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Two female-specific DSX proteins are encoded by the sex-specific transcripts of *dsx*, and are required for female sexual differentiation in two wild silkmoth species, *Antheraea assama* and *Antheraea mylitta* (Lepidoptera, Saturniidae)

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ABSTRACT

doublesex (*dsx*) is the bottom most gene of the sex-determination cascade of *Drosophila melanogaster*. The pre-mRNA of *dsx* splices to produce male- and female-specific transcripts which code for the male- and female-specific proteins, respectively. *dsx* homologues have been characterized from different (many in Diptera, two in Hypmenoptera and only one in Lepidoptera) insect species. Sex-specific splice forms of *dsx* pre-mRNA in all these species code for one male- and one female-specific DSX proteins, which regulate the downstream target genes responsible for sex-specific characters. In the present study we have cloned and characterized the *dsx* homologues from two saturniid silkmoths, *Antheraea assama* and *Antheraea mylitta*. The divergence time between Saturniidae and Bombycidae to which the domesticated silkworm, *Bombyx mori* belongs is estimated to be around 160.9 MY. Interestingly, the *dsx* pre-mRNA of these wild silkmoths sex-specifically splices to generate multiple splice variants. On the basis of their open reading frame (ORF) and conceptual translation, two female-specific (DSX^{F1} and DSX^{F2}) and one male-specific (DSX^{M}) proteins could be inferred, in both the moths. Presence or absence of a 15 bp stretch within the ORF of the two groups of female-specific transcripts resulted in the production of two distinct female-specific DSX proteins. The sex-specific DSX proteins have common amino-terminal sequence but sex-specific carboxy termini. The two female-specific DSX proteins (DSX^{F1} and DSX^{F2}) share common DNA binding domain (DM domain) and oligomerization domain (OD domain) and differ only at their extreme C-termini by 21aa. Functional analysis of *dsx* transcripts in *A. assama* by *ds*RNA mediated knock-down resulted in complete abolition of expression of *vitellogenin* and *hexamerin* genes, the direct targets of the DSX proteins, irregular differentiation of gonads, and drastic reduction in fecundity and hatchability. Together, these results suggest the involvement of both the female-specific DSX proteins in the process of female sexual differentiation. Further, conservation of the 4th exon sequence, especially the PESS sequence responsible for the sex-specific splicing of *Bmdsx* in the female-specific transcripts of *Aadsx* and *Amydsx*, indicated the existence of a common mechanism of sex-specific splicing of *dsx* homologues in silkmoths. To our knowledge this is the first report of existence of multiple splice forms of *dsx* pre-mRNA encoding two female-specific DSX proteins.

1. Introduction

Sex determination is a fundamental biological process by which sexual fate of an individual, either to become a male or female, is encrypted very early during the development. Sex-determination mechanisms are highly diverse in animal kingdom (Bull, 1983; Zarkower, 2001); insect kingdom embodies examples of some of the best studied sex-determination mechanisms (Sanchez, 2008). The most well studied sex-determination pathway among insects is exemplified by *Drosophila melanogaster* (Cline and Meyer, 1996; MacDougall et al., 1995; Sanchez, 2008; Schutt and Nothiger, 2000) where sex is determined by a well characterized sex-determination cascade comprising of hierarchy of regulatory genes. The ratio of X chromosomes to the autosomal set (X:A ratio) provides a primary cue to activate this sex-determination cascade (Bridges and Anderson,

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1925; Cline, 1984; Keyes et al., 1992; Sanchez and Nothiger, 1983). Recently, it has been proposed that the double dose of X signaling elements (XSEs) rather than X:A ratio signals the sex-determination cascade in *Drosophila* (Erickson and Quintero, 2007). An autoregulatory feedback loop is established to maintain the continuous supply of functional SXL protein in females throughout the development (Bell et al., 1991; Cline, 1984; Keyes et al., 1992; Penalva and Sanchez, 2003). SXL transmits the sex-determination signal to an SR protein encoding gene *transformer* (*tra*). SXL acts on the *tra* pre-mRNA to produce functional TRA protein (Belote et al., 1989; Boggs et al., 1987; Inoue et al., 1990; McKeown et al., 1987). TRA, together with the product of the constitutive gene *transformer-2* (*tra-2*) and with some other general splicing factors, forms a complex on the *dsx* repeat element (*dsx*RE) in the *dsx* pre-mRNA, and allows the use of a non-canonical weak female-specific 3′ splice site to execute female-specific splicing of the *dsx* pre-mRNA encoding DSX^F protein (Hedley and Maniatis, 1991; Hoshijima et al., 1991; Ryner and Baker, 1991; Tian and Maniatis, 1993).

In males, single dose of XSEs is insufficient to activate the *Sxl-pe* thus resulting in the absence of SXL protein (Erickson and Quintero, 2007). As a result the *tra* pre-mRNA splices in a default male mode producing a truncated non-functional TRA protein due to presence of a premature stop codon in the mature *tra* mRNA (Boggs et al., 1987; Valcarcel et al., 1993). In the absence of functional TRA protein in males, *dsx* pre-mRNA splices in default male mode resulting in male-specific mature mRNA encoding the DSX^M protein (Hoshijima et al., 1991). The female- and male-specific DSX proteins, DSX^F and DSX^M, respectively are transcription factors which share common amino termini but differ in their sex-specific carboxy termini (Burtis and Baker, 1989; Slee and Bownes, 1990; Steinmann-Zwicky et al., 1990). Sex-specific DSX proteins regulate the genes involved in various aspects of somatic sexual differentiation in an antagonistic manner (Burtis and Baker, 1989; Burtis et al., 1991). According to Wilkin's 'bottom-up' model of evolution (Wilkins, 1995), the downstream genes are more conserved in sex-determination pathway as compared to the upper hierarchy genes. Consistent with the Wilkin's hypothesis, upstream genes and signals in the sex-determination cascade are very diverse even among closely related species whereas *dsx* homologues characterized in a number of diverse insect taxa have been found to be functionally conserved. Outside *drosophilids*, *dsx* homologues have been found in many other dipterans including *Megaselia scalaris* (Kuhn et al., 2000), *Musca domestica* (Hediger et al., 2004) and *Anopheles gambiae* (Scali et al., 2005), in the tephritids *Bactrocera tryoni* (Shearman and Frommer, 1998), *Bactrocera oleae* (Lagos et al., 2005), *Ceratitis capitata* (Saccone et al., 2008) and *Anastrepha obliqua* (Ruiz et al., 2005). *dsx* genes of all the dipterans reported till date show conservation in the *cis*-regulatory elements required for sex-specific splicing of their pre-mRNA: i) a weak polypyrimidine tract at the 3′ splice acceptor site before the female-specific exon (exon 4) and ii) TRA/TRA-2 binding sites in the female-specific 3′ untranslated region (Sanchez, 2008). *dsx* homologues have also been characterized recently in the hymenopterans, *Apis mellifera* (Cristino et al., 2006) and *Nasonia vitripennis* (Oliveira et al., 2009), and a lepidopteran *Bombyx mori* (Ohbayashi et al., 2001). A generalized fact that emerges from the available data on *dsx* genes in different organisms is that *dsx* produces sex-specific alternatively spliced transcripts encoding one male- and one female-specific DSX proteins which differ only at their extreme C-termini. *dsx* gene is emerging as a potential candidate gene to be used in sterile insect technique (SIT) programs for the control and eradication of harmful insect pests (Saccone et al., 2002). In spite of the fact that the order Lepidoptera embraces a vast array of coloured butterflies, beneficial moths and innumerable pest species, sex-determination studies have confined only to the domesticated silkmoth, *B. mori*. The Lepidopteran insects (moths and butterflies) follow female-heterogametic sex chromosome system; females are ZW or ZO whereas males are ZZ. In *B. mori* a hypothetical dominant epistatic factor present on the W chromosome determines female development whereas in its absence male development ensues (Hasimoto, 1933). Screening of the *B. mori* genome for sex-determination cascade genes has revealed that except *dsx* no other genes could be identified as potential candidates of sex-determination pathway except for the recent reports suggesting the involvement of W-borne zinc finger motifs as upstream regulators of sex-determination pathway (Ajimura et al., 2006; Satish et al., 2006, manuscript under preparation). *B. mori doublesex* (*Bmdsx*) gene is present on autosomes and like *dsx*, the pre-mRNA of *Bmdsx* is sex-specifically spliced to produce male- and female-specific mRNAs encoding $BmDSX^M$ and $BmDSX^F$ proteins, respectively (Ohbayashi et al., 2001). Sex-specific BmDSX proteins differ only at their C-terminal OD2 domains, and are known to regulate many aspects of somatic sexual differentiation (Suzuki et al., 2003, 2005). Despite these similarities, sex-specific splicing of *Bmdsx* is different from that of *dsx*. Unlike *Drosophila dsx*, default form of *Bmdsx* splicing is the female form (Suzuki et al., 2001). In addition, in *Bmdsx* the 3′ splice site preceding the upstream female-specific exon 3 is not weak and there is no *dsx*RE sequence found within the female-specific exons. These results led to the speculation that processing of *Bmdsx* pre-mRNA needs splicing repressor(s) rather than splicing activators such as TRA and TRA-2 (Suzuki et al., 2001). Recently it has been shown that the BmPSI, a *Bombyx* homologue of PSI (P-Element Somatic Inhibitor), binds to the 'Putative Exonic Splicing Silencer' (PESS) sequence in exon 4, and is essential for repressing female-specific splicing and skipping of exons 3 and 4 of *Bmdsx* in males (Suzuki et al., 2008). These results show that *Bmdsx* is very different from Dipteran *dsx* in terms of *cis*-acting element it harbors and the mechanism of sex-specific splicing of its pre-mRNA.

To validate further the Wilkin's hypothesis of bottom-up evolution of sex-determination pathways and to investigate whether the features of *Bmdsx* is unique to *B. mori* alone or it is universal among silkmoths, *dsx* homologues from two saturniid wild silkmoth species *Antheraea assama* (*Aadsx*) and *Antheraea mylitta* (*Amydsx*) were cloned and characterized for their functional properties. *A. assama* and *A. mylitta* possess different chromosome numbers and distinct sex chromosome compositions. *A. assama*, considered to be the progenitor species of silkmoths (Arunkumar et al., 2008a), has low chromosome number ($n = 15$) (Deodikar et al., 1962) as compared to other known silkmoths, and female lacks W chromosome (ZO), which makes it interesting for sex-determination studies. *A. mylitta*, another economically important silk secreting saturniid moth ($n = 31$), has ZW females and ZZ males (Arunkumar et al., 2008b; Mahendran et al., 2006). The study reported here shows that *Aadsx* and *Amydsx* do not have TRA/TRA-2 binding sites, instead, contain PSI binding sequences in all the female-specific transcripts similar to *Bmdsx*. Contrary to the reports on *dsx* in other insects, our study revealed multiple isoforms of *Aadsx and Amydsx* transcripts, which encode two female-specific and one male-specific putative DSX proteins. The two female-specific *Aadsx and Amydsx* ORFs differ by the presence or absence of a 15 bp stretch. Functional analysis of *Aadsx* through RNAi experiments suggests that both the female-specific AaDSX proteins are required for expression of downstream genes involved in female sexual differentiation. Further, PESS sequence required for the sex-specific splicing of *Bmdsx* is 100% conserved in all the female-specific transcripts of *Aadsx* and *Amydsx*, which is an indirect evidence for existence of a universal mechanism of sex-specific splicing of *dsx* in silkmoths. Also, comparative studies of *dsx* homologues from silkmoths (*A. assama*, *A. mylitta* and *B. mori*) led us to the identification of a novel female-specific splice form of *Bmdsx* containing the same 15 bp stretch found in a few female-specific transcripts of *Aadsx* and *Amydsx*, after exon 3 of *Bmdsx* (Shukla, Jadhav and Nagaraju,

manuscript communicated). Thus the study reported here suggests the presence of two female- and one male-specific DSX proteins in silkmoths. Further studies are required to decipher the mode of action of the two female DSX proteins on the downstream target genes in female sexual differentiation in silkmoths. Also, it remains to be investigated whether multiple splice forms encoding two female-specific DSX proteins are the characteristic feature of only silkmoths or widespread among lepidopterans. The studies are underway in our laboratory to characterize the *dsx* homologues from other lepidopteran insects.

2. Materials and methods

2.1. Degenerate primers

DSX protein sequences of seven insect species downloaded from the database (www.ncbi.nlm.nih.gov) were aligned using ClustalW program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The two conserved regions, one in OD1 domain and the other in OD2 domain (Supporting information, Fig. S1A), were selected for designing forward and reverse degenerate primers, respectively. Nucleotide sequences of the corresponding conserved region of OD1 and OD2 domains were manually aligned with each other (Supporting information, Fig. S1B) and the degenerate primers were designed on the basis of consensus nucleotide sequences. Codon usage database (http://www.kazusa.or.jp/codon/) was used to select the preferential nucleotide (for *A. assama* and *A. mylitta*) in case of highly degenerate codons. The forward and reverse degenerate primers thus designed are:

Dsx_1aF {5′-CAACTGCGCCCGGTG(Y)(M)(R)(RY)AA(Y)CA-3′} and New_DsxR {5′-CA(Y)(W)AG(B)GGCATC(R)TCTC-3′}, respectively. These primers were used to obtain the *dsx* sequences from both the wild silkmoths. To obtain the full length transcripts, 5′ and 3′ RACE primers were designed on the basis of specific *dsx* sequences of *A. assama* and *A. mylitta*.

2.2. Primers for A. assama specific hexamerin and vitellogenin genes

Primers (Aa_Hex_4F and Aa_Hex_4R) for *A. assama hexamerin* gene were designed based on the template obtained (Unigene_Aa00258) from the EST database of *A. assama* (http://www.cdfd.org.in/wildsilkbase/home.php) through tBLASTn using Hexamerin protein (GI: 100134931) of *B. mori* as a query sequence. For designing *A. assama vitellogenin* gene primers, the *vitellogenin* gene sequences of six lepidopteran insects (*B. mori* – GI: 60391273, *B. mandarina* – GI: 32526657, *Antheraea yamamai* – GI: 123299275, *A. pernyi* – GI: 152002197, *Samia cynthia ricini* – GI: 12862882, *S.C. pryeri* – GI: 61651633) were aligned using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html), and the primers (Aa_Vg_F2 and Aa_Vg_R2) were designed from the conserved region. These primer sequences are given in Table S2 (Supporting information).

2.3. Silkmoths, RNA isolation and RT-PCR

Fifth instar larvae of *A. assama* and *A. mylitta* were sex separated based on visualization of sex-specific glands present at their ventral surface. The sexual markings appear as a pair of milky white spots in the eighth and ninth larval abdominal segments in female, and are referred to as Ishiwata's Fore Gland and Ishiwata's Hind Gland, respectively. In males a small milky white body known as Herold's Gland appears ventrally in the centre, between eighth and ninth segments (Hiaso, 1994). Tissues were obtained from 3 days old 5th instar larvae, frozen in liquid nitrogen and stored at −70 °C till further use. RNA was isolated using Trizol method (Invitrogen Corporation, USA). DNAse treated total RNA was denatured at 75 °C for 10 min and immediately chilled on ice. First strand cDNA was synthesized by SuperScript III reverse transcriptase (Invitrogen, USA) using 17-mers polyT primer, according to the manufacturer's instructions. The conditions for DOP-PCR to obtain *dsx* sequences from *A. assama* and *A. mylitta* were initial denaturation at 94 °C for 2 min, 32 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min, and final extension at 72 °C for 10 min.

The smear obtained in the primary PCR was diluted 50 fold in water, and 1 μl was used for secondary PCR using the same degenerate primers (Dsx_1aF and New DsxR) and the same PCR conditions as mentioned above.

2.4. Rapid amplification of 5′ and 3′ ends of Aadsx and Amydsx

5′ and 3′ RACE reactions were performed using the RLM RACE kit (Ambion, USA) according to manufacturer's instructions, using one adapter-specific and the other gene-specific primers. The outer and the inner gene-specific primers for 5′ RACE of *Aadsx* were Aad_5′RACE1 (5′-ATCGATACTTGCAGTACCGTTTGTGGCC-3′) and Aad_5′RACE 2 (5′-AGTACCGTTTGTGGCCTTTCAGCTCGAC-3′) whereas for *Amydsx* were ML_5′RACE2 (5′-TGCTTTCACTATAGGCGGCTCCG GTC-3′) and Aad_5′RACE 2 (5′-AGTACCGTTTGTGGCCTTTCAGCTCG AC-3′), respectively. The outer and inner gene-specific primers used for 3′ RACE of *Aadsx* and *Amydsx* were Aad_3′RACE1 (5′-TTGCCACAAACTGCTGGAGAAGTTCCAC-3′) and Aad_3′RACE2 (5′-TGCTTTCACTATAGGCGGCTCCGGTC-3′), respectively. Both primary and secondary RACE-PCR reactions were performed on an Eppendorf master cycler with the PCR conditions of initial denaturation at 94 °C for 2 min, 35 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 2 min, and final extension at 72 °C for 10 min. Amplicons of different sizes were gel-eluted, sequenced and the genuine 5′ and 3′ *dsx* sequences were selected.

2.5. Southern blot analysis

A. assama genomic DNA isolated from male and female was digested separately with two restriction enzymes, *Nde*I or *Spe*I (NEB, USA) which have no recognition sites in the *Aadsx* cDNA sequences. Male and female genomic DNA (20 μg) was incubated with *Nde*I or *Spe*I enzymes at a final concentration of 5 U/μg, along with 1× NEBuffer 2, at 37 °C for 16 h and the digested DNA was electrophoresed overnight at 40 V on a 0.8% agarose gel. After capillary gel transfer under alkaline conditions (0.4 M NaOH, 1 M NaCl) to the Hybond N^+ nylon membrane (Amersham Biosciences, USA), pre-hybridization was done for 2 h at 65 °C in 10 ml pre-hybridization solution containing 5× Standard Saline Citrate (SSC), 5× Denhardt's solution (0.1% w/v bovine serum albumin, 0.1% w/v Ficoll, 0.1% w/v polyvinylpyrrolidone) and 0.5% SDS. 50 ng of purified probe DNA (the first exon) was radioactively labeled with α-^{32}P-dATP using Strip EZ kit (Ambion, USA) and added to the pre-hybridization solution at a concentration of $\sim 1 \times 10^6$ cpm/ml. Hybridization was carried out overnight at 65 °C and the hybridized membrane was washed thrice in 2× SSC, 0.1% Sodium Dodecyl Sulfate (SDS), at 60 °C for 10 min followed by two washes in 0.1× SSC, 0.1% SDS, at 60 °C for 5 min. The radioactive membrane was exposed to PhosphorImager (Amersham Biosciences, USA) screen and the results were analyzed with Image-Quant 5.0 software (Amersham Biosciences, USA).

2.6. Expression profile of Aadsx

RT-PCR was performed to analyze the expression pattern of *Aadsx* in different tissues including fatbody, midgut, silkgland, epidermis, gonads and head. The forward and reverse primers used

were – "3′RACE_Aad" (5′-GCCGTCGGTTCCGCCTTTACAGGCC-3′) and "Aad3′m3" (5′-ATTTATGTCCCACACGCTTC-3′). Primers for *Aa*Actin were used as endogenous control. PCR was carried out on an Eppendorf PCR master cycler using PCR conditions of 94 °C for 2 min (initial denaturation), 40 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 2 min, and final extension at 72 °C for 10 min. All the female- and male-specific amplicons were sequenced and were confirmed to be *dsx* transcripts. RT-PCR was done using combinations of common forward primer (F1) located in the first exon and eight different reverse primers (R1, R2, R3 R4, R5, R6, R7, R8) located in different exons of *Aadsx* (Fig. 1), using cDNA from fatbody as template. Core mix for a 10 μl PCR reaction was composed of 4.7 μl H_2O, 1 μl 10× PCR reaction buffer, 0.4 μl $MgCl_2$ (25 mM), 0.5 μl dNTP mix (5 mM), 1 μl each of forward and reverse primers (5 pm/μl), 0.8 μl cDNA, 4% DMSO and 0.2 μl Taq DNA polymerase (5 U/μl). The amplified products were separated on 1.5% agarose gel. The PCR product was cloned into pCR®II-TOPO® cloning vector (Invitrogen Life Technologies, USA). The cloned DNA fragments were sequenced with the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit and the ABI PRISM 3100 Genetic Analyzer. The gene-specific and the control primers used in different RT-PCR and PCR experiments are listed in Table S1 (Supporting information).

2.7. Sequence analysis

The sex-specific *Aadsx* transcripts were aligned using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Exon boundaries were marked based on the alignment results, and were further confirmed by genomic PCR using primers on the adjacent exons. The sex-specific *Amydsx* transcripts were also aligned using ClustalW. The sequences of *Aadsx* and *Amydsx* splice forms have been submitted to GenBank (Accession no. for *Aadsxf1*, *Aadsxf2*, *Aadsxf3*, *Aadsxf4*, *Aadsxf5*, *Aadsxf6* and *Aadsxm* are GU930279, GU930280, GU930281, GU930282, GU930283, GU930284 and GU930278, respectively. Accession no. for *Amydsxf1*, *Amydsxf2* and *Amydsxm* are GU930285, GU930286 and GU930287, respectively).

2.8. Target regions and double stranded RNA (dsRNA) synthesis

Two regions of the *Aadsx* transcripts i.e., 574 bp of exon 1 (T1) (present in all the splice forms of *Aadsx*) and 116 bp (T2) which includes 10 bp in exon 2 common to all the female-specific forms, 15 bp of exon 2 specific to *Amydsxf1*, *Aadsxf3* and *Aadsxf5* and 91 bp of exon 3 specific only to *Aadsxf1* (Fig. 1) were amplified using the specific primers, T1F–T1R and T2F–T2R, respectively (Supporting information, Table S2). The amplicons were cloned in pCR®II-TOPO® (Invitrogen Life Technologies, USA) followed by amplification with M13 forward and reverse primers. These templates with flanking T7 and SP6 promoters were used for *in-vitro* synthesis of sense and antisense RNA strands using T7 and SP6 Megascript kits (Ambion, USA). The synthesized RNA was treated with DNAse followed by purification by Trizol treatment (Invitrogen Life Technologies, USA) and isopropanol precipitation. The RNA was dissolved in DEPC treated water, combined in equimolar amounts in 1× insect buffer saline (NaCl-160 mM, KCl-10 mM, $CaCl_2$-4 mM) and annealed by heating to 95 °C for 10 min followed by overnight incubation at room temperature. dsRNA specific to ORF (718 bp) of green fluorescent protein (GFP) was used as a non-target control.

2.9. Injections of dsRNA and knock-down of Aadsx gene

70 μg of dsRNA per larva was injected into a set of 16 (8 males and 8 females) one day old fifth instar *A. assama* larvae at their fourth abdominal legs using insulin syringe. GFP-dsRNA injected ($n = 15$), saline injected ($n = 15$) and uninjected larvae of the same developmental stage were maintained as experimental controls. The larvae were reared on their natural host plants (*Litchia polyantha*). Six days post injection, six (3 males and 3 females) larvae from each set of injected and uninjected batches were dissected and their gonads were observed under microscope. Fatbodies of the dissected larvae were collected separately in 'RNA-later' (Ambion, USA) and stored at −70 °C until further use. The moths eclosed from the remaining injected and uninjected larvae were allowed to mate and lay eggs. These eggs were observed till hatching. Mean values of eggs laid in each group were compared using student's *t*-test.

3. Results

3.1. Analysis of A. assama dsx

DOP-PCR generated 490 bp of *dsx* sequences from *A. assama* and *A. mylitta*. 5′ and 3′ RACE-PCRs, performed on total RNA samples from fatbody of 5th instar male and female larvae, yielded full length transcripts including UTR sequences. In *A. assama*, the 5′ RACE-PCR yielded 225 bp sequence upstream of the gene-specific

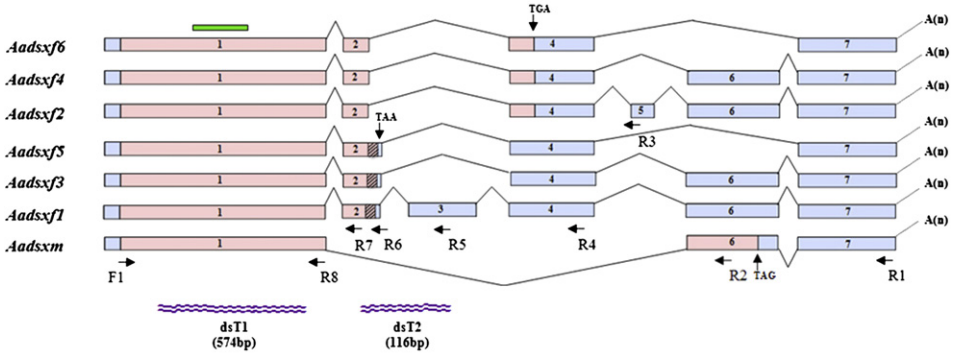

Fig. 1. Schematic representation of splice forms of *Aadsx* pre-mRNA, showing the primer positions, probe region and regions used for the preparation of dsRNA for the RT-PCR, Southern hybridization and RNAi experiments, respectively. Boxes are exons and lines are introns. Pink coloured portion is the ORF whereas blue coloured regions are UTRs. Seven different splice products of *Aadsx* pre-mRNA, six female-specific (*Aadsxf1 Aadsxf2 Aadsxf3 Aadsxf4 Aadsxf5* and *Aadsxf6*) and one male-specific (*Aadsxm*), are produced. A (n) represents the polyadenylation site. Vertical arrows represent stop codon sites whereas horizontal arrows represent primer positions. Hatched boxes indicate 15 bp additional sequences in the exon 2 present only in *Aadsxf1, Aadsxf3 and Aadsxf5*. dsRNA produced for exon 1 (T1 region) is of 574 bp whereas it was of 116 bp for T2 region which includes 10 bp in exon 2 common to all the female splice forms, 15 bp in exon 2 specific to *Aadsxf1, Aadsxf3 and Aadsxf5* splice forms and 91 bp of exon 3 specific only to *Aadsxf1*. Green colour bar represents the probe region used for Southern hybridization and wavy lines represent the regions used for dsRNA synthesis.

primer binding site. The 3′ RACE, on the other hand, amplified 6 amplicons of 1092 bp, 1022 bp, 959 bp, 944 bp, 744 bp and 729 bp in female and one amplicon of 690 bp in male. These were further confirmed to be genuine stretches of *Aadsx* transcript by aligning the overlapping sequences and performing BLASTx searches. All the seven *dsx* (six female- and one male-specific) sequences revealed overlapping and non-overlapping regions. The full length sequences of sex-specific *Aadsx* transcripts were confirmed after the assembly of the RACE sequences with the sequence obtained through DOP-PCR. RT-PCR with the specific primers, 3′RACE_Aad and Aad3′m3 in the first and the last exons, respectively using male and female fatbody cDNAs as template generated six amplicons (*Aadsxf1*, *Aadsxf2*, *Aadsxf3*, *Aadsxf4*, *Aadsxf5* and *Aadsxf6*) in female and one in male (*Aadsxm*). RT-PCR using cDNA as template from different tissues of *assama* also yielded similar results (Fig. 2). In addition, we observed very faint male-specific transcript in all the female tissues examined (Fig. 2).

Alignment of sequences of full length *Aadsx* transcripts (Supporting information, Fig. S2), RT-PCR results and genomic PCR results (not shown) confirmed that *Aadsx* gene is comprised of seven exons of variable lengths (Table 2). Exons 2–5 are skipped in males, leading to change in the reading frame (Fig. 1). Exons 1, 2, 4 and 7 are common to all the female-specific *dsx* transcripts; exons 3 and 5 are specific to *Aadsxf1* and *Aadsxf2*, respectively and exon 6 is present in *Aadsxf1*, *Aadsxf2*, *Aadsxf3* and *Aadsxf4* and absent in *Aadsxf5* and *Aadsxf6* (Table 1). A 15 bp stretch (GTACGGACTTTAATA), generated as a result of alternative 5′ splice site selection at exon 2, is present in the female-specific transcripts *Aadsxf1*, *Aadsxf3* and *Aadsxf5* (Fig. 1). Tissue-specific expression profile showed no difference in the expression pattern of different *Aadsx* splice forms in different tissues (Fig. 2) and in different developmental stages tested (data not shown). RT-PCR, using the common forward primer (in the first exon) in combination with different reverse primers (in different exons) (Fig. 1), gave the expected numbers and sizes of the amplicons in both male and female (Fig. 3). The primer combinations, the amplified transcripts and the lengths of the amplicons in each RT-PCR experiments are summarized in Table 3.

3.2. Analysis of A. mylitta dsx

In *A. mylitta*, a single sequence of 229 bp (upstream of the primer binding site) was obtained from 5′ RACE-PCR product, in both the sexes whereas 3′ RACE-PCR products yielded two sequences (downstream of primer binding site) of 845 bp and 860 bp in female and one sequence of 590 bp in male. Two female (*Amydsxf1* and *Amydsxf2*) and one male-specific (*Amydsxm*) sequences were assembled. The RT-PCR using primers (F1 and Amydsx_R2) designed from the beginning (F1) to the end (Amydsx_R2) of the *Amydsx* transcripts confirmed the RACE results (Fig. 4). The full length *Aadsx* and *Amydsx* transcript sizes are given Table 1.

We could not deduce the exon–intron boundaries for *Amydsx* gene. Alignment of sex-specific *Amydsx* transcripts (Supporting information, Fig. S3) confirmed the existence of two female splice forms whose difference is attributed to the same stretch of 15 bp sequence as observed in *Aadsx*.

3.3. Fourth exon sequence of Bmdsx is conserved in Aadsx and Amydsx

As described earlier, 4th exon of *Bmdsx* contains the *cis*-acting element (PESS-Putative Exonic Splicing Silencer) responsible for the sex-specific splicing of *Bmdsx* pre-mRNA and skipping of exon 3 and exon 4 in *Bmdsx*m splice form. On analyzing the nucleotide sequence of *Aadsx* and *Amydsx* transcripts, we found that the nucleotide sequence of 4th exon of *Bmdsx* is relatively well conserved in *Aadsx* and *Amydsx*. Notably, the PESS sequence of *Bmdsx* is 100% conserved (Fig. 5). This sequence is present in all the female splice variants of *Aadsx* as well as in the female splice forms of *Amydsx*, but not in the male splice variants.

3.4. Genomic copy number of Aadsx

To test whether the multiple sex-specific *Aadsx* transcripts are produced as a consequence of alternative splicing of pre-mRNA from a single gene or are transcribed by more than one gene, copy number of *Aadsx* gene was determined by Southern hybridization. Genomic DNA from male and female larvae was digested separately using two different restriction enzymes (*Nde*I or *Spe*I). 370 bp from the common region (first exon) of *Aadsx* transcripts (Fig. 1) was

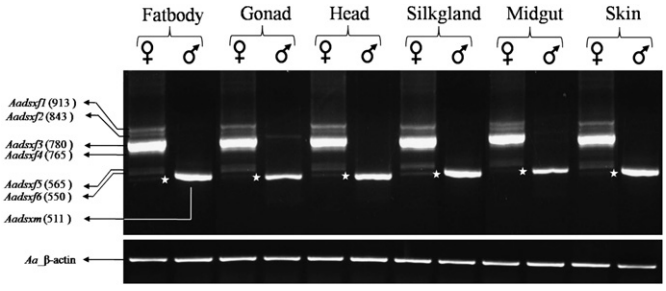

Fig. 2. RT-PCR using cDNA from different *A. assama* tissues as template and forward (3′RACE_Aad) and reverse (Aad3′m3) primers designed in the common region of the first and last exons of *Aadsx* (see text). Multiple bands were amplified in all the female organs whereas only single band was amplified from the male organs which were subsequently confirmed to be *Aadsx* transcripts. The splice variants are mentioned on the left of the diagram. *A. assama* β-actin gene was amplified as a control. Faint male-specific band (indicated by star) amplified in all the female tissues.

Table 1
Transcript lengths of *Aadsx* and *Amydsx*, presence of different exons in sex-specific transcripts of *Aadsx*.

Serial No.	Transcript	Length of the transcript (bp)	Exons present
1	*Aadsxf1*	1746	1, 2, 3, 4, 6, 7
2	*Aadsxf2*	1676	1, 2, 4, 5, 6, 7
3	*Aadsxf3*	1614	1, 2, 4, 6, 7
4	*Aadsxf4*	1598	1, 2, 4, 6, 7
5	*Aadsxf5*	1398	1, 2, 4, 7
6	*Aadsxf6*	1383	1, 2, 4, 7
7	*Aadsxm*	1344	1, 6, 7
8	*Amydsxf1*	1587	–
9	*Amydsxf2*	1603	–
10	*Amydsxfm*	1333	–

Note: The second exon of *Aadsxf1*, *Aadsxf3* and *Aadsxf5* differs from that of *Aadsxf2*, *Aadsxf4* and *Aadsxf6* by the presence of 15 bp stretch generated as a result of alternative 5′splicing.

Table 2
Exons of *Aadsx* and their corresponding length.

Serial No.	Exon No.	Exon length (bp)
1	Exon 1	741
2	Exon 2	84 or 99[a]
3	Exon 3	133
4	Exon4	170
5	Exon 5	78
6	Exon 6	215
7	Exon 7	388

[a] Exon 2 of transcripts *Aadsxf1*, *Aadsxf3* and *Aadsxf5* is of 99 bp whereas exon 2 of *Aadsxf2*, *Aadsxf4* and *Aadsxf6* is of 84 bp.

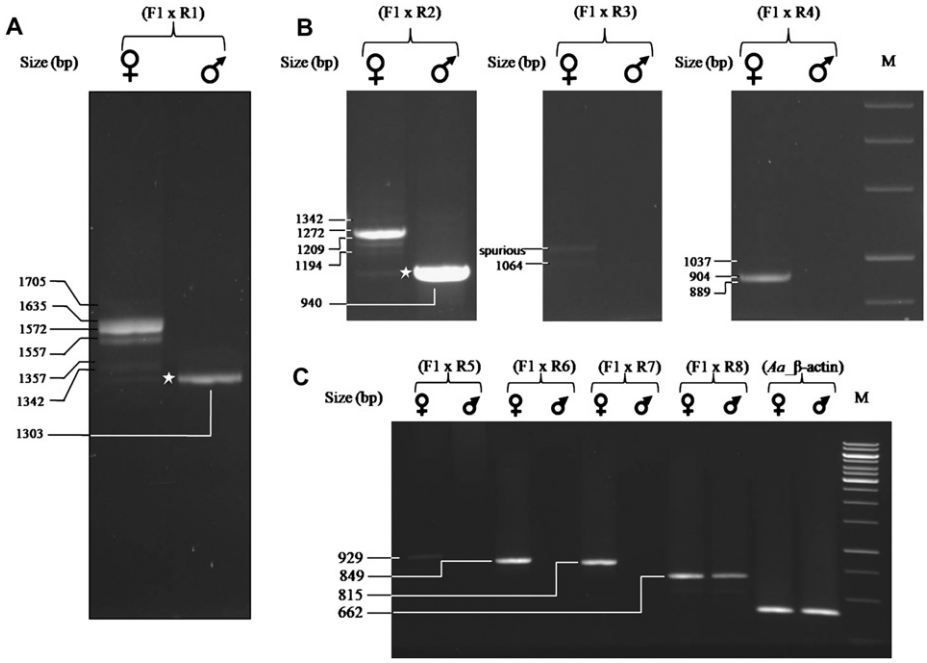

Fig. 3. RT-PCR using cDNA from *A. assama* fatbody as template and the combinations of one common forward (F1) and eight different reverse (R1-R8) primers (Fig. 1). A) RT-PCR of the first combination (F1 × R1) has been shown in an enlarged view for clarity and the molecular sizes of the amplified bands has been indicated on left of the figure. B) RT-PCR products generated by primer combinations F1 × R2, F1 × R3 and F1 × R4 resolved on the same gel. M is the 1 kb marker C) RT-PCR by the primer combinations F1 × R5, F1 × R6, F1 × R7, F1 × R8 and *A. assama* β-actin. M is the 1 kb marker.Number of amplicons is consistent with the primer set used. The primer combinations, the amplified transcript(s) and the length(s) of the amplicons in each of the RT-PCR experiments are summarized in Table 3.

used as a probe for hybridization. A single band, common to both the sexes hybridized to the probe (Fig. 6), suggesting that *Aadsx* is present as a single copy per haploid genome.

3.5. Sex-specific proteins encoded by Aadsx and Amydsx transcripts

On the basis of virtual translation, the six female-specific *Aadsx* transcripts can be grouped into two, three transcripts (*Aadsxf2*, *Aadsxf4* and *Aadsxf6*) coding for the female-specific protein, AaDSX^{F1} and the other three transcripts (*Aadsxf1*, *Aadsxf3* and *Aadsxf5*) coding for the second female-specific protein, AaDSX^{F2} (Fig. 7). The male-specific splice form, *Aadsxm*, on the other hand, codes for only one male-specific protein, AaDSX^{M} (Fig. 7). The two female-specific *Amydsx* transcripts encode two female-specific putative proteins (AmyDSX^{F1} and AmyDSX^{F2}) whereas the male-specific transcript encodes male-specific putative protein AmyDSX^{M} (Fig. 7). The putative DSX proteins of *A. assama* and *A. mylitta*, DSX^{F1}, DSX^{F2} and DSX^{M} are of 265aa, 247aa and 279aa, respectively.

The deduced amino acid (aa) sequences of DSX proteins encoded by sex-specific *Aadsx* and *Amydsx* transcripts can be divided

Table 3
Aadsx transcripts amplicon size profile using different primer-pairs.

S. No.	Primer combinations	Transcripts amplified	Amlicon size (bp)
1	F1 × R1	Aadsxf1	1705
		Aadsxf2	1635
		Aadsxf3	1572
		Aadsxf4	1557
		Aadsxf5	1357
		Aadsxf6	1342
		Aadsxm	1303
2	F1 × R2	Aadsxf1	1342
		Aadsxf2	1272
		Aadsxf3	1209
		Aadsxf4	1194
		Aadsxm	940
3	F1 × R3	Aadsxf2	1064
4	F1 × R4	Aadsxf1	1037
		Aadsxf2, Aadsxf4, Aadsxf6	889
		Aadsxf3, Aadsxf5	904
5	F1 × R5	Aadsxf1	929
6	F1 × R6	Aadsxf1, Aadsxf3, Aadsxf5	849
7	F1 × R7	Aadsxf1, Aadsxf2, Aadsxf3, Aadsxf4, Aadsxf5, Aadsxf6	815
8	F1 × R8	Aadsxf1, Aadsxf2, Aadsxf3, Aadsxf4, Aadsxf5, Aadsxf6, Aadsxm	662

Note: All the different *Aadsx* transcripts in this table are represented by f1–f6 (female-specific transcripts) and m (male-specific transcript).

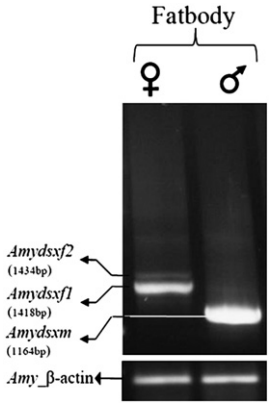

Fig. 4. RT-PCR using cDNA from *A. mylitta* fatbody as template and forward (F1) and reverse primers (Amydsx_R2), designed in the first and last exons of *Amydsx* (see text). Two *Amydsx* bands are amplified in female whereas only single band is amplified in male. *Amydsx* splice variants and their respective sizes are indicated on the left side of the diagram.

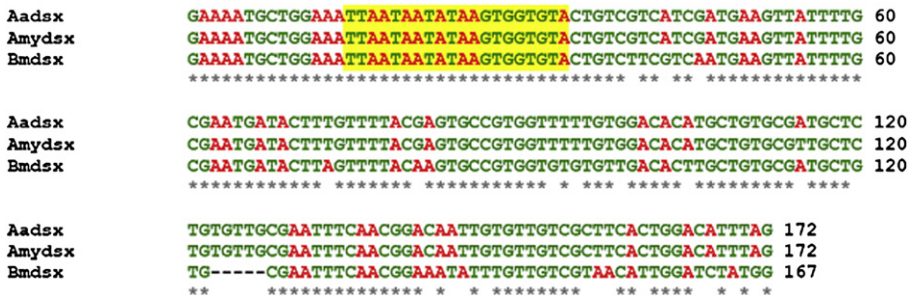

Fig. 5. ClustalW alignment of 4th exon of *Bmdsx* and *Aadsx* and the corresponding sequence of *Amydsx*. The *Bmdsx* nucleotide stretch in the yellow background is the *cis*-acting element responsible for the skipping of *Bmdsx* exon 3 and exon 4 in males (Suzuki et al., 2008).

into three parts: the region common to both the sexes (aa 1–216), the female-specific regions (aa 217–265 of DSX^{F1} and aa 217–247 of DSX^{F2}), and the male-specific region (aa number 217–279) (Fig. 7). The female-specific (DSX^{F1} and DSX^{F2}) and male-specific (DSX^{M}) proteins are similar at their N-termini but differ at their C-termini, starting from the C-terminal region of DBD/OD2 domains. Both the female proteins (DSX^{F1} and DSX^{F2}) differ at their C-termini by 21aa (Fig. 7). The predicted AmyDSX and AaDSX proteins in both male and female were 100% identical at OD1 and OD2 domains but at nucleotide level their identity was 92 and 93% respectively. But both the proteins differed from BmDsx by 4 out of 63 residues at OD1 domain and 5 out of 61 residues at OD2 domain (90% identity) with 80% identity at nucleotide level (Fig. 7, Fig. S3A). In contrast to their female counterparts, the male-specific regions were remarkably poorly conserved (Fig. 7). Both AaDSX and AmyDSX male-specific regions differed from that of BmDSX by 31 out of 51 residues. The otherwise remarkably well conserved AaDSX and AmyDSX also differed from each other by as many as 5 out of 63 residues in this region (Fig. 7).

3.6. *In vivo knock-down of Aadsx by RNAi inhibits the expression of downstream genes required for female sexual differentiation*

In order to establish the functional conservation of *Aadsx* gene, the dsRNA specific to different regions (Fig. 1) of *Aadsx* transcripts were administered into the haemocoel of *A. assama* larvae. Four different parameters were checked for the effectiveness of RNAi experiments: a) Absence of target transcript/s, b) Change in the expression profile of *Aadsx* downstream target genes, c) Change in the anatomy and morphology of sex organs and secondary sexual characters, and d) Mating behaviour, egg laying and hatching of the eggs. To check the downstream effect of *Aadsx* transcript/s knock-down, the expression profile of two *A. assama* genes i.e., *vitellogenin* and *hexamerin* were analyzed by semi-quantitative RT-PCR. In insects, *vitellogenin* and *hexamerin* genes are the direct targets of the DSX proteins, and are predominantly expressed in females (Burtis et al., 1991; Coschigano and Wensink, 1993; Izumi et al., 1988; Suzuki et al., 2003; Yano et al., 1994; Zakharkin et al. 2001). Expression of *vitellogenin* and *hexamerin* homologues in *A. assama* is also female-specific as confirmed by RT-PCR results (Fig. 8). RT-PCR of cDNA from female larval fatbody showed complete abolishment of the target transcripts in the *Aadsx*-specific dsRNA treated larvae whereas in male organs *Aadsx* transcript remained unaffected (Fig. 8). Though no significant morphological defect was observed in the moths eclosed from the RNAi-treated larvae, at molecular level, expression of *vitellogenin* and *hexamerin* gene was completely abolished in all the *Aadsx*-dsRNA treated female larvae (Fig. 8). The gonads of the dsRNA injected female larvae were deformed and shrunken compared to those of control female larvae (Fig. 9). No knock-down effect was observed seen at molecular level in the treated males. We also checked *Aadsx* knock-down effect in the next generation offspring by crossing female and male moths derived from *Aadsx*-dsRNA injected larvae; T1 females with T1 males, T2 females with T2 males and control females with control males. The number of eggs laid by the knock-down females mated with knock-down males reduced drastically in all the *Aadsx*-dsRNA injections compared to that of control females ($P > 0.0001$) (Table 4). Moths derived from the dsGFP and saline injected larvae laid the same number of eggs as that of uninjected control (Table 4). The eggs laid by moths derived from different crosses of treated individuals failed to hatch, whereas moths derived from uninjected females mated with *Aadsx*-dsRNA injected males recorded normal fecundity and hatchability (Table 4).

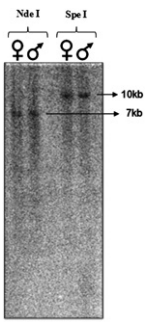

Fig. 6. *Aadsx* is present as a single copy per haploid genome. Southern hybridization was performed with a radiolabeled probe common to both male and female *Aadsx* (Fig. 1), that has no restriction site recognized by any of the two enzymes used. The molecular sizes of the hybridized bands are indicated on the right side of the figure.

4. Discussion

Production of sex-specific *doublesex* (*dsx*) transcripts is a general feature reported till date in insects where *dsx* has been characterized. In all the dipteran *dsx* reported so far, one male- and one female-specific transcripts are produced using alternative splicing and alternative polyadenylation. The same is also true for the lepidopteran, *B. mori dsx* gene (*Bmdsx*) (Ohbayashi et al., 2001). One exception is the *A. mellifera dsx* (*Amdsx*) gene, which produces 4 alternatively spliced transcripts, two specific to female, one specific to male and one common to both the sexes. Both the female-specific transcripts share a common ORF, coding for the similar putative protein (Cho et al., 2007). A generalized fact that emerges from the available data on *dsx* genes and their splice forms in different organisms is, *dsx* produces sex-specific alternatively spliced transcripts to produce one male- and one female-specific DSX proteins which differ only at their extreme C-termini.

In the study reported here we show that both *Aadsx* and *Amydsx* produce sex-specific transcripts. Unlike the case of *dsx* splicing in

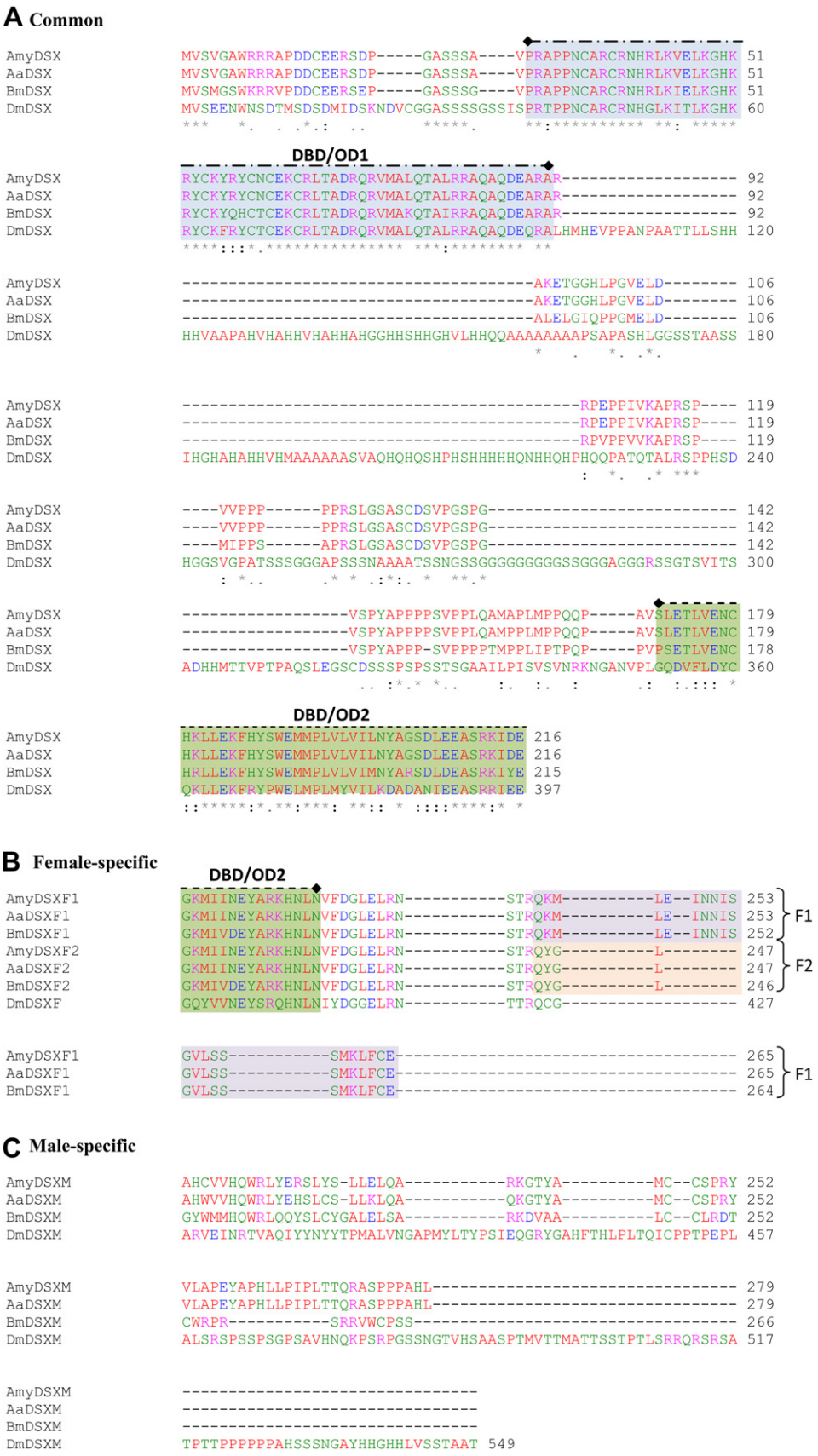

Fig. 7. Amino acid sequence alignment of DSX proteins of *A. mylitta* (*Amy*), *A. assama* (*Aa*), *B. mori* (*Bm*) and *D. melanogaster* (*Dm*). The sequence is divided into (A) the region common to males and females, (B) the conserved female-specific region and (C) the male-specific region. The DNA binding domain (OD1) is boxed in blue and oligomerization domain (OD2) is shown in green. Three female splice forms (*Aadsxf2*, *Aadsxf4* and *Aadsxf6*) of *Aadsx* code for AaDSXF1 whereas the other three splice forms (*Aadsxf1*, *Aadsxf3* and *Aadsxf5*) code for AaDSXF2. The two female splice forms of *Amydsx* code for two female-specific proteins, AmyDSXF1 and AmyDSXF2.The female splice forms of *Bmdsx* also encode two female-specific proteins, BmDSXF1 and BmDSXF2 (Shukla, Jadhav and Nagaraju, In press). The female splice forms of *Dmdsx* code for single female-specific protein, DmDSXF. The male-specific transcripts of *Aadsx, Amydsx, Bmdsx* and *Dmdsx* encode only one male-specific protein, AaDSXM, AmyDSXM, BmDSXM and DmDSXM, respectively. The two female-specific proteins, F1 (purple shaded) and F2 (orange shaded) in all the three species of silkmoths differ in their size. The male-specific proteins differ from the female-specific proteins in their C-terminal regions.

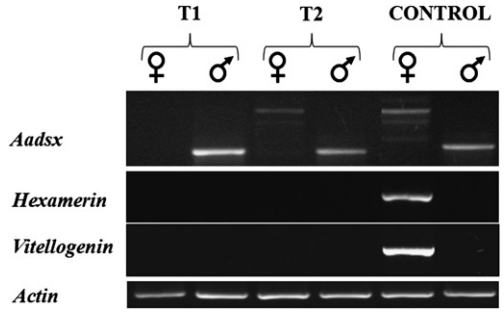

Fig. 8. Expression profile of *Aadsx*, *hexamerin* and *vitellogenin* genes in the fatbody tissue of knock-down and control larvae. Complete abolishment of *vitellogenin* and *hexamerin* expression was observed in females with the knock-down of *Aadsx* transcripts. Males were unaffected by the dsRNA injections. *A. assama* β-actin gene was used as a loading control.

Table 4
Effect of dsRNA injections on egg laying and their hatching.

Injection type	No. of eggs laid	No. of eggs hatched
T1	≈16	No hatching
T2	≈19	No hatching
Control (dsGFP)	≈170	170 (all)
Control (Uninjected)	≈170	171 (all)

There is a significant difference ($P < 0.0001$) in the number of eggs laid by control moths and the treated moths.

other insect species, the pre-mRNA of *Aadsx* gene sex-specifically splices to produce six splice variants in female and one in male whereas the pre-mRNA of *Amydsx* gene splices to produce two female- and one male-specific transcripts. As in the case of *Bmdsx*, there is no *dsx*RE (TRA/TRA-2 binding sequence) and PRE sequences in any of the *Aadsx* and *Amydsx* transcripts. Analysis of different

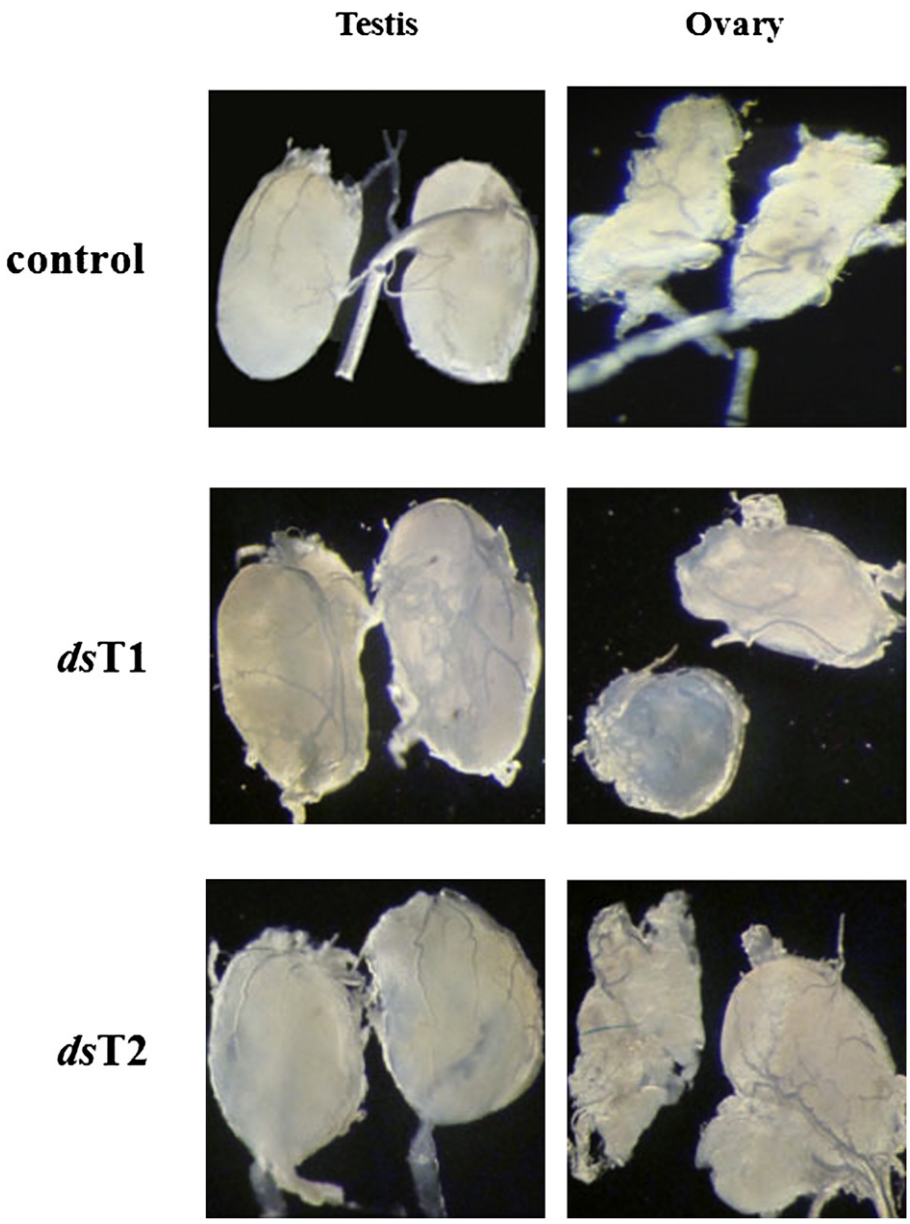

Fig. 9. Effect of *Aadsx*-dsRNA injections on the larval gonads. Control gonads are from the larvae injected with dsGFP. Testis of the experimental larvae seems to be normal (consistent with the expression of *Aadsxm* – Fig. 8) whereas the ovaries are deformed compared to the ovaries of control larvae.

splice variants of *Aadsx* revealed splice junctions which were further confirmed through genomic PCR results. All these results indicated the presence of 7 exons in *Aadsx* gene. The PESS sequence, responsible for sex-specific splicing of *Bmdsx* (Suzuki et al., 2008), was found to be 100% conserved in *Aadsx* and *Amydsx* which points to the existence of a common mechanism of sex-specific splicing of *dsx* in silkmoths. It remains to be investigated whether or not these sequences are involved in splicing regulation of *Aadsx* and *Amydsx*. The female-specific *Aadsx* transcripts having common ORF differ in their 3′ UTR region which might have some regulatory significance since UTRs are the known targets of proteins or miRNAs for spatial and temporal regulation of translation (Ambros, 2004; Beckmann et al., 2005). Difference in the ORFs of two groups of female-specific *Aadsx* transcripts and two female-specific *Amydsx* transcripts is due to the presence or absence of a 15 bp stretch which results in the alternative 5′ selection at exon 2. The two female-specific DSX proteins (DSX^{F1} and DSX^{F2}) of *A. assama and A. mylitta* share all the features of a functional DSX protein. They have common DNA binding (OD1 domain) and oligomerization domain (OD2 domain) but differ in their amino acid composition at their extreme C-terminal ends; the longer of the two female-specific Dsx proteins ($AaDSX^{F1}$ and $AmyDSX^{F1}$) has additional 21aa whereas the shorter one ($AaDSX^{F2}$ and $AmyDSX^{F2}$) has an additional stretch of 3aa, after the common region (1–244 aa). As is the case with sex-specific DSX proteins of the other insect species, the $AaDSX^{M}$ and $AmyDSX^{M}$ proteins differ from female DSX proteins at their C-termini such that the C-terminal region of OD2 domain is different in the sex-specific DSX proteins.

In light our finding of two female splice forms of *Aadsx* and *Amydsx* encoding two female-specific DSX proteins, we revisited the *Bmdsx* splice forms. We searched for the 15 bp stretch, which results in two female splice forms in *Anthearea*, in the *B. mori* EST database, which led to the identification and confirmation of existence of a novel female splice form of *Bmdsx* (*Bmdsxf1*) having this stretch. The protein ($BmDSX^{F1}$) encoded by *Bmdsxf1* is identical to $BmDSX^{F}$ protein reported earlier except for the difference in their extreme C-terminal regions such that after the identical region till aa 243, $BmDSX^{F1}$ contains 3 additional aa whereas $BmDSX^{F}$ contains 21aa (Shukla, Jadhav and Nagaraju, manuscript communicated).

Existence of two female-specific DSX proteins in silkmoths (*A. assama*, *A. mylitta* and *B. mori*) raises a possibility that both are required to exert effect on their downstream genes involved in sexual differentiation. Our results on RNAi mediated knock-down experiments in *A. assama* corroborate our contention. The complete abolishment of expression of *vitellogenin* and *hexamerin* genes that are downstream target genes of *dsx*, deformed gonads of the injected female larvae, reduced fecundity and complete lethality of eggs derived from the *Aadsx*-dsRNA treated larvae underscore the importance of *Aadsx* as a terminal regulatory gene in the hierarchy of regulatory genes controlling the sexual differentiation of *A. assama*. The failure of knock-down in male larvae may be because of the secondary structure of male-specific transcript *Aadsxm*, which does not allow the dicer components to access the transcript (Shao et al., 2007). The effect of knock-down seen at the molecular level is consistent with the complete failure of egg hatching in the treated batches; however, no visible morphological changes were noticed. It may be because of the involvement of additional factors in the sexual differentiation of the organism, governed by the common upstream signal but making a separate path other than the DSX, to regulate all aspects of sexual differentiation. The knock-down of the transcripts having 15 bp additional sequence (contributing to the change in the ORF) also gave the same results as that of the knock-down of all the *Aadsx* transcripts together. This raises two possibilities: (i) Both the female-specific AaDSX proteins are essential for the process of female sexual differentiation. (ii) Alternatively, the group of transcripts that code for the longer protein ($AaDSX^{F1}$) may not have any function, as a result, either knock-down of all the transcripts at a time or only the knock-down of transcripts of second group (*Aadsxf1*, *Aadsxf3* and *Aadsxf5*) produces similar results. The first possibility seems reasonable considering the existence of two female-specific transcripts having two different ORFs in another wild silkmoth, *A. mylitta*, similar to that of *Aadsx*. Further functional characterization of these proteins and their binding to the regulatory sequences of the downstream genes may shed light on this aspect. Another evidence for the requirement of both the female-specific DSX proteins in the sexual differentiation process comes from *B. mori* transgenesis experiments where silkworm transgenic lines ectopically expressing $BmDSX^{F}$ showed less pronounced sexual differentiation process and change in the morphology and expression levels of *vg*, *Sp1* and *PBP* genes compared to those that ectopically expressed $BmDSX^{M}$. These results suggest the recruitment of additional factor by the $BmDSX^{F}$ protein to exert its effect fully on female sexual differentiation (Suzuki et al., 2003, ,2005). The $BmDSX^{F1}$ that we have identified may indeed be this additional factor. The existence of two DSX^{F} forms in all the silkmoths we studied, and the results obtained with the *Aadsx* knock-down suggest that both the DSX proteins are important in sexual differentiation in silkmoths. We believe that our finding of *dsx* splice forms encoding two female-specific proteins (DSX^{F1} and DSX^{F2}), opens up avenue to gain insight into the sexual differentiation process governed by DSX proteins in the lepidopterans.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ibmb.2010.06.008.

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