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Drosophila *intersex* orthologue in the silkworm, *Bombyx mori* and related species

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Abstract *Intersex* (*ix*), a gene required for female sexual development in *Drosophila*, acts in concert with *doublesex (dsx)* at the end of the sex determination pathway. In the present study a homologue of *ix* was identified in *Bombyx mori*. Expression analysis of this gene by RT–PCR and RNase protection assay revealed a diagnostic alternative splice form present only in testis, whereas the most common splice form was found to express in all other tissues from early embryonic developmental stages. The present study provides evidence for the presence of an alternative splice form of *ix* in three species of silkmoths examined. Taken together with the results of an earlier study on *ix* in piralid moth, *Maruca vitrata* (Cavaliere et al. 2009), the present study suggests that the testis-specific splice form may be a characteristic feature of lepidopterans. Though *ix* lacks a conserved splicing pattern it appears to have retained its functional conservation in terminal sexual differentiation. We speculate that the presence of an additional splice form, perhaps encoding non-functional protein only in testis, may prevent the feminizing effects exerted by the functional IX protein.

Keywords *Bombyx mori* · *Intersex* · Transcriptome · ESTs · *Antheraea*

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Introduction

The insect order Lepidoptera, in contrast to most other animal groups, has a female heterogametic system. It has a WZ/ZZ (female/male) sex chromosome system, with some exceptions (Traut et al. 2007). The sex of the domesticated silkworm, *Bombyx mori*, is strongly controlled by the presence of the W chromosome. There is a striking difference in the mode of sex determination between silkworms (Bombycids and Saturniids) and *Lymantria*, even though both belong to the same insect order. In *Lymantria*, intersexes have been found in abundance. In contrast, all sexual abnormalities in the silkworm are gynandromorphs, apart from a few rare examples of intersexes (Tanaka and Matsuno 1929). These reported intersexes may have been gynandromorphs for the following reasons: they appeared in a hereditary mosaic strain, and no evidence had been presented of sex alteration proceeding with development (Tazima 1947). These observations suggest that there may not be an occurrence of intersexes in silkworms, and *Bombyx* may possess a sex determination mechanism that is quite distinct from other insect species.

Intersex (*ix*), a gene implicated in female sexual development in *Drosophila*, is expressed and likely produces functional proteins in both sexes and functions together with *doublesex* (*dsx*) to regulate terminal sex differentiation (Waterbury et al. 1999). In *Drosophila*, unlike *Sxl*, *tra* and *dsx*, *ix* produces only one splice form in all tissues of both males and females, and females express higher levels of *ix* (Garrett-Engele et al. 2002). The IX contains proline-, glycine-, glutamine- and serine-rich region similar to known transcriptional activating domains (Garrett-Engele et al. 2002) which forms a complex that binds to the regulatory regions of DSX target genes, *Yp1* and *Yp2*. Much of the work on

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functional aspect of *ix* has been done in *Drosophila*, whereas among lepidopterans, *ix* has been reported in *B. mori* and the piralid agricultural pest, *Maruca vitrata*. In *M. vitrata*, a female-specific *ix* transcript is found only in pupae, which appears to be the first described sex-specific transcript of an *ix* homologue characterized to date (Cavaliere et al. 2009). In another study, a *B. mori* homologue of *ix (Bmix)* was identified using Expressed Sequence Tags (ESTs) resources, and it was found that *ix*-mutant *Drosophila* females expressing *Bmix* cDNA restored partial characteristics of the wild type phenotype (Siegal and Baker 2005). *Ix*-mutant *Drosophila* expressing *M. vitrata intersex* (*Mvix*) cDNA also resulted in partial restoration of wild type female features as in the case of rescue with *Bmix* (Cavaliere et al. 2009). This is in contrast to the complete rescue of the wild female phenotype with the expression of *Megaselia scalaris ix* cDNA that is almost indistinguishable from the expression of *ix* cDNA of *D. melanogaster* (Siegal and Baker 2005). These results suggest that *ix,* similar to *dsx,* is broadly conserved.

Recent high throughput genomics projects have focused on the construction, annotation and analysis of cell- and tissue-specific transcriptomes that have provided fundamental insights into biological processes. Data gathered from ESTs and microarray-based gene expression profiles provide an important methodology for discovery of novel genes that are expressed in a tissue-specific manner. In this context, we investigated the testis transcriptome of the domesticated silkworm, *B. mori*, to identify and analyze testis-specific genes. This study led to the identification of a testis- specific splice form of *ix* mRNA in addition to the common form expressed in all tissues. RT–PCR and RNase protection assays confirmed the two splice forms; one form accumulated exclusively in the testis and the second form was present ubiquitously in all tissues including testis. This finding provides an opportunity to investigate the pleiotropic role of *ix* in sexual differentiation.

Materials and methods

Sequence source and analysis

More than 100,000 ESTs, are available for *B. mori* in NCBI dbEST (Mita et al. 2003; Xia et al. 2004). We downloaded 9,614 testis ESTs and a total of 95,051 ESTs derived from tissues other than testis. ESTs generated from other tissues were also downloaded to identify testis-specific genes in *B. mori*. Since many ESTs may be derived from the same gene, the sequences were assembled into clusters with the TGICL program (Pertea et al. 2003).

Expression analysis of *B. mori intersex* (Bmix) gene

In silico analysis of the testis transcriptome led to the identification of two splice forms of *Bmix* gene. Complementary DNA was synthesized from total RNA isolated from midgut, fatbody, head, silk gland, epidermis and gonads of 5th instar silkworm larvae and, gonads and other tissues of pupae and moths, by oligo(dT) priming using MMLV reverse transcriptase (Invitrogen). To determine the tissue distribution of *Bmix* expression, PCR experiments were performed using cDNAs from multiple tissues of male and female. *Bmix* gene specific primers were designed using primer 3 software (Rozen and Skaletsky 2000). Five primers designed for exons (Table 1) are shown in Fig. 1a. A pair of primers that bind to first and second exons was also designed to specifically amplify the second splice form (Table 1). PCR was performed for 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The primers for β-actin, actin-F CACTGAGGCTCCC CTGAAC and actin-R GGAGTGCGTATCCCTCGTAG, were used to amplify the endogenous control. PCR products amplified using *Bmix* gene specific primers were cloned into pCRII- TOPO vector (Invitrogen) and then sequenced to confirm the testis-specific splice form.

RNase protection assays were carried out to study the expression pattern of *Bmix* splice forms. A radiolabelled anti-sense RNA probe of 284 bp was synthesized for the region comprising first and second exons using MegaScript in vitro transcription kit (Ambion, Austin, Texas, USA)

Table 1 Details of primers used in RT–PCR expression analysis of *Bmix* gene and primers used to sequence *A. assama* and *S. c. ricini ix* transcripts

Species	Primer name	Sequence
B. mori	ixA	ATGCACGTACCAATGAACCA
	ixB	TCTGCATTGTGATTTTGGTG
	ixC	TTCGGGCGGTGATATATTCT
	ixD	TTTTTCAAATCTAGGAACAGGATT
	ixE	CGCTTGCTGGATACACGTT
	ixT-F	TCCAAATGTAGCCATGCAAA
	ixT-R	GGAAGATGACCAGGGTTTCA
A. assama	Aix-A	ATATGCACGTCCCGATGAAT
	Aix-B	AATATTTGAGCGGCTGACTTC
	AixD	CAGGTCATCGCTGTTCTCAA
	Aix-T	GGTTCAACTCGGTCAGCAGT
	Aix-Tr	ACTGCTGACCGAGTTGAACC
S. c. ricini	Scix_1	AATATGCACGTGCCAATGAA
	Scix_2	AGTTAGCGTTGTTTCCTGTGC
	Scix_3	TTCAAATCCACAGAAAGCCATA

The sequences of *A. assama* and *S. c. ricini ix* cDNA can be downloaded from online database 'WildSilkBase'

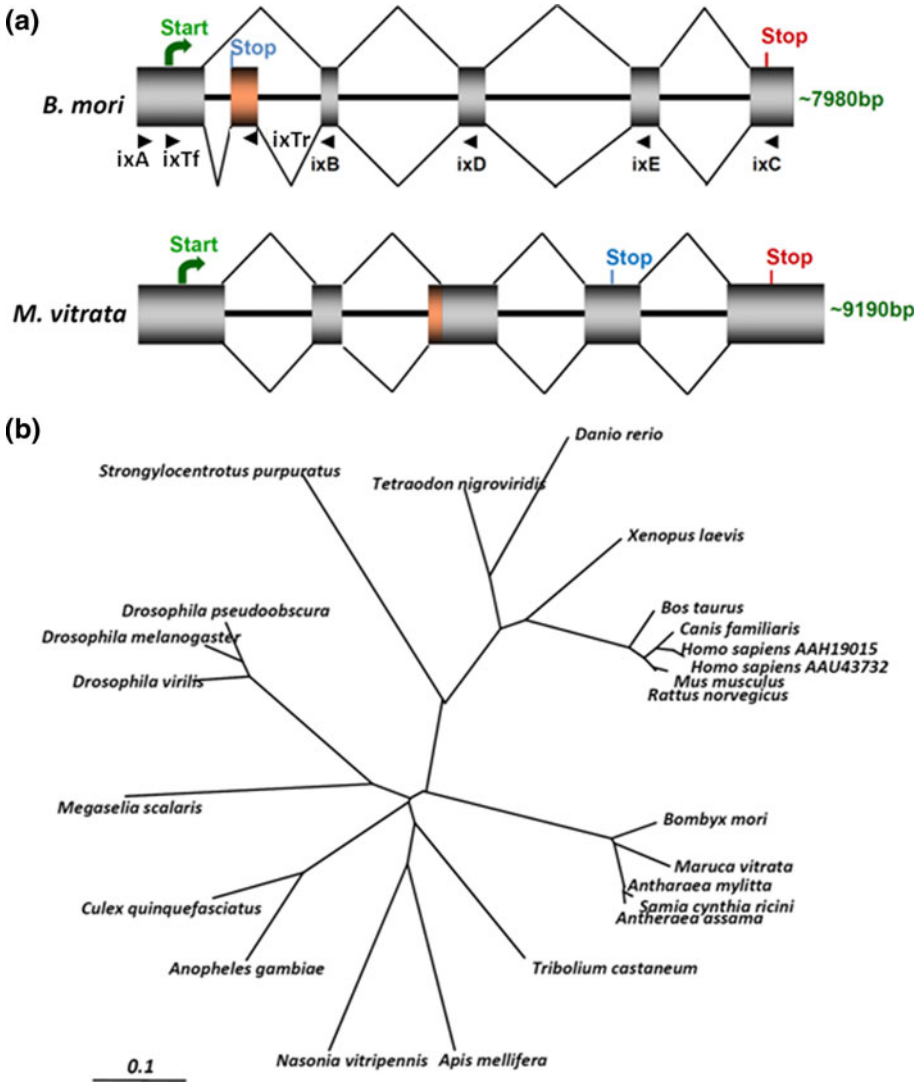

Fig. 1 **a** Comparison of gene structure and alternative splice forms of *Bmix* and *Mvix*. The primers *ixA*, *ixB*, *ixC*, *ixD* and *ixE*, were designed for different exons and *black arrows* indicate their location and direction of primer extension. Primers *ix-T* and *ix-Tr* were designed to amplify testis-specific splice form. *Thick black line* in the gene structure indicates intronic region whereas *grey* and *orange blocks* indicate positions of exons. The *lines joining* the exons above gene structure form common splice form and *lines joining below* the gene indicate testis splice form in *B. mori* and second splice form in *M. vitrata*. Second splice form in both cases leads to introduction of premature stop codon (represented in *blue colour*). **b** Dendrogram showing the relationship of IX protein between different species

after cloning the fragment into pCRII- TOPO vector (Invitrogen). This probe contains 89 bp sequence complementary to the second exon and the remaining 195 bp complementary to a part of the first exon. RNA samples (10 µg each) and aliquots containing 2×10^5 cpm of the RNA probe were hybridized overnight at 45°C in 20 µl hybridization solution containing 75% formamide/0.5 M NaCl/10 mM Tris HCl, pH 7.5. After addition of 300 µl of 300 µM NaCl/5 mM EDTA containing RNase A at 60 µg/ml, the mixture was incubated for 1 h at 37°C and subsequently treated with proteinase K (300 µg/ml) for 30 min at 37°C, in the presence of 0.1% SDS, extracted twice with 1:1 phenol/chloroform and once with chloroform, precipitated with ethanol, and subjected to electrophoresis on a 8% polyacrylamide/8 M urea gel in 90 mM Tris borate buffer, pH 8.3. RNase protected fragments were quantified with a PhosphorImager.

Sequencing and phylogenetic analysis of *intersex* homologs in other Lepidoptera

Putative homologs of *ix* in lepidopteran insects were identified either by searching the EST databases or by sequencing with primers designed for conserved regions. DNA sequences found to encode proteins with significant similarity to BmIX amino acid sequence were identified by translated BLAST (tblastn) search against WildSilkbase, an EST database of wild silkmoths (Arunkumar et al. 2008). The search resulted in 6 ESTs from *Antheraea assama* (Ac. Nos. Aaov1642, Aaov0427, Aaov0387, Aaov1877 and Aaov2957 from ovary ESTs, and Aabr4525 from brain ESTs) and 1 EST from *Samia cynthia ricini* (Ac. No. Sc_96Hrs4747). These ESTs were individually inspected and assembled which returned partial sequences of *ix* homologs in these species.

To identify putative *ix* homolog in *Antheraea mylitta*, we performed reverse transcription of total RNA from *A. mylitta*, followed by PCR amplification of cDNA using *Aix-A* and *Aix-D* primers of *A. assama* (Table 1). PCR amplicons were then sequenced after cloning into TA vector. Based on the partial sequence, RACE (Rapid Amplification of cDNA Ends) primers were designed and 5′ end sequence was obtained. All the three putative *ix* cDNA sequences obtained had complete 5′ end sequences. These sequences were conceptually translated and aligned with other homologs from several taxa.

The amino acid sequences of IX homologs available in other organisms (*Maruca vitrata*, ACQ90246; *Nasonia vitripennis*, XP_001601927; *Culex quinquefasciatus*, XP_001849607; *Rattus norvegicus*, XP_214868; *Mus musculus*, NP_080318; *Homo sapiens*, AAH19015; *H. sapiens*, AAU43732; *Canis familiaris*, XP_855358; *Bos taurus*, XP_871568; *Xenopus laevis*, AAH78525; *Tetraodon nigroviridis*, CAF9084; *Danio rerio*, AAM34654; *Strongylocentrotus purpuratus*, XM_001178265; *Apis mellifera*, XP_395989; *Tribolium castaneum*, XP_970547; *Anopheles gambiae*, XP_321918; *D. melanogaster*; *D. pseudoobscura*, EAL26072; *Drosophila virilis*, AAV65894; *Megaselia scalaris*, AAV65895) were retrieved from NCBI protein database. The protein sequences of newly identified homologs were aligned with BMIX and other IX homologs downloaded from NCBI. Phylogenetic analysis was carried out using ClustalX and an unrooted tree was constructed.

Results and discussion

Intersex is alternatively spliced in *B. mori*

Through BLAST analysis of a non-redundant set of *B. mori* testis transcripts, we identified a homolog of *ix*. Further mining of this transcript in other tissue ESTs revealed a transcript in the ovary. Pair-wise alignments showed distinct alternative splice forms among ovaries and testis. A contig of 16,856 bp with accession number AADK01005119, harboring all the six exons was obtained by searching the *B. mori* WGS database. The complete gene structure was deduced by aligning ovary and testis *Bmix* ESTs to this contig. The total gene size was found to be 7.8 kb which includes a total of ~7.2 kb intronic region (Fig. 1a).

Expression profile of *Bmix* alternative splice forms was carried out by designing a common forward primer for exon 1 (primer name: *ixA*) and separate reverse primers for exons 3 (*ixB*), 4 (*ixC*), 5 (*ixD*) and 6 (*ixE*). RT–PCR analysis using different combinations of the above mentioned primer sets (*ixA-ixB*, *ixA-ixC*, *ixA-ixD*, and *ixA-ixE*) revealed the presence of different exons in the tissues tested (data not shown). The common splice form of *Bmix* containing all but exon 2 was present in all tissues tested. However, in testis it showed an additional splice form containing all exons in larva (Fig. 2a), pupa and moth (Fig 2c). The RT–PCR amplicons were cloned into a TA vector and sequenced to verify the sequence of the two splice forms. Further confirmation of the testis-specific splice form was done through an RNase protection assay (Fig. 2b). Conceptual translation of both the transcripts showed that the common splice form encodes a protein product of 192 amino acids (aa) and the testis-specific splice form encodes a protein product of only 72 aa as a result of a stop codon at the beginning of exon 2 (Fig. 1a). Cavaliere and co-workers (Cavaliere et al. 2009) also reported the identification of a second splice form in *B. mori*. Their RT–PCR experiments with three tissues from both sexes failed to establish the presence of testis-specific splice form. However, our data clearly demonstrated the presence of the testis-specific splice form in larval, pupal and moth stages using RT–PCR and an RNase protection assay. We also did not observe different *ix*

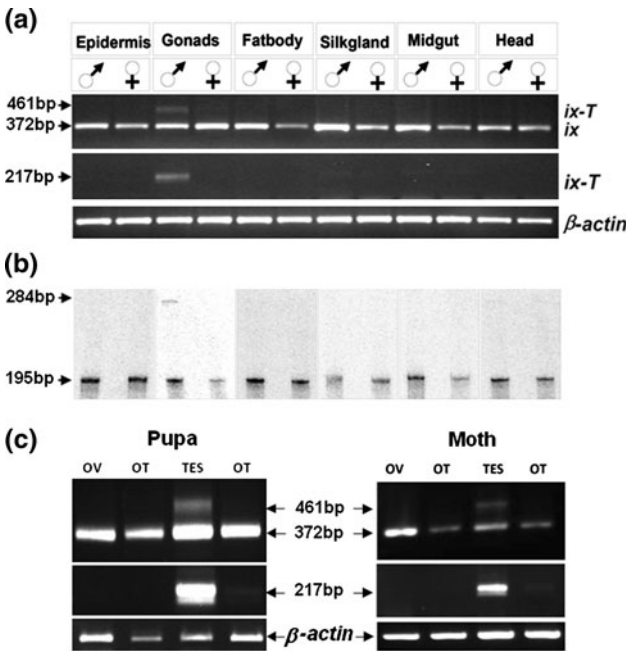

Fig. 2 : Expression of *Bmix* mRNA during different developmental stages of *B. mori* as revealed by RT–PCR. **a** *Upper panel* shows RT–PCR profile of different tissues from 5th instar larvae amplified using ix-A and ix-E primers, which will amplify both common (*Bmix*) and testis-specific (*Bmix-T*) splice forms. *Middle panel* is the RT–PCR profile, obtained by using primers binding to first and second exons which will specifically amplify *Bmix-T*. *Lower panel* shows *β*-actin levels as control. **b** Shows the RNase protection assay for six tissues of both sexes separately, of fifth instar larvae, wherein only testis is showing the second splice form. **c** Shows the expression pattern of *ix* in gonads and other tissues of pupae and moths (*OV* ovary, *TES* testis, *OT* other tissues)

transcripts in pupal and adult stages as reported in piralid moth, *M.vitrata* (Cavaliere et al. 2009).

In contrast to *Drosophila*, where *ix* is a single exon gene and not sex-specifically spliced, *Bmix* possesses six exons of which five (exons 1, 3–6) are included in the transcripts expressed in all tissues, and all six exons are present in the testis-specific splice form (Fig. 1a). Studies in *Drosophila* (Baker and Ridge 1980; Chase and Baker 1995, Garrett-Engele et al. 2002) suggested that the IX is required to function with the female-specific product of the *dsx* gene to implement female sexual differentiation in diplo-X animals. However, other studies have favoured that IX might function not only with DSX to control many aspects of somatic sex, but also independently of DSX to regulate other aspects of somatic sex (McRobert and Tompkins 1985). Chase and Baker (1995) observed that, while DSX proteins are capable of binding to the sex-specific enhancer site of the *yolk protein* (YP) genes, the IX may not be required to achieve high levels of YP transcription (Chase and Baker 1995). Another study (Acharyya and Chatterjee 2002), revealed the possible functions of *ix* in *Drosophila* males by characterizing an allele of *ix* (*ix5*). Attempts made to rescue the wild type phenotype in *ix* mutants of *Drosophila* using *Bmix* cDNA (Siegal and Baker 2005) and *Mvix* cDNA (Cavaliere et al. 2009) resulted in partial rescue of the *ix*-mutant females. These results suggest that IX proteins of the dipterans and lepidopterans have diverged functionally at least partially. In IX homologues, down stream to the amino N- terminal region that constitutes the transcriptional activation domain there are several conserved stretches of amino acids rich in glutamine, methionine, proline, glycine and asparagine. In *D. melanogaster*, one of these conserved stretches corresponding to 94–116 aa residues, harbours four aminoacid residues that bind DsxF (Yang et al. 2008). It is shown that only two of these four aminoacids are conserved between MvIX and BmIX and *Drosphila* IX probably resulting in reduced binding of these IX proteins to DsxF (Cavaliere et al. 2009).

The previous study (Cavaliere et al. 2009) reported sequences of alternative splice forms obtained through RT–PCR of male adult tissues samples. But subsequent expression analysis using primers specific to the specific splice form did not show any tissue specificity. We speculate that this splice form might also be male specific as expression analysis missed essential negative controls. Further confirmation is needed to conclude these speculations.

An earlier study (Siegal and Baker 2005) hypothesized that potential diversity of biological processes in which IX homologs participate is at least as great as that in which DSX homologs participate. This is possible because IX homologs have interaction partners beyond the DM protein family. Though the authors report in their study partial rescue of wild type female phenotype, they also opine that mere ability of a homolog to replace *D. melanogaster ix* does not guarantee that the homolog is functioning in the sex determination pathway in the donor species.

Sequencing and phylogenetic analysis of *intersex* homologs in other lepidopterans

We also identified putative homologs of IX in three saurniid silkmoths, *Antheraea assama*, *A. mylitta* and *Samia cynthia ricini* either by searching the WildSilkbase (Arunkumar et al. 2008) or by de novo sequencing. Other homologs were downloaded from NCBI. Protein sequences were aligned and an unrooted tree was constructed. The resulting tree grouped the insect species together in one group and mammals in another group. *M. vitrata*, a lepidopteran pest species clustered with silkmoths. The phylogenetic analysis showed close nesting of lepidopterans. The tree showed that IX is highly conserved within these species when compared to that in *Drosophila* species (*D. melanogaster*, *D. pseudoobscura* and *D. virilis*). Though all the three *Drosophila* species belong to the family Drosophilidae, they showed loose clustering compared to the members of Bombycoidae (Fig. 1b).

To study whether the *ix* homolog in *A. assama* also shows alternative splice forms, RT–PCR products were obtained from whole larval RNA extracts and sequenced. We amplified 2 kinds of sequences with a stretch of overlapping sequence between them. The alignment revealed the presence of an additional exon of 51 bp sequence in one of the isoforms. Conceptual translation yielded a protein with an additional 17 aa and the additional sequence did not possess stop codons. The extra exon is in the same region as the testis-specific exon in *Bmix* (Fig. 3). However, there is no sequence similarity between the second exon of *B. mori* (present only in the testis-specific splice form) and the extra exon of *A. assama*.

Comparison of gene structure and alternative splicing patterns between *Bmix* and *Mvix* hinted at rapid evolution of this gene in lepdipteran insects. Available data for *Bmix* and *Mvix* showed that there are differences in length and number of exons, gene size and splicing pattern between these two *ix* genes. Though an extra exon was introduced at the same region of the second splice form of *Bmix* and *A. assama ix*, it was different from the second splice form of *Mvix* (Figs. 1a, 3). In *Mvix* an additional 17 bp was added at the third exon. Analysis of *ix* homologs from other lepidopteran species would shed more light regarding evolution of *ix* in this order.

Alternative splicing is a widespread mechanism in eukaryotes for regulating gene expression, and for increasing protein diversity (Black 2000; Graveley 2001). Most metazoan genes encode pre-mRNAs that undergo

Fig. 3 Amino acid sequence alignment of IX proteins of *B. mori*, *A. mylitta*, *A. assama*, *S. c. ricini* and *M. vitrata*. Except for *B. mori* and *M. vitrata*, we don't have complete C-terminal region for remaining three species. Sequences of only normal splice form are shown here. *Green arrow* indicates the corresponding region of transcript where an extra exon is introduced in the second splice form of *ix* in *B. mori* and *A. assama*. *Blue arrow* shows the corresponding region of transcript at which an extra exon is introduced in *M. vitrata*. *M. vitrata* has an additional 38 amino acid C-terminal sequence (indicated in *red letters*) that is unique to this species

alternative splicing. Alternative splicing controls expression of many genes, including the *B. mori* sex determination gene *dsx*. Since the *ix* phenotype is supposed to be female-specific, and some genes in the somatic sex determination hierarchy are regulated at the level of splicing, it was conceivable that the *ix* pre-mRNA would be sex specifically spliced.

It has been reported that *B. mori dsx* is more closely related to *Drosophila dsx* than to honey bee *dsx*. The *B. mori dsx* transcript is sex-specifically spliced but appears to lack splicing regulation mediated by TRA/TRA-2 (Suzuki et al. 2001). The mechanism of sex-specific splicing at the *dsx* gene is very different between *D. melanogaster* and *B. mori*. These findings suggest that the structural features of the sex-specific splicing patterns of *Bmdsx* pre-mRNA are similar to those of *Drosophila dsx* but the regulation of sex-specific alternative splicing of *Bmdsx* pre-mRNA appears to be different (Suzuki et al. 2001). In a similar way, splicing patterns are also not conserved between *Drosophila* and *B. mori* for *ix*. Though *ix* lacks a conserved splicing pattern, it may be functionally conserved to exhibit its role in sex differentiation either in co-ordination with DSX or independent of it to control many aspects of somatic sex as observed in *Drosophila* (McRobert and Tompkins 1985). Our assumptions draw support from the fact that the *Drosophila* and *Bombyx* mRNAs encode highly related and similarly sized proteins. BLAST analysis revealed 72% similarity and 50% identity between these two proteins. These scores are quite high, considering that Lepidoptera and Diptera shared a common ancestor 250 million years ago. Interestingly, only the first 50 aa did not show similarity, while the remaining amino acid sequences showed two main conserved regions corresponding to the amino and carboxy termini, respectively.

BmIX protein shows 89% sequence identity over the entire length of *M. vitrata* IX (Cavaliere et al. 2009). This is corroborated by phylogenetic analysis where lepidopteran IX homologues are closely clustered. In addition there are similarities in the more complex splicing regulation observed in these two lepidopteran species. These results tend to suggest that BmIX is a structural homologue of *Drosophila* and *M. vitrata* IX. The earlier observations of Siegal and Baker (2005) in *Drosophila* transgenic flies, and the recent observation of a female-specific *ix* mRNA (most probably somatic) in lepidopteran pest, *M. vitrata*, (Cavaliere et al. 2009), suggest that BmIX is indeed involved in sexual differentiation together with DSX, as in *Drosophila* and possibly in *Maruca*.

Presence of alternative splice forms of *ix* in the three lepidopterans analysed (*M. vitrata*, *B. mori* and *A. assama*) and absence of such *ix* splice forms in any other reported insect species suggests that this unique feature is perhaps confined to lepidopterans. *Ix* is alternatively spliced in all the three lepidopterans, where the amino acid sequence of the ubiquitously expressed common splice form is similar in all the three species examined, however, there is no similarity either at nucleotide or amino acid sequence of alternative splice forms. *Mvix* has a unique C-terminal region of 38 amino acids that is not present in any other lepidopteran homologues of *ix* (Fig. 3). The observation of a longer and probably non-functional testis-specific mRNA in addition to the common *Bmix* mRNAs, suggests that quantitative down-regulation of the IX may be required in testis to protect this tissue from "feminizing" or other

effects possibly exerted by the full-length IX protein, as the transcripts containing premature stop codons are marked for degradation by the Non-sense Mediated Decay pathway (Hansen et al. 2009). The presence of additional splice forms, probably producing functional proteins, in female pupae of *M. vitrata* suggests that additional *ix* isoforms are required in later stages of female differentiation.

Conclusion

The present study provides evidence for the presence of an alternative splice form of *ix* in silkmoths. This study, together with the previous report (Cavaliere et al. 2009), indicates the occurrence of tissue-specific splice forms of *ix* in lepidopterans. It appears that the presence of *ix* alternative splice forms is unique to lepidopterans. However, further studies should be carried out to investigate the function of this gene in both sexes, by using techniques such as RNAi and transgenesis to suppress or block the expression of *ix* thereby studying the role of IX in sexual differentiation. The studies on the role of *ix* in terminal sex differentiation are important in light of the ongoing efforts to develop genetic sexing strains either by killing females at embryonic stages or reversing sex from female to male using genetic approaches.

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