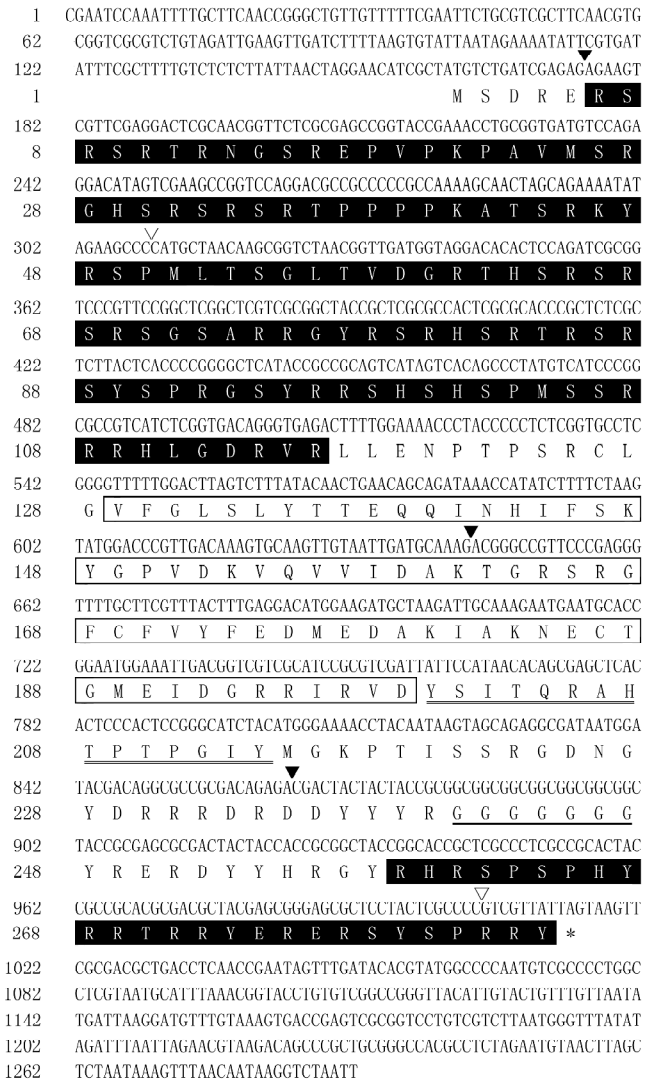

Fig. 1 Nucleotide and deduced amino acid sequences of *Bombyx mori* transformer-2 gene (*Bmtra-2*) cDNA (PA isoform) cloned by blasting *B. mori* EST database

Both strands were completely sequenced. Serine/arginine-rich domains (white text in black boxes) and an RNA recognition motif (black text in an open box) are shown. The linker region is double underlined and the glycine-rich region has a thick underline. *, termination codon; ▼, sites of introns; ▽, intron sites with alternative splicing acceptor sites.

to many other RNA binding proteins. PA showed 80% homology to that of *Apis mellifera* (GenBank accession number XP_396858), 68% to that of *Bactrocera oleae* (CAD67988), 65% to that of *Drosophila virilis* (AAB58113), 64% to that of *Musca domestica* (AAW34233), 60% to that of *Drosophila melanogaster* (AAA28953) and 58% to that of *Anopheles gambia* (EAA13826) (**Fig. 2**).

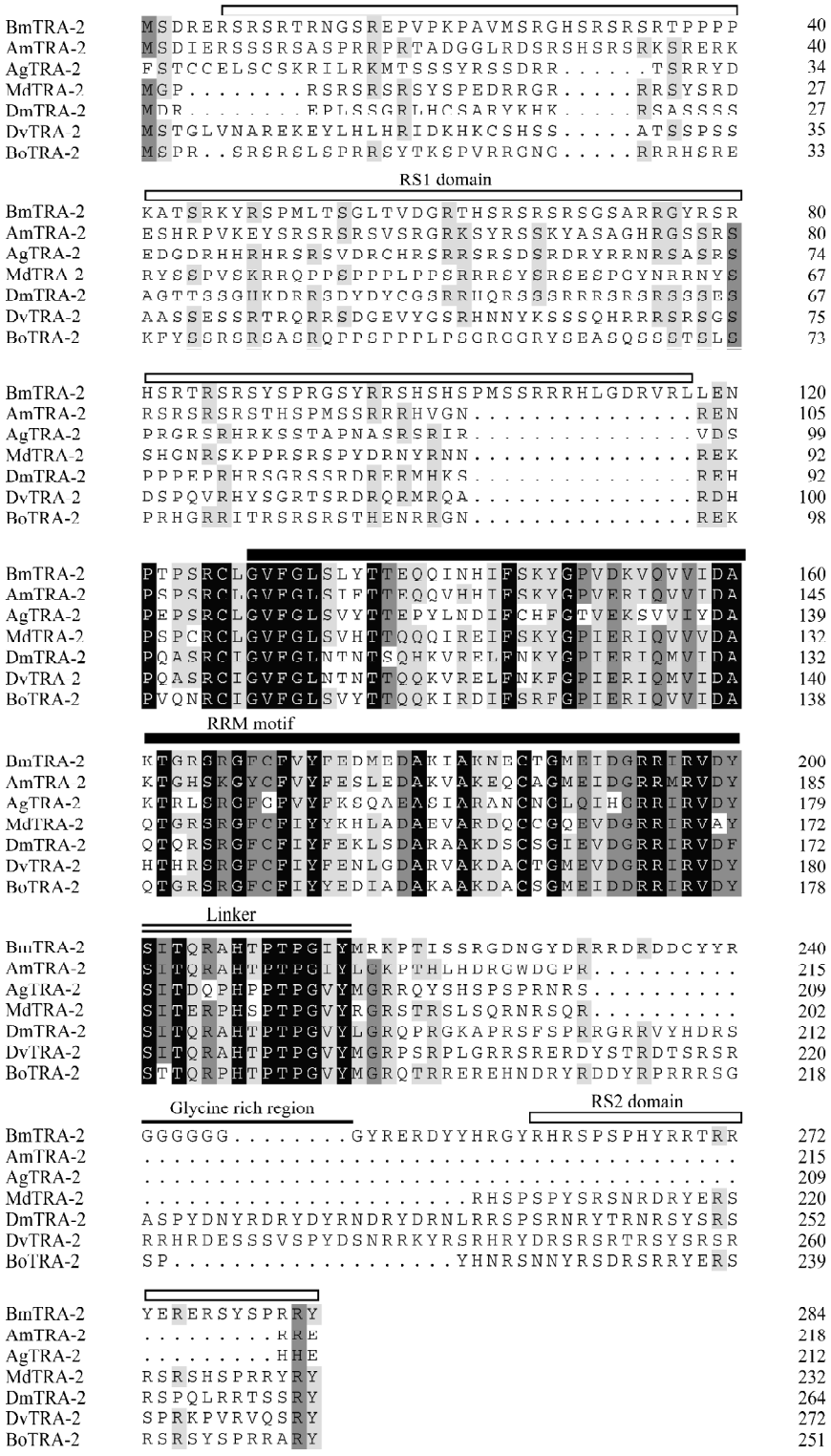

Fig. 2 Alignment of amino acid sequences of *Bombyx mori* transformer-2 gene (*Bmtra-2*) (PA isoform) and its homologs

The deduced amino acid sequence of the *Bmtra-2* gene was aligned with its homologs of other insects using Clustal X (DDBJ, Japan) with the default settings. Conserved residues in all homologs are shaded in black boxes and partly conserved residues in gray boxes. The organization of BmTRA-2 is shown: the RNA recognition motif RRM is indicated by a black box; the arginine/serine-rich (RS) domains are indicated by open boxes; the linker region is indicated by double lines; the glycine-rich region is indicated by a single line. BmTRA-2, *Bombyx mori* TRA-2 (GenBank accession number AAT42220); AmTRA-2, *Apis mellifera* TRA-2 (XP_396858); AgTRA-2, *Anopheles gambiae* TRA-2 (EAA13826); DmTRA-2, *Drosophila melanogaster* TRA-2 (AAA28953); DvTRA-2, *Drosophila virilis* TRA-2 (AAB58113); MdTRA-2, *Musca domestica* TRA-2 (AAW34233); BoTRA-2, *Bactrocera oleae* TRA-2 (CAD67988).

The conserved regions were the RRM and RRM-linker junction region. Although similarity extended throughout the entire protein, it should be noted that the RS domains were of low sequence complexity, diminishing the significance of the matches in these regions. The RS domain sequences in the silkworm and the fly TRA-2 proteins aligned only in areas of alternating arginines and serines, suggesting that the arginine/serine-rich composition of these domains, rather than the primary sequence, is conserved. In addition, there was a glycine-rich region similar to that of human TRA-2α (hTRA-2α; GenBank accession number AAC50658) [8], but it was not contained in the known TRA-2 proteins of other insects (**Fig. 2**). Based on the organizational and sequence similarities of this silkworm TRA-2 to *Drosophila* TRA-2, we designated this gene the silkworm *Bmtra-2*.

Bmtra-2 gene structure

To determine the exon/intron organization of the *Bmtra-2* gene, its DNA sequence was obtained (GenBank accession number AY626066) by combining many PCR fragments with no intervening sequences. The PCR fragments were produced with the silkworm genomic DNA as the template and the primer pairs were designed according to the sequence of *Bmtra-2* cDNA (**Table 1**). It was revealed that there were eight exons and seven introns in the *Bmtra-2* gene. All splicing sites of exons/introns conformed to the GT/AG rule.

Three genomic clones, BAAB01121891, BAAB01077090 and BAAB01073639, were obtained by blasting the *B. mori* genomic database with the *Bmtra-2* DNA sequence. They overlapped with each other and all showed a perfect match with the *Bmtra-2* DNA sequence in the corresponding regions. The sequence upstream of the *Bmtra-2* gene was included in the clone BAAB01121891. The promoter region was on the forward strand between –2007 and –1757 bp upstream of the *Bmtra-2* gene transcriptional initiation site. A TATA-like element was at –1785 bp. The positions of other putative TFB sites are also shown in **Table 3**. Whether the presence of these sites is relevant to the transcriptional regulation of the *Bmtra-2* gene remains to be analyzed in future studies.

Alternative splicing of *Bmtra-2* pre-mRNA

By comparing the sequences of the resulting 27 ESTs from blasting the *B. mori* EST database with the RRM to the *Bmtra-2* DNA sequence, three acceptor sites were found in the second intron and two acceptor sites in the seventh intron. The nucleotide sequences of alternative splicing ESTs from *Bmtra-2* pre-mRNA are shown in **Fig. 3(A,B)**. The specific primers were designed according to the two alternative splicing sites, and six specific primer pairs were used for detecting the different transcripts. All six RT-PCR reactions with mRNAs, which were extracted separately from five organs of 3-d fifth instar male and female larvae (the fat body, Malpighian tubule, silk gland, testis and ovary) showed positive results [**Fig. 3(C)**]. These results indicated that there are six mRNAs produced from *Bmtra-2* pre-mRNA using the alternative splicing pathway. The gene structure of *Bmtra-2* and its six alternatively spliced mRNAs are shown in **Fig. 4**, with deduced amino acid numbers in parentheses.

All six isoforms (PA, PB, PC, PD, PE and PF) deduced from six alternative splicing mRNAs of *Bmtra-2* pre-mRNA

Table 3 Potential transcriptional factor-binding (TFB) motifs for corresponding elements upstream of *Bombyx mori* transformer-2 gene (*Bmtra-2*)

Transcriptional factor	TFB motif	Strand	Location (bp)	Weight
Sp1	CCCGCC	+	–1994	2.755000
Sp1	GGCGGG	–	–1989	2.772000
E2F	TTTTGGCGG	–	–1985	6.454000
TFIID	TATAAAA	+	–1783	2.920000
HSV_IE_repeat	GCGGAA	–	–1160	1.363000
EIIF	GCGCGAAA	+	–1148	50.000000
Element_II_rs-4	TTTCGCG	–	–1141	25.816999
E2F	TTTCGCGC	–	–1141	50.000000
IFN-inducible_CS	AGAAAATGAAACC	+	–1037	1.434000
ICSBP	TGGTTTCATTTTCT	–	–1024	34.423000

All information was predicted from website http://thr.cit.nih.gov/molbio/proscan/. +, forward strand; –, reverse strand.

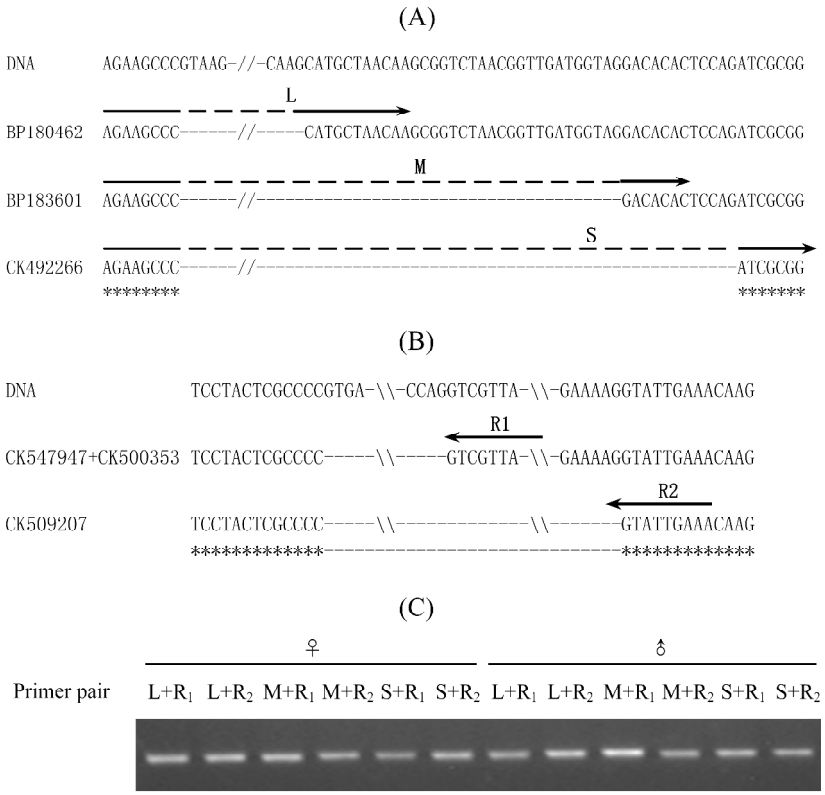

Fig. 3 Comparative analysis of multiple mRNAs alternatively spliced from *Bombyx mori* transformer-2 gene (*Bmtra-2*) pre-mRNA

(A) The second intron. Comparison of the nucleotide sequences of ESTs produced by alternative splicing of *Bmtra-2* pre-mRNA at three different acceptor sites in the second intron. (B) The seventh intron. Comparison of the nucleotide sequences of ESTs produced by alternative splicing of *Bmtra-2* pre-mRNA at two different acceptor sites in the seventh intron. (C) The reverse transcription-polymerase chain reaction products produced with the different primer pairs shown in (A) and (B) and mRNA extracted from the fat body of male and female larvae, respectively. The results produced with the mRNAs from the Malpighian tubule, silk gland, testis and ovary were similar to those of the fat body. The GenBank accession number of each EST is shown on the left. L, M, S, R1 and R2 are the primers designed according to the nucleotide sequence of each EST indicated by the line with arrowheads.

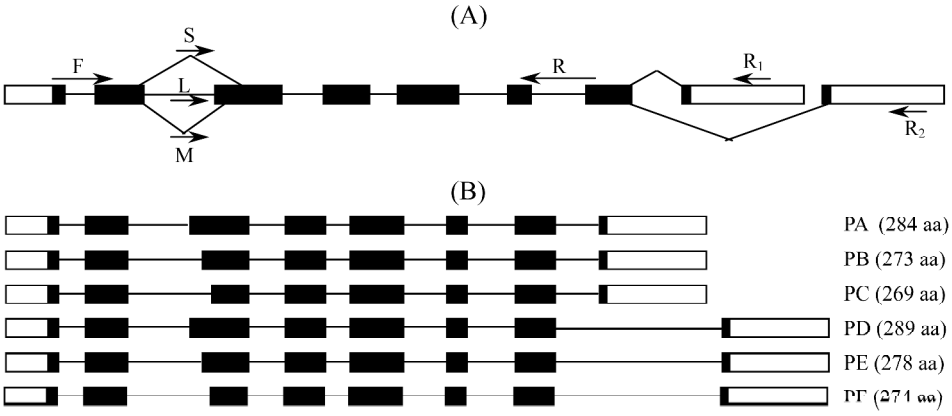

Fig. 4 Gene structure and alternative splicing mRNAs of *Bombyx mori* transformer-2 gene (*Bmtra-2*)

(A) The gene structure of *Bmtra-2*. The three different splicing sites were on the second intron, L, M and S, and two were on the seventh intron, R_1 and R_2. These sites are also indicated as the specific primers for each alternative splicing mRNA. The positions of the primers are indicated with arrowheads. (B) Six different alternative splicing mRNAs of *Bmtra-2* pre-mRNA. PA, PB, PC, PD, PE and PF represent the protein names, and the residue numbers of the deduced amino acid sequences are shown in parentheses. Exons are shown as boxes and introns as lines. Untranslated regions are shown as open boxes and coding regions as black boxes. aa, amino acid.

contained two RS domains at each end and one RRM. There were no major differences between them. PD, PE and PF have a different C-terminus, with a tyrosine phosphorylation site, compared with PA, PB and PC. PA and PD have 11 amino acid residues more than PB and PE respectively; and have 15 amino acid residues more than PC and PF, respectively. PA, PB, PD and PE have one threonine phosphorylation site more than PC and PF respectively. Whether these differences in the phosphorylation site bring about different roles will be analyzed in the future.

Northern blot hybridization

To determine the size of *Bmtra-2* mRNA transcripts, Northern blot analysis was conducted using the digoxigenin-labeled product produced by PCR reaction, with digoxigenin as the probe. Only a band of nearly 1.3 kb was observed. It was expressed in all stages of eggs, larvae, pupas and adults of the male and female silkworm (**Fig. 5**). According to the results of the *in silico* clone and RT-PCR reaction, there should be six isoforms of *Bmtra-2* mRNA. However, these isoforms had no significant differences in length and could not be distinguished using polyacrylamide gel electrophoresis, so that the six RT-PCR reactions with six different pairs of primers seemed to possess the same band. It is for this reason that there was only one band in Northern blot hybridization.

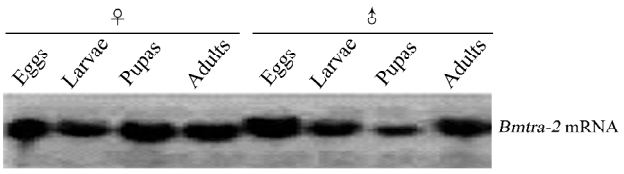

Fig. 5 Northern blot hybridization of *Bombyx mori* transformer-2 gene (*Bmtra-2*) mRNA

All samples were isolated separately from eggs, larvae, pupas and adults of the male and female silkworm with the digoxigenin-labeled probe produced by polymerase chain reaction using digoxigenin-labeled and primer F and R.

Discussion

In this report, we used a bioinformatic (or *in silico*) strategy to quickly clone and identify the *Bmtra-2* gene. This is different from the time-consuming, traditional homologous gene cloning approach, which needs degenerate-priming RT-PCR or low stringency screening of both the cDNA and genomic libraries of silkworm. The bioinformatic approach takes advantage of genetic and sequence information available for silkworm from public databases [13,14]. By searching those databases with the blast program, it was found that the silkworm EST clone CK496349 contains an RRM that is similar to the corresponding domain of RNA-binding proteins. Through further blasting, many cDNA sequences identified to the original sequence were obtained. The genomic DNA sequence was also cloned. By comparing all the relative ESTs to the DNA sequence, it was revealed that there were eight exons and seven introns in the *Bmtra-2* gene, and three acceptor sites in the second intron and two acceptor sites in the seventh intron. RT-PCR reactions with six different pairs of the specific primers, designed according to the two alternative splicing sites, revealed that there are six mRNAs produced from *Bmtra-2* pre-mRNA using the alternative splicing pathway in all tested tissues of the male and female silkworm. Six isoforms all contain one RRM similar to the corresponding domain of RNA binding proteins and two RS domains at each end. These organizations were similar to that of TRA-2 [10–12]. The most conserved regions were the RRM and RRM-linker junction regions. The similarities in the RS domains are low, as the matches in these regions align only in areas of alternating arginines and serines. The phosphorylation sites are different among the six isoforms. They may have different rules to affect the splicing of different pre-mRNAs in silkworm.

In *Drosophila*, *tra-2* pre-mRNA can produce multiple mRNAs encoding three distinct isoforms of TRA-2 protein (TRA-2^{264}, TRA-2^{226} and TRA-2^{179}) using the alternative splicing pathway during development [2,5]. The *tra-2* gene plays a key role in the "sex-determination cascade". TRA-2 is one of the two factors known from genetic analysis to be directly required for processing of *dsx* pre-mRNA along the female-specific pathway in *Drosophila* [4–6]. It functions in combination with TRA to direct female-specific *dsx* splicing [17,18].

B. mori dsx (*Bmdsx*) acts as a double-switch gene at the final step in the sex-determination cascade in the same way as in *Drosophila dsx* [19]. Although *Bmtra-2* can produce multiple mRNAs encoding six distinct isoforms just like that of *tra-2* in *Drosophila*, BmTRA-2 proteins do not seem to be required in the sex-specific splicing of *Bmdsx* pre-mRNA, because the TRA/TRA-2 binding motif-related sequence is not present in the *Bmdsx* genomic sequence, and *Bmdsx* pre-mRNA processing would need splicing repressor(s) rather than splicing activator(s), such as TRA and TRA-2 [20–23]. Given that *Bmtra-2* can not affect *Bmdsx* pre-mRNA splicing, it is surprising that the RRM,

which is thought to constitute the major RNA binding domain for this protein, is only 65% identical to the *Drosophila virilis* TRA-2 (**Fig. 2**). Of the 71 identical residues in the RRM, 32 are conserved at the positions that make up the RRM consensus (**Fig. 1**) and thus are very similar to sequences found in many proteins that do not interact specifically with *dsx* pre-mRNA, such as the RRM in the U1A and U2B″ proteins which contain residues shown to be essential for RNA binding specificity [24–26]. The RRM-linker junction region is similar to the known TRA-2 proteins in other insects and is likely to perform conserved functions that are specific to TRA-2. But the seven-glycine region similar to that of hTRA-2α is not contained in other insects' known TRA-2 protein (**Fig. 2**). hTRA-2α protein is able to recognize and affect the splicing of the *dsx* pre-mRNA in a manner to that of TRA-2 expressed in *Drosophila* [8]. However, there have been no natural human targets for hTRA-2α found in the human genome. HTRA-2β (GenBank accession number AAB08701), another human SR-like splicing factor and human homolog of *Drosophila tra-2*, which has many isoforms generated by alternative splicing [27,28], is involved in the regulation of alternative splicing processes during neural development, particularly the splicing of fibroblast growth factor receptor 2 (FGF-2R) and glutamate receptor subunit B (GluR-B) genes. The results therefore suggest that TRA-2β plays an important role in neural differentiation by regulating the FGF-2R and GluR-B genes [29,30]. So it can be proposed that BmTRA-2 may interact with specific silkworm pre-mRNAs to affect their splicing patterns, just as hTRA-2 does, in a manner analogous to the way TRA-2 affects *dsx* splicing.

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