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REVIEW ARTICLE

Doublesex: a conserved downstream gene controlled by diverse upstream regulators

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Abstract

Sex determination, an integral precursor to sexual reproduction, is required to generate morphologically distinct sexes. The molecular components of sex-determination pathways regulating sexual differentiation have been identified and characterized in different organisms. The *Drosophila doublesex* (*dsx*) gene at the bottom of the sex-determination cascade is the best characterized candidate so far, and is conserved from worms (*mab3* of *Caenorhabditis elegans*) to mammals (*Dmrt-1*). Studies of *dsx* homologues from insect species belonging to different orders position them at the bottom of their sex-determination cascade. The *dsx* homologues are regulated by a series of upstream regulators that show amazing diversity in different insect species. These results support the Wilkin's hypothesis that evolution of the sex-determination cascade has taken place in reverse order, the bottom most gene being most conserved and the upstream genes having been recruited at different times during evolution. The pre-mRNA of *dsx* is sex-specifically spliced to encode male or female-specific transcription factors that play an important role in the regulation of sexually dimorphic characters in different insect species. The generalization that *dsx* is required for somatic sexual differentiation culminated with its functional analysis through transgenesis and knockdown experiments in diverse species of insects. This brief review will focus on the similarities and variations of *dsx* homologues that have been investigated in insects to date.

[Shukla J. N. and Nagaraju J. 2010 *Doublesex*: a conserved downstream gene controlled by diverse upstream regulators. *J. Genet.* **89**, 341–356]

Introduction

Sex determination, the fundamental biological process required for shaping the sexual fate of organisms into either sex, comes into action very early during embryonic development (Bull 1983; Zarkower 2001). The sex-determination pathway of *Drosophila melanogaster* has been studied in great detail, and now much is known about the players involved in this pathway (MacDougall *et al*. 1995; Cline and Meyer 1996; Schutt and Nothiger 2000). The ratio of number of X chromosomes to autosomal sets (X : A ratio) is thought to provide the initial cue for the activation of *Sex-lethal* (*Sxl*), the primary gene of the sex-determination cascade (Cline 1984; Penalva and Sanchez 2003). Recently, it has been proposed that *Sxl* is activated through its early promoter (*Sxl-pe*) in females in response to the double dose of X-linked genes typically called X-signalling elements (XSEs), rather than the X : A ratio (Erickson and Quintero 2007). The double dose of X-linked genes leads to the synthesis of an early pulse of Sxl protein in females whereas a single dose of XSEs in males is unable to activate the *Sxl-pe*, resulting in the absence of early Sxl protein (Erickson and Quintero 2007). Transcripts from the late promoter (*Sxl-pm*) of *Sxl* are produced in both the sexes but an in-frame stop codon is present only in males. The early pulse of Sxl protein is required for processing of transcript from *Sxl-pm*. An autoregulatory feedback loop maintains the continuous supply of functional Sxl protein in females throughout development (Bell *et al*. 1991; Keyes *et al*. 1992; Penalva and Sanchez 2003). Absence of early pulse of Sxl protein in males leads to the default processing of *Sxl-pm* transcript, producing non-functional Sxl protein. The functional Sxl in females acts on the pre-mRNA of *transformer* (*tra*) gene resulting in the removal of the in-frame stop codon and production of functional Tra protein (Boggs *et al*. 1987; McKeown *et al*. 1987;

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Keywords. *doublesex*; alternative splicing; ODI domain; OD2 domain.

Belote *et al*. 1989; Inoue *et al*. 1990). The *tra* pre-mRNA in males, on the other hand, splices in a default manner leading to the retention of the in-frame stop codon, resulting in non-functional Tra (Boggs *et al*. 1987; Valcarcel *et al*. 1993). In females, the functional Tra protein, along with the protein product of a constitutive gene *tra-2*, ensures *doublesex* (*dsx*) pre-mRNA to follow the female splicing pathway, producing female-specific *dsx* mRNA (Hoshijima *et al*. 1991; Tian and Maniatis 1992). In males, the *dsx* pre-mRNA splices in a default manner to produce male-specific mRNA (Hoshijima *et al*. 1991).

Being well investigated at molecular level, the *Drosophila* sex determination pathway provided a reference system to follow the sex-determination pathway in other insects. The comparative evaluation of sex-determination mechanisms operative in different organisms not only helped us to understand the evolution of genes involved in sex determination in different insect species but also provided us with the basic information of the molecules required for the generation of two sexes. From the studies of several homologues of the sex-determination cascade genes of *D. melanogaster* one interesting fact has emerged: a diverse array of upstream genes and signals in the sex-determination cascade are used whereas genes at the bottom of the cascade are relatively few and are comparatively more conserved (Sanchez 2008); for e.g., *Sxl* acts as the upstream gene of sex-determination cascade only in drosophilids, and its role as a master switch gene has been validated in three *Drosophila* species: *D. melanogaster*, *D. virilis* (Bopp *et al*. 1996) and *D. subobscura* (Penalva *et al*. 1996). The *tra* gene of *Drosophila* is rapidly evolving but relatively more conserved than *Sxl*, occupying its place upstream to *dsx* in the sex-determination cascade of various dipterans (O'Neil and Belote 1992). As compared to *Drosophila* where splicing of *tra* pre-mRNA is regulated by Sxl protein, in tephritids and calliphorids (Acalyptratae) *tra* acts as upstream gene of their sex-determination cascade and its sex-specific splicing is brought about by an autoregulatory feedback loop to make functional protein only in females (Pane *et al*. 2002; Lagos *et al*. 2007; Concha and Scott 2009). Recently it has been shown that a similar splicing mechanism operates for *tra* pre-mRNA (*F* factor) of *Musca domestica* (Calyptratea species) (Hediger *et al*. 2010). These observations suggest that the conventional mechanism of sex determination in dipterans is represented by the system where *tra* acts as the primary switch gene and is controlled via an autoregulatory-feedback loop (Hediger *et al*. 2010).

The *tra-2* has been characterized in *D. virilis* (Chandler *et al*. 1997), which encodes a set of protein isoforms analogous to those of *D. melanogaster*, and can rescue *tra-2* mutations in the latter species. Outside drosophilids, the role of *tra-2* in *tra* autoregulation and *dsx* pre-mRNA splicing has been unambiguously demonstrated in *Musca* (Burghardt *et al*. 2005) and *Ceratitis* (Salvemini *et al*. 2009). Also, very recently in *Anastrepha* (Sarno *et al*. 2010) where autoregulation of *tra* has not been directly tested, the presence of Tra-Tra2 binding sites in the *tra* pre-mRNA (Ruiz *et al*. 2007) and the results of the *tra-2* knockdown analysis (Sarno *et al*. 2010) support the idea that the *Anastrepha tra* also shows autoregulation. The role of *tra-2* in *Musca*, *Ceratitis* and *Anastrepha* sex determination is evident by the fact that dsRNA mediated knockdown of *tra-2* in these insects results in complete reversal of genotypically female embryos into fertile adult pseudomales. As in the case of *Drosophila*, *tra-2* is transcribed in both the sexes in these insects, and its function is required for the female-specific splicing of *dsx* pre-mRNA. This system *tra* (+*tra -2*) → *dsx* of sex determination is prevalent in most dipterans compared to the *sxl* → *tra* (+*tra -2*) → *dsx* mode of sex determination which is confined only to drosophilids (Hediger *et al*. 2010).

In hymenopterans (wasps and bees), the upstream regulators of sex-determination cascade are different from that of dipterans. In some of these insects (e.g. *Apis mellifera*), heterozygosity at the '*complementary sex determining* (*csd*) locus' determines the femaleness whereas hemizygous or homozygous individuals at this locus develop into males (Beye *et al*. 2003). In *A. mellifera*, *csd* acts as the switch gene to initiate the female determination cascade. The functional CSD protein (an SR-type protein) in females serves as a potential splicing factor to execute splicing of pre-mRNA of the *feminizer* (*fem*) gene to produce functional Fem protein only in females (Gempe *et al*. 2009). Fem, an ortholog of *Drosophila* Tra, is an SR protein which, besides acting on *A. mellifera dsx* (*Am-dsx*) pre-mRNA to produce female splice variant (*Am-dsxf*), maintains its functional state through an autoregulatory-feedback mechanism throughout development to retain the female determined state (Gempe *et al*. 2009). Absence of functional CSD protein in males leads to default splicing of *fem* pre-mRNA resulting in truncated non-functional protein and hence default splicing of *Am-dsx* pre-mRNA takes place to generate *Am-dsxm*.

In another hymenopteran insect, *Nasonia vitripennis*, *csd* does not act as a primary signal for sex determination as seen in *A. mellifera* (Werren *et al*. 2010). Instead, the sex of the individual is signalled by maternal imprinting effect. Imprinting in the maternal genome prevents the zygotic transcription of maternally derived *Nvtra* allele in unfertilized haploid males, which receive only the maternal genome. On the other hand, in females, fertilization triggers the transcription of *Nvtra* allele from the paternal genome. The zygotic Tra protein, in females, initiates and establishes an autoregulatory loop of *tra*, providing a continuous supply of functional Tra protein which results in the splicing of *dsx* pre-mRNA in the female mode (Verhulst *et al*. 2010). Due to the absence of initial zygotic Tra protein, male embryos fail to establish the *tra* autoregulatory loop thus resulting in male-specific splicing of *tra* and *dsx* pre-mRNA.

Among lepidopterans (moths and butterflies) only silkmoths have been investigated in some detail for their sex-determination pathway genes. In the domesticated silkmoth,

Bombyx mori, regardless of the number of Z chromosomes, the presence of a W-chromosome ensures female development whereas in its absence male development takes place. This led to the prediction that the W chromosome carries a dominant epistatic factor that determines femaleness (Hasimoto 1933; Tazima 1964). Recently two genes (*Z1* and *Z20*), encoding Zn finger protein motifs have been identified in the minimal *fem*-region of the W chromosome which appear to act as upstream regulatory signals in the silkworm sex-determination pathway (Ajimura *et al.* 2006; Satish *et al.* 2006). As expected from the divergence time between lepidopterans and dipterans, a *tra* homologue has not been found in the whole genome sequence of *Bombyx* (Mita *et al.* 2004; Xia *et al.* 2004). Since *tra* evolves rapidly it is quite possible that the *tra* homolog in *Bombyx* has diverged to such an extent that the sequence homology searches have not been able to pick it up. Also, the sequence corresponding to the *dsx*RE/PRE to which Tra binds in *Drosophila* is probably much diverged that it is beyond identification through simple sequence homology searches. Example for such a possibility comes from the recent studies on sex-determination cascade in *A. mellifera*, where the *dsx*RE/PRE sites were reported to be absent in the *Am-dsx* (Cho *et al.* 2007), but a functional *tra* ortholog (termed *feminizer*) was subsequently found (Hasselmann *et al.* 2008; Gempe *et al.* 2009). The *Sxl* homologue is present in the *Bombyx* genome but is not sex-specifically spliced suggesting that it may not have any role in sex determination (Niimi *et al.* 2006). Also, the homologue of *tra-2* has been characterized in *Bombyx* where its pre-mRNA produces multiple mRNAs through alternative splicing encoding six distinct BmTra-2 proteins (Niu *et al.* 2005), but their role in sex-specific splicing of *Bmdsx* remains to be demonstrated. Besides, no generalized sex-determination mechanism can be assumed to be operative in silkmoths since the wild silkmoth *Antheraea assama*, which has diverged from *B. mori* approximately 160.9 million years ago does not contain a W chromosome (ZZ male and ZO females) (Deodikar *et al.* 1962).

We speculate that different upstream regulators exist among lepidoperans with W and without W chromosomes. Even though the upstream regulators of sex-determination cascade are different in lepidopterans and dipterans, the homologue of *dsx* (*Bmdsx*) is functionally conserved in *B. mori* (Suzuki *et al.* 2001). Recently, *dsx* homologues *Aadsx* and *Amydsx* from two wild silkmoths, *A. assama* and *A. mylitta* respectively, have been characterized (Shukla and Nagaraju 2010). The *dsx* of lepidopterans have some conserved features that are very different from that of dipteran *dsx* (Suzuki 2010, this issue pp. 357–363). The characteristic features of *dsx* homologues from dipterans, lepidopterans and hymenopterans are discussed in this review.

Doublesex (dsx) gene

As mentioned earlier, *dsx* is the most downstream gene of the *Drosophila* sex-determination cascade. *dsx* is transcribed in both sexes but its pre-mRNA sex-specifically splices to produce a male (*dsxm*) and a female-specific (*dsxf*) splice form which code for a male (DsxM) and female-specific (DsxF) protein, respectively (Baker and Wolfner 1988; Burtis and Baker 1989). Sex-specific Dsx proteins have two functional domains, an N-terminal DNA binding domain (DM or OD1 domain) and C-terminal dimerization domain (OD2 domain). The male and the female Dsx proteins share a common OD1 domain (Erdman and Burtis 1993) but differ at their OD2 domains (Erdman *et al.* 1996). The OD2 domains of DsxF and DsxM differ only in their sex-specific C-terminal regions attributing the antagonistic effect of sex-specific Dsx proteins on their downstream target genes. The DM domain contains a non-classical Zn module (Zhu *et al.* 2000) and its DNA binding activities are enhanced by a C-terminal dimerization domain (Cho and Wensink 1998) mediated by a novel α-helical dimer containing ubiquitin-associated (UBA-like) folds (Bayrer *et al.* 2005). As compared to the upstream genes (*Sxl* and *tra*) of the sex-determination cascade which produce their functional products only in females, *dsx* encodes functional proteins in both the sexes, and is essential for sexual differentiation. Some of the sexually dimorphic features controlled by Dsx proteins include genitalia and sex combs, differences in pigmentation of fifth and sixth abdominal tergites and certain aspects of courtship behaviour (Villella and Hall 1996), nervous system development (Taylor and Truman 1992), and female-specific expression of *yolk protein* genes (Bownes 1994) in *Drosophila*. Two major factors that govern the production of sex-specific splicing of *dsx* pre-mRNA are *cis*-regulatory elements located on *dsx* pre-mRNA and the *trans*-acting proteins encoded by the upstream genes of the sex-determination cascade, in different species. In other words, the *cis*-element required for the sex-specific splicing of *dsx* pre-mRNA decides the type of *trans*-acting protein which will be acting upstream to *dsx* in the hierarchy of the sex-determination cascade. Owing to the presence of diverse upstream regulators of *dsx* in different insect orders, the *dsx* in these orders bear specific molecular signatures, the significance of some of which have been investigated and some are yet to be understood. Before gaining insight into the molecular mechanism of sex-specific splicing of *dsx* pre-mRNA, a brief visit to the regulators of alternative splicing may help in better understanding of this topic.

Default and forced splicing

The pattern of pre-mRNA splicing in the presence of general splicing factors is known as default pattern. But when the presence of any *trans*-factor deviates the splicing of pre-mRNA from its normal mode, it is called forced splicing. For example, the pre-mRNA of *dsx* in all the insect species studied so far produces sex-specifically spliced transcripts, following the default splicing in one sex and forced splicing in the other. The *trans*-acting proteins exert their effect by binding to the regulatory elements, called splicing silencers and enhancers, present on pre-mRNA.

Splicing enhancers and silencers

Splicing enhancer and silencer elements play an important role both in alternative and constitutive splicing (Schaal and Maniatis 1999; Fairbrother *et al*. 2002; Cartegni *et al*. 2003). These elements, present in RNA as distinct motifs, are recognized by the members of the serine–arginine (SR), hnRNP, or other RNA-binding protein families (Graveley 2000; Wang and Burge 2008). SR proteins generally have two domains: an RS domain, rich in arginine–serine repeats and a RNA-recognition motif (RRM). RRM recognizes exonic splicing enhancer (ESE) sequences which are purine rich and are typically located within exons. Upon binding to ESEs, the SR protein recruits general splicing factors through its RS domains (Graveley and Maniatis 1998) and facilitate the assembly of spliceosomal complex at the adjacent intron. The *Drsophila* Tra is also an SR protein but does not have RRM domain, and as mentioned in the previous paragraphs it needs Tra2 to provide the RRM domain to recognize the ESE motifs present on *dsx* pre-mRNA (Hedley and Maniatis 1991; Hoshijima *et al*. 1991). Proteins devoid of RS domains have also been found in the ESE-dependent stimulation of splicing (Shaw *et al*. 2007). Some recent studies suggest the role of SR proteins in multiple steps of pre-mRNA splicing (reviewed by Blencowe 2000; Hertel and Graveley 2005). Many non-SR proteins are also shown to improve splicing through binding to ESEs e.g., a non-SR protein, YB-1 recognizes AC-rich (ACE) enhancers (Coulter *et al*. 1997).

By contrast, hnRNP proteins act as splicing silencers since binding of these proteins to splicing silencer motifs of pre-mRNA typically leads to inhibition of splicing (Wang *et al*. 2006) mainly by counteracting the effects of the SR protein (Mayeda and Krainer 1992; Caceres *et al*. 1994); although not much is known about the mechanisms underlying such inhibition. 5′-splice site (5′SS) like sequences present in the pre-mRNA have also been found to modulate splicing. For example, 5′SS-like sequence in the pre-mRNA of *Drosophila P*-element transposase (Rio 1991; Siebel *et al*. 1992) is required for intron retention in somatic cells. Besides, 5′SS-like motifs can also improve splicing; a 5′SS can functionally substitute for a purine-rich enhancer to promote *trans*-splicing in HeLa extracts (Chiara and Reed 1995), and a screen for *trans*-splicing enhancers in nematodes identified similar 5′SS-like sequences (Boukis and Bruzik 2001). The effect of splicing enhancers and silencers are additive, the magnitude of which increases with the increase in the copy number of these elements (Huh and Hynes 1994; McCullough and Berget 1997; Graveley and Maniatis 1998; Chou *et al*. 2000). Also, the activity of enhancers and silencers is shown to be position dependent; exon enhancers can inhibit splicing whereas exon silencers can activate it, when placed in an intron (Ibrahim *et al*. 2005; Ule *et al*. 2006; Buratti *et al*. 2007). The description of mechanism by which these *cis*-regulatory elements (ESEs and ESSs) and *trans*-acting factors modulate the splicing and affect a particular splicing step is beyond the scope of this review.

Dipteran dsx

The pre-mRNA of the *Drosophila dsx* gene contains six exons: the first three exons are common to both male-specific and female-specific *dsx* transcripts, exon 4 is female-specific whereas exons 5 and 6 are male-specific (figure 1) (Burtis and Baker 1989). The 3′ splice site preceding the female-specific exon 4 contains a stretch of purine nucleotides which makes it a weak splicing acceptor that is overlooked by the spliceosomal machinery in males resulting in the splicing of exon 3 to exon 5 (default male mode of splicing). The female-specific exon 4 contains six copies of identical tridecamer (13 nucleotide (nt)) repeat sequences called *dsx* repeat elements (*dsx*RE) which act as ESE. The regulatory protein Tra promotes cooperative binding of an SR protein, Rbp1 and an SR-like protein, transformer 2 (Tra2) to *dsx*RE in the *dsx* pre-mRNA (figure 2) (Lynch and Maniatis 1996). This binding leads to the formation of a complex of SR proteins which in turn interact with the U2AF small subunit through its RS domain, stimulating the use of a non-canonical weak female-specific 3′ splice site. As a result, the *dsx* pre-mRNA follows the female mode of splicing encoding DsxF (Hedley and Maniatis 1991; Hoshijima *et al*. 1991; Ryner and Baker 1991; Tian and Maniatis 1992; Tacke and Manley 1999). The efficient female-specific splicing of *dsx* pre-mRNA is attributed to the additive contribution of each of the six copies of 13 nt elements, suggesting that each enhancer complex interacts with a single target during spliceosomal assembly (Hertel and Maniatis 1998). Absence of Tra protein in males leads to the use of an alternative conventional splice site due to which *dsx* pre-mRNA splices in a default mode, producing DsxM protein.

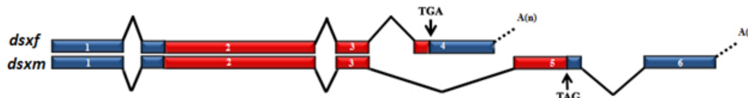

Figure 1. Schematic representation of male and female splice forms of *Drosophila dsx*. *dsxf* and *dsxm* represent the female and male forms, respectively. Boxes are exons and lines are introns. Red coloured portion is the ORF whereas blue coloured regions are UTRs. A(*n*) represents poly A tail and arrows represent stop codon sites.

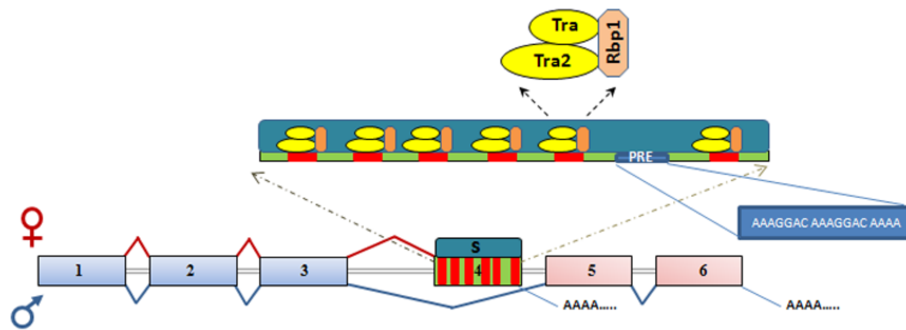

Figure 2. The sex-specific splicing pattern of *dsx* pre-mRNA. Boxes are exons and lines are introns. Red colour introns show the splicing pattern followed by female whereas blue colour introns show the splicing pattern followed by males. Assembly of female-specific heterotrimeric protein complexes on ESEs regulate the splicing of *dsx* pre-mRNA in female mode. Exons 1, 2 and 3 are constitutively spliced in both sexes. The weak 3′ splice site preceding exon 4 is not recognized by the splicing machinery in males, resulting in the exclusion of this exon, and splicing of exon 3 to exon 5. In females, the female-specific Tra protein promotes the binding of the SR protein Rbp1, and the SR-like protein Tra2 to six copies of exon splicing enhancers (ESE) (indicated by red rectangles). A purine-rich element (PRE) located between 5th and 6th ESEs is required for specific binding of Tra 2 to the ESE. The Tra and SR proteins bind co-operatively in the presence or absence of PRE (Lynch and Maniatis 1995). These splicing enhancer complexes then recruit the splicing machinery to the 3′ splice site preceding exon 4, leading to its inclusion in the mRNA. In females, polyadenylation (AAA. . .) occurs downstream of exon 4, whereas in males it occurs downstream of exon 6. 'S' designates the splicing machinery. Enlarged view of exon 4 is shown for clarity.

Outside drosophilids, homologues of *dsx* have been found in many other dipterans, including *Megacelia scalaris* (Sievert *et al*. 1997; 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), *B. oleae* (Lagos *et al*. 2005), *Ceratitis capitata* (Saccone *et al*. 2008, cited in Pane *et al*. 2002) and *A. obliqua* (Ruiz *et al*. 2005, 2007). The pre-mRNAs of all the dipteran *dsx* studied to date sex-specifically splice to produce sex-specific transcripts that encode putative male-specific and female-specific Dsx proteins having common N-terminal but different C-terminal regions. As in the case of *Drosophila dsx*, the *dsx* pre-mRNA of other dipterans also contain Tra-Tra2 binding sites (*dsx*RE elements) in the female-specific exon, suggesting a common mode of sex-specific splicing of *dsx* in dipterans; default splicing in males and Tra-mediated splicing of female-specific exon (Sanchez 2008). An exception to the sex-specific splicing of *dsx* pre-mRNA among dipterans is seen only in *M. domestica*, where the male-specific exon (exon m) of *M. domestica dsx* (*Mddsx*) lies within the female-specific intron (intron 3) (figure 3). A similar type of splicing is also found in the Australian sheep blowfly, *Lucilia cuprina* (Calliphoridae) (Concha *et al*. 2010, this issue pp. 279–285).

Lepidopteran dsx

Among lepidopterans, *dsx* homologues have been characterized in *B. mori* (Ohbayashi *et al*. 2001), *A. assama* and *A. mylitta* (Shukla and Nagaraju 2010), but the molecular mechanism involved in the sex-specific splicing of *dsx* pre-mRNA has been investigated only for *B. mori dsx* (*Bmdsx*). In an earlier study, pre-mRNA of *Bmdsx* was shown to be sex-specifically spliced to generate one female (*Bmdsxf*) and one male (*Bmdsxm*) specific mRNA which is expressed in various tissues at larval, pupal and adult stages of the silkworm (Ohbayashi *et al*. 2001). Recently, one more female-specific splice form of *Bmdsx* pre-mRNA has been characterised which differs from the *Bmdsxf* by the presence of additional stretch of 15 bp (GTACGGACTTTAATA) after exon 3 as a result of alternative 5′ splice site selection (Shukla *et al*. 2010). This novel splice form expresses in various tissues and at different developmental stages. We refer to this newly identified female splice form of *Bmdsx* as *Bmdsxf2* and the *Bmdsxf* reported earlier by Ohbayashi *et al*. (2001) as *Bmdsxf1* and proteins encoded by these two forms as BmDsxF2 and BmDsxF1, respectively.

The finding of this novel female splice form in *B. mori* is an outcome of characterization of *Aadsx* and *Amydsx*, which is described in the later section of this article. *Bmdsx* gene consists of six exons; the female-specific *Bmdsx* transcripts (*Bmdsxf1* and *Bmdsxf2*) contain all the six exons out of which the first four exons constitute its ORF. In male-specific *Bmdsx* transcript (*Bmdsxm*), exons 3 and 4 are skipped and the resulting ORF includes exons 1, 2 and 5 (Suzuki *et al*. 2001) (figure 4). Exon 5 encodes male-specific C-terminal amino acid sequence, while it is transcribed as a 3′-UTR in females. The ORF of *Bmdsxf2* differs from that of *Bmdsxf1* by the early occurrence of stop codon in the former resulting in the putative protein (BmDsxF2) which differs from BmDsxF1 at its extreme C-termini.

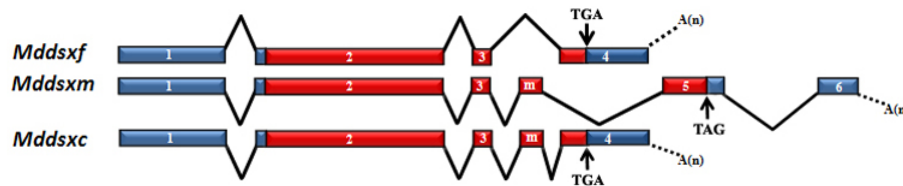

Figure 3. Schematic representation of male and female splice forms of *Musca dsx*. *Mddsxf* and *Mddsxm* represent the female and male forms, respectively. *Mddsxc* is common to both male and female. Boxes are exons and lines are introns. The male-specific exon (exon 4) of *M. domestica dsx* (*Mddsx*) lies within the female-specific intron (intron 3). Red coloured portion is the ORF whereas blue coloured regions are UTRs. A(*n*) represents poly A tail and arrows represent stop codon sites.

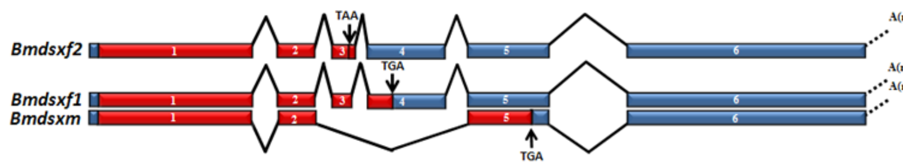

Figure 4. Pre-mRNA of *Bmdsx* splices to produce three products i.e., *Bmdsxf1*, *Bmdsxf2* and *Bmdsxm*. Boxes represent exons, whereas lines represent introns. Exons have been numbered. A small stretch of 15 nt (GTACGGACTTTAATA) after exon 3 (small box after exon 3) generated as a result of alternative 5′ splicing, leads to the production of second female splice form i.e., *Bmdsxf2*.

The molecular mechanism of sex-specific splicing of *Bmdsx* pre-mRNA is very different from that of dipteran *dsx*. Unlike in *Drosophila*, the default form of *Bmdsx* splicing is the female form, as suggested from the splicing pattern of the *Bmdsx* mini gene in the HeLa nuclear extract (Suzuki *et al*. 2001). *Bmdsx* neither has a weak female-specific splice site nor contains Tra/Tra-2 binding motif related sequences although we cannot exclude the possibility that the latter have been missed because of sequence divergence. Hence, it is assumed that *tra* and *tra-2* genes are not required for the sex-specific splicing of *Bmdsx* pre-mRNA. Since *Bmdsx* has a strong female splice site, splicing repressor(s) is needed for skipping of the female-specific exons 3 and 4 in *Bmdsxm* (Suzuki *et al*. 2001). Recently it has been shown that binding of the BmPSI, a *Bombyx* homolog of PSI (*P*-element somatic inhibitor) to the exonic splicing suppressor (ESS) sequence on exon 4 is essential for repressing female-specific splicing and skipping of exons 3 and 4 in males (Suzuki *et al*. 2008). In male cells, interaction of BmPSI with the *cis*-element (CE1) on exon 4, splices exon 2 to exon 5 resulting in the skipping of exons 3 and 4 to produce *Bmdsxm* (figure 5). *Bmpsi* transcripts have been detected in both the sexes but either because of insufficient levels of BmPSI or the presence of a *trans*-acting factor(s) that counteracts the activity of BmPSI, exons 3 and 4 are spliced to generate female-type *Bmdsx* mRNA only in females (Suzuki *et al*. 2008).

The *Aadsx* and *Amydsx* have been identified and characterised recently from two wild silkmoth species (Shukla and Nagaraju 2010). The sex-specific splicing of the *Amydsx* pre-mRNA produces two female and one male-specific mRNAs where as *Aadsx* sex-specifically splices to produce six splice variants in female and one in male. *Aadsx* gene consists of seven exons of which exons 1, 2, 4 and 7 are common to all the female-specific transcripts; exons 3 and 5 are specific to *Aadsxf1* and *Aadsxf2*, respectively and exon 6 is present in *Aadsxf1, Aadsxf2*, *Aadsxf3* and *Aadsxf4*. The six female-specific splice variants of *Aadsx* can be grouped into two on the basis of their ORFs: *Aadsxf2*, *Aadsxf4* and *Aadsxf6* (group I) include exons 1, 2 and 4 whereas *Aadsxf1*, *Aadsxf3* and *Aadsxf5* (group II) harbour a 15-bp stretch (GTACGGACTTTAATA) after exon 2 generated as a result of alternative 5′ splice site selection which results in the early occurrence of stop codon and hence exon 4 constitutes as the UTR region (figure 6). The female-specific transcripts of each group having common ORF differ from each other only in their 3′UTR regions, making only one kind of protein; thus the two groups of female-specific ORFs encode two types of female-specific proteins. The differences in the UTR regions may be of regulatory significance since 3′UTRs are the known target of the proteins or miRNAs (Ambros 2004; Beckmann *et al*. 2005). In males, exons 2 to 5 are skipped to generate male-specific *Aadsx* transcript (*Aadsxm*) which includes exons 1, 6 and 7; the ORF is comprised of only exons 1 and 6 (figure 6) where exon 6 encodes a male-specific C-terminal amino acid sequence. As in the case of *Bmdsx*, neither *dsx*RE (Tra/Tra2 binding sequence) nor PRE are found in any of the transcripts of *Aadsx* and *Amydsx*. *Aadsx* exon 4 which is specific to all

PSI (P-element Somatic Inhibitor) binding site

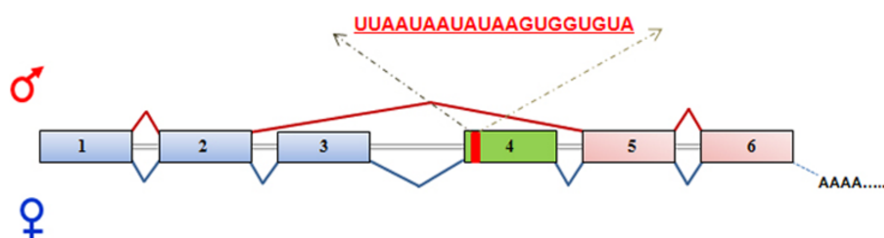

Figure 5. Organization of BmPSI binding site and sex-specific splicing of *Bmdsx* pre-mRNA. Boxes are exons and lines are introns. Red colour introns show the male splicing pattern whereas blue colour introns show the splicing pattern followed by females. All the splice sites of *Bmdsx* pre-mRNA are strong and hence default splicing takes place in females; *Bmdsxf1* includes all the six exons. Assembly of male-specific BmPSI protein on exonic splicing silencer (ESS) sequence (UUAAUAAUAUAAGUGGUGUA) on exon 4 regulates the splicing of *Bmdsx* pre-mRNA in male mode; exons 3 and 4 are skipped hence *Bmdsxm* includes exons 1, 2, 5 and 6. The polyadenylation (AAA…) site is same for *Bmdsxf1* and *Bmdsxm*. Small red box in exon 4 represents BmPSI binding site and is indicated by an arrow.

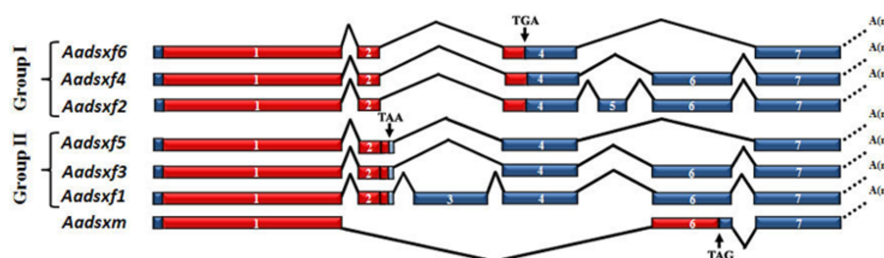

Figure 6. Schematic representation of splice forms of *Aadsx* pre-mRNA. Boxes are exons and lines are introns. Red coloured portion is the ORF whereas blue coloured regions are UTRs. Seven different splice products of *Aadsx* pre-mRNA, six female-specific (*Aadsxf1*, *Aadsxf2*, *Aadsxf3*, *Aadsxf4*, *Aadsxf5* and *Aadsxf6*) and one male-specific (*Aadsxm*), are produced. A(*n*) represents the polyadenylation site. Arrows represent stop codon sites. Exons from 2 to 5 are skipped in males leading to the production of *Aadsxm*. The female-specific transcripts, on the basis of their open reading frame, can be divided into two groups; transcripts *Aadsxf2*, *Aadsxf4* and *Aadsxf6* (Group I) encode AaDsxF1 whereas transcripts *Aadsxf1*, *Aadsxf3* and *Aadsxf5* (Group II) encode AaDsxF2. Presence (in group II splice variants) or absence (in group I splice variants) of a 15 nt stretch immediately after exon 2 as a result of alternative 5′ splicing is responsible for the difference in the ORF of two groups of female-specific transcripts. Note that the female-specific transcripts of each group having common ORF differ from each other in their 3′UTR regions.

the female splice forms shows 90% sequence similarity with *Bmdsx* exon 4. As mentioned earlier, *Bmdsx* exon 4 contains the ESS sequence stretch (UUAAUAAUAUAAGUGGUGUA) which, on binding with BmPSI, acts as splicing suppressor leading to the skipping of exons 3 and 4 in males (Suzuki *et al*. 2008). This sequence stretch, responsible for sex-specific splicing of *Bmdsx* is 100% conserved, both in *Aadsx* and *Amydsx*, suggesting the existence of a common mechanism of sex-specific splicing of *dsx* in silkmoths. However it remains to be investigated whether or not these sequences are involved in splicing regulation of *Aadsx* and *Amydsx*.

The two female-specific splice forms of *Amydsx* also differ from each other by the presence or absence of the same 15 nt stretch which alters the ORF of two groups of female-specific *Aadsx* mRNAs. On the whole, both in case of *A. assama* and *A. mylitta* two female and one male-specific Dsx are encoded by their sex-specific splice variants.

The deduced amino acid (aa) sequences of Dsx proteins encoded by sex-specific transcripts of *Aadsx* and *Amydsx* can be divided into three parts: the region common to both sexes (aa 1–216), the female-specific regions (aa 217–265 of DsxF1 and aa 217–247 of DsxF2) and the male-specific region (aa number 217–279). The female-specific (DsxF1 and DsxF2) and male-specific (DsxM) 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 (DsxF1 and DsxF2) differ at their C-termini by 21 aa (figure 7).

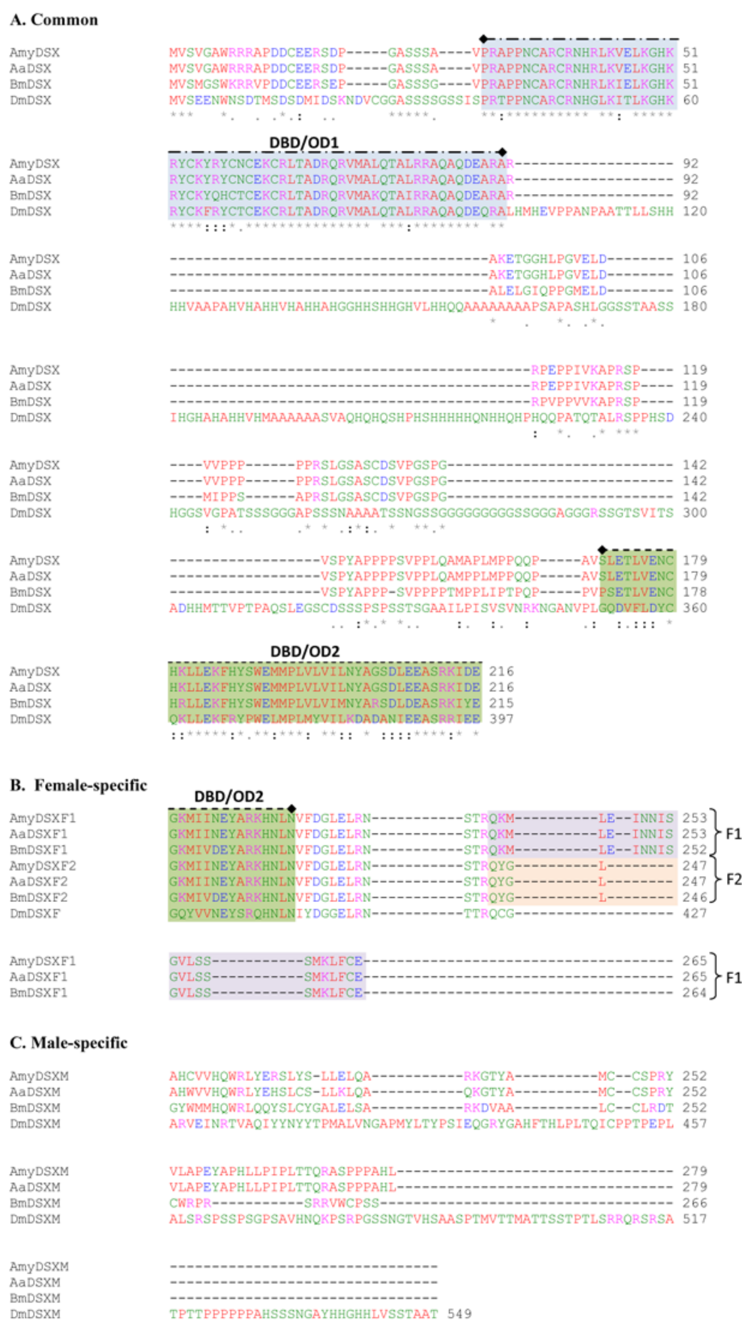

Figure 7. Sequence alignment of Dsx proteins of *A. mylitta* (*Amy*), *A. assama* (*Aa*), *B. mori* (*Bm*) and *D. melanogaster* (*Dm*). A, region common to males and females; B, region conserved in female; C, the male-specific region. The DNA binding domain (OD1) is boxed in blue and oligomerization 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 and Nagaraju 2010). The female splice forms of *Dmdsx* code for single female-specific protein, DmDsxF. The male-specific transcripts of *dsx* of all the three species, code for only one male-specific protein. 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.

Searching for the conserved stretch of 15 nt of *Aadsx* and *Amydsx* in the EST database of *B. mori* led to the identification and confirmation of the second female splice form of *Bmdsx* (*Bmdsxf2*) (Shukla *et al*. 2010). *Bmdsxf2* encodes another female-specific protein i.e., BmDsxF2 which differs from BmDsxF1 at its C-terminal by 21 aa, similar to the female-specific AaDsxF2 and AmyDsxF2.

Existence of two female-specific Dsx proteins in silkmoths (*B. mori*, *A. assama* and *A. mylitta*) raises the possibility that the single female Dsx protein alone may not be able to execute the functions related to somatic sexual differentiation. Perhaps the activity of one Dsx protein requires the help of the other Dsx protein to exert its effect on the downstream genes involved in sexual differentiation. In *D. melanogaster*, the Ix protein is required for the proper functioning of the female-specific Dsx protein to regulate terminal sex differentiation in females. Together with the recent studies on Ix proteins isolated from *Maruca vitrata* (Cavaliere *et al*. 2009) and *B. mori* (Arunkumar and Nagaraju manuscript communicated) and their partial rescue of *Drosophila ix* mutant (Siegal and Baker 2005; Cavaliere *et al*. 2009) we cannot exclude the role of BmIx in sex differentiation in co-ordination with BmDsx. Further, both the female-specific Dsx proteins in wild silkmoths, as demonstrated by knockdown experiments, are required for the female sexual differentiation (Shukla and Nagaraju 2010). This can be further proved by the molecular interactions of the Dsx protein(s) with its partner(s) and with its downstream target genes.

Hymenopteran dsx

The pre-mRNA of honeybee (*A. mellifera*) *dsx* gene (*Am-dsx*) splices to produce four types of transcripts (Cho *et al*. 2007): one male-specific (*Am-dsxm*), two female-specific (*Am-dsxf1* and *Am-dsxf2*), and one common to both male and female (*Am-dsxb*) (figure 8).

ORFs of both the female-specific *Am-dsx* transcripts (*Am-dsxf1* and *Am-dsxf2*) are identical leading to the production of the same putative protein from both transcripts. In this way, *A. mellifera* is similar to dipteran insects where *dsx* has been characterized to produce one male- and one female-specific Dsx protein. Initial studies by Cho *et al*. (2007) suggested that the splicing pattern of *Am-dsx* shows similarity to that of *Drosophila dsx* as well as to that of *Bombyx dsx* (*Bmdsx*). Comparison of the female-specific transcript *Am-dsxf1* with the male-specific transcript (*Am-dsxm*) indicates that the splicing pattern of *Am-dsx* is similar to that of *Bmdsx*; the female-specific exon is having a strong splice site thus leading to the female default splice form, and skipping of the female-specific exons occurs in males because of the presence of splicing suppressor. Also, as in the case of *Bmdsx*, there is no *dsx*RE or PRE in the female-specific exons and the 3′ splice site preceding the female-specific exon is supposedly not weak (probably leading to the female splice form as the default form) (Cho *et al*. 2007). Similarity in splicing pattern of *Am-dsx* and *Drosophila dsx* is evident from the comparison of another female-specific transcript, *Am-dsxf2* with the male-specific transcript (*Am-dsxm*); the sex-specific splice forms differ from each other by the differential polyadenylation sites (Cho *et al*. 2007). Later, the knockdown studies by Hasselmann *et al*. (2008) discarded the possibility of any of the female splice form of *Am-dsx* to be the default form (and hence any similarity of *Am-dsx* with *Bmdsx*) since RNAi-induced knockdown of *fem* and *csd* alleles in separate experiments resulted into male-specific *Am-dsx* splice variant in females, whereas no change was observed in males.

Unlike *Apis*, the haplodiploid wasp, *N. vitripennis* produces one male and one female-specific *dsx* (*Nvdsx*) splice form (Oliveira *et al*. 2009). Although *Nvdsx* clusters together with that of *Apis*, the DM domains of these two hymenopterans show a high degree of amino acid divergence (Oliveira *et al*. 2009). As mentioned above *Nvdsx* is sex-specifically spliced, in females, by a zygotically active *transformer* gene that is autoregulated by maternally provided *Nvtra* transcript (Verhulst *et al*. 2010). The *Nvdsx* in haploid embryos which are unable to activate the autoregulatory loop of *tra*, splices in a default male mode (Verhulst *et al*. 2010).

Recently, putative binding sites for sex-specific splicing factors found in *Nasonia fruitless* and *doublesex* as well as *A. mellifera doublesex* transcripts have been identified, which suggest that similar factors in both hymenopteran species (*A. mellifera* and *N. vitripennis*) could be responsible for sex-specific splicing of both the genes

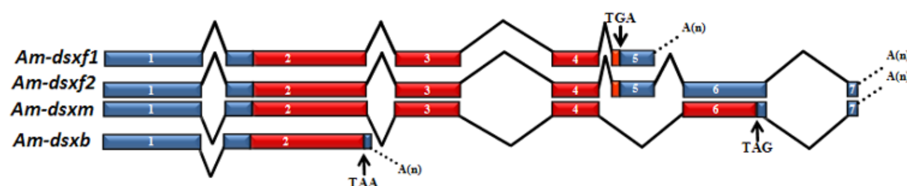

Figure 8. Splice forms of *Apis dsx* (*Am-dsx*). Boxes are exons and lines are introns. Red coloured portion is the ORF whereas blue coloured regions are UTRs. The *Am-dsx* pre-mRNA splices to generate four different splice products, two female-specific (*Am-dsxf1* and *Am-dsxf2*), one male-specific (*Am-dsxm*) and one common to both the sexes (*Am-dsxb*). A(*n*) represents the poly-adenylation site. Arrows represent stop codon sites.

(Bertossa *et al.* 2009), though a functional demonstration is required to confirm this point.

Functional assays of Dsx proteins of different insect species

In *Drosophila*, sex-specific Dsx proteins contribute to the sex-specific antagonistic functions owing to differences at their C-termini. The Dsx proteins act as transcriptional regulators which control the genes responsible for differentiation of sexually dimorphic traits. *Drosophila yolk protein* genes (*Yp1* and *Yp2*) are by far the best characterized target genes of sex-specific Dsx proteins, and are affected by any change in the sex-determination pathway (Postlethwait *et al.* 1980; Ota *et al.* 1981; Belote *et al.* 1985). The Dsx protein alters the basal level expression of these genes; DsxM represses basal transcription of these genes whereas DsxF enhances their transcription (An *et al.* 1996; Erdman *et al.* 1996). Several specific sites in the enhancer sequence of yolk protein encoding genes, designated as fat body enhancer elements (FBE), are utilized by sex-specific Dsx proteins to exert their antagonistic effects on these genes. DsxM and DsxF proteins bind to three sites within the 127-bp enhancer that directs sex-specific and tissue-specific transcription of *yolk protein* genes (Coschigano and Wensink 1993). Binding of DsxF and DsxM to these enhancer elements has been shown in *in vitro* studies as well (Burtis *et al.* 1991). Ectopic expression of DsxF in XY males results in appearance of some of the female characters such as enhanced expression of *yolk protein* genes and the production of female profile of pheromones (Baker *et al.* 1989; Waterbury *et al.* 1999). The female-specific differentiation in *D. melanogaster* is not achieved by the DsxF activity alone rather it requires to interact with the *intersex* (*ix*) gene product to execute its function (Waterbury *et al.* 1999). The *ix* gene is expressed in both males and females and functions together with the DsxF to regulate terminal sexual differentiation (Garrett-Engele *et al.* 2002). Ix protein is not required for the activity of DsxM. *Hermaphrodite* (*her*) is another gene expressed in a non-sex-specific manner, and acts together with the Dsx and Ix to control female sexual differentiation (Li and Baker 1998). The expression of the pheromone binding protein gene is also regulated in sex-specific manner by Dsx proteins.

Another known target gene of Dsx proteins is the '*take-out*' gene, expression of which is activated by DsxM and Fru (product of *fruitless* gene), and is repressed by DsxF in a Fru independent manner (Dauwalder *et al.* 2002). DsxM and Fru together govern the male-specific development of neural circuitry, a separate lineage of sex-determination pathway.

Several studies have shown the function of Dsx protein of different insect species through its overexpression in transgenic background or through RNAi mediated knockdown of the *dsx* transcripts. Ectopic expression of female-specific *M. domestica* Dsx protein, MdDsxF in the males of *Musca* and *Drosophila* activated their *vitellogenin* gene expression, whereas the ectopic expression of male-specific *M. domestica* Dsx protein, MdDsxM in *Drosophila* females produced male-like pigmentation of posterior tergites, suggesting that sex-specific splice forms of *Mddsx* are not only structurally but also functionally conserved (Hediger *et al.* 2004).

The dsRNA mediated knockdown of female-specific Dsx protein in the fruit fly *Bactrocera dorsalis*, hinders the *yolk protein* gene expression and affects its reproductive ability (Chen *et al.* 2008). Partial masculinization of both somatic and germ line tissues was observed on ectopic expression of male-specific Dsx protein of *C. capitata*, CcDsxM in *Drosophila* female (XX) flies (Saccone *et al.* 2008). Male-like pigmentation of the posterior tergites was observed in *Drosophila* female transgenic flies expressing CcDsxM. This pigmentation was more pronounced compared to that in flies expressing MdDsxM (*M. domestica* male Dsx protein), probably because of higher sequence similarity of CcDsxM to DmDsxM and phylogenetically closer relationship of *Ceratitis* to *Drosophila* (both belong to Acalyptratae), than to *Musca* (Calyptratae) (Saccone *et al.* 2008).

Functional conservation of sex-specific *Anastrepha* Dsx proteins, DsxF and DsxM was established through overexpression of these proteins in *dsx* intersexual *Drosophila* flies (Alvarez *et al.* 2009). The sexual differentiation of *dsx* intersexual *Drosophila* flies followed the female or male pathway on the expression of *Anastrepha* Dsx proteins, DsxF and DsxM, respectively. However, this transformation was not complete and the effect of heterologous protein expression on the differentiation of sexually dimorphic features varied. The expression of *Drosophila yolk protein* genes was also affected by the sex-specific *Anastrepha* Dsx proteins similar to the effect of the sex-specific Dsx protein of *Drosophila* itself (Alvarez *et al.* 2009).

Apart from dipteran Dsx proteins, studies have also been carried out to functionally characterize the sex-specific Dsx proteins of *B. mori*. Three different genes of *B. mori* i.e., *vitellogenin, storage protein* (*SP1*) and *pheromone-binding protein* (*PBP*) express in sex-specific manner. *Vitellogenin* gene of *B. mori* is expressed in the female fat body cells during the larval–pupal ecdysis and the protein is released into the hemolymph subsequently to be taken up by the developing oocytes (Mine *et al.* 1983; Yano *et al.* 1994). SP1, one of the plasma proteins termed hexamerins, accumulates in the hemolymph of female larva of *B. mori* (Mine *et al.* 1983) and its expression is sex-specifically regulated at the level of transcription in the female fat body (Izumi *et al.* 1988). The PBP is predominantly expressed in male antennae and accumulates in the pheromone-sensitive sensilla (Steinbrecht *et al.* 1995). PBP binds to the sex-attractant pheromone released by female moths of *B. mori* and conveys the signal to a membrane-bound receptor on a nerve cell (Pelosi and Maida 1995).

Studies have shown that the ectopic expression of *Bmdsxf* from a ubiquitous promoter in the males of *B. mori*, activated the expression of female-specific genes, *vitellogenin* and *SP1* whereas *PBP* gene was repressed (Suzuki *et al.* 2003). Gel mobility shift assays revealed the binding of BmDsx pro-

teins to the enhancer sequence (ACATTGT) located between −95 and −89 nt relative to the transcriptional start site of the *vitellogenin* gene (Suzuki *et al.* 2003). Ectopic expression of *Bmdsxm* in the females of *B. mori* led to abnormal differentiation of some female-specific genital organs and caused partial male differentiation in female genitalia (Suzuki *et al.* 2005). Transgenic females expressing BmDsxM showed increased expression of PBP and decreased expression of *vitellogenin* (Suzuki *et al.* 2005). In contrast, the effect was less pronounced in males ectopically expressing BmDsxF (Suzuki *et al.* 2003, 2005). The authors have reasoned that the BmDsxF protein may recruit additional factors to exert its effect fully on female sexual differentiation (Suzuki *et al.* 2005). The recent finding of a novel female-specific splice form of *dsx* in lepidopterans, which codes for an additional female-specific putative protein, opens up a new avenue to unravel the sexual differentiation process governed by BmDsx proteins in lepidopterans. Authors propose a model for the probable action of two female-specific BmDsx proteins on its downstream target genes. Either the two female-specific BmDsx proteins make homodimers and bind to the different regulatory elements on the downstream target genes (figure 9a) or there could be a common target governed by the heterodimer of female BmDsx proteins (BmDsxF1 and BmDsxF2) (figure 9b).

Conclusion

A molecular picture of insect sex determination is emerging at least with respect to the players involved in the sex-determination cascade of respective species. The upstream genes are more diverged and their mode of action is very different from each other whereas downstream genes are relatively conserved. It has been observed that the sex-determination pathways discovered in insects follow three basic rules: (i) an initial signal for the activation of the sex-determination cascade in one sex; the other sex follows the default pathway in the absence of any signal, (ii) some gene (*Sxl* in case of drosophilids, *tra* in other dipterans and *N. vitripennis* and *fem*, the equivalent of *tra*, in *A. mellifera*) of the pathway should have an autoregulatory function to maintain the determined, but not the default state of sex, and (iii) a downstream-most gene produces its functional product in both sexes to regulate the sex-differentiation genes in an antagonistic fashion. The basic outline of existing knowledge of sex-determination mechanism in different insect species is summarized in figure 10. Data accumulated so far for a number of model organisms have shown a relative economy in the molecular regulation of sex determination; evolution of the sex-determination pathway has emerged in reverse order and the most downstream gene, i.e. *dsx*, is functionally conserved in all insect species examined to date. Nevertheless, variations in the *dsx* splicing pattern and the *cis*-regulatory element harboured by *dsx* exist within and outside different species. *Mddsx* shows deviation from the general *dsx* splicing features as compared to that found in other dipteran insects owing to the presence of (i) a common splice variant present in both male and female, (ii) The male-specific exon is found within the female-specific introns.

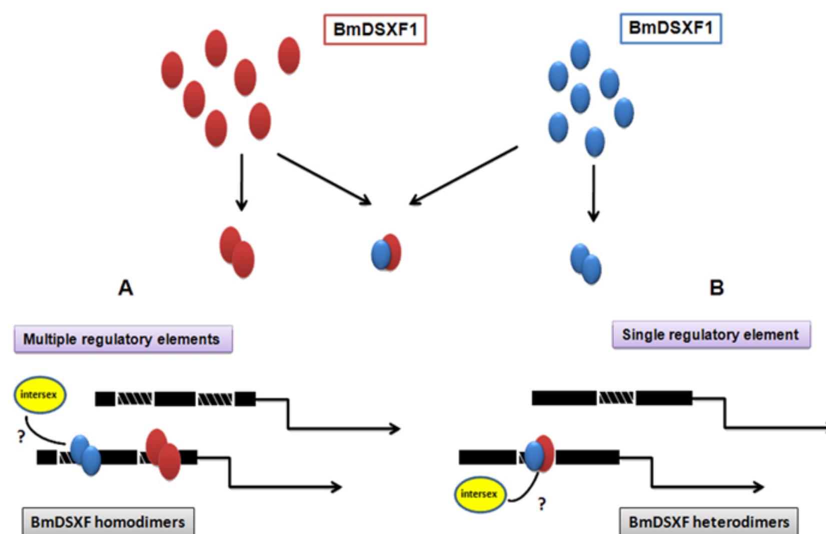

Figure 9. Model explaining the mode of action of two BmDsx proteins in females. A, two different regulatory elements (RE) are present in the promoters of downstream target genes (*vitellogenin*, *SP1* and *PBP*). Homodimers of BmDsxF1 and BmDsxF2 bind separately to these regulatory elements to independently regulate them. B, alternatively, heterodimers of BmDsxF1 and BmDsxF2 bind to the single regulatory element present in the promoters of downstream target genes to regulate their expression. Ix represents protein products of *intersex* gene which may also possibly interact with BmDsx.

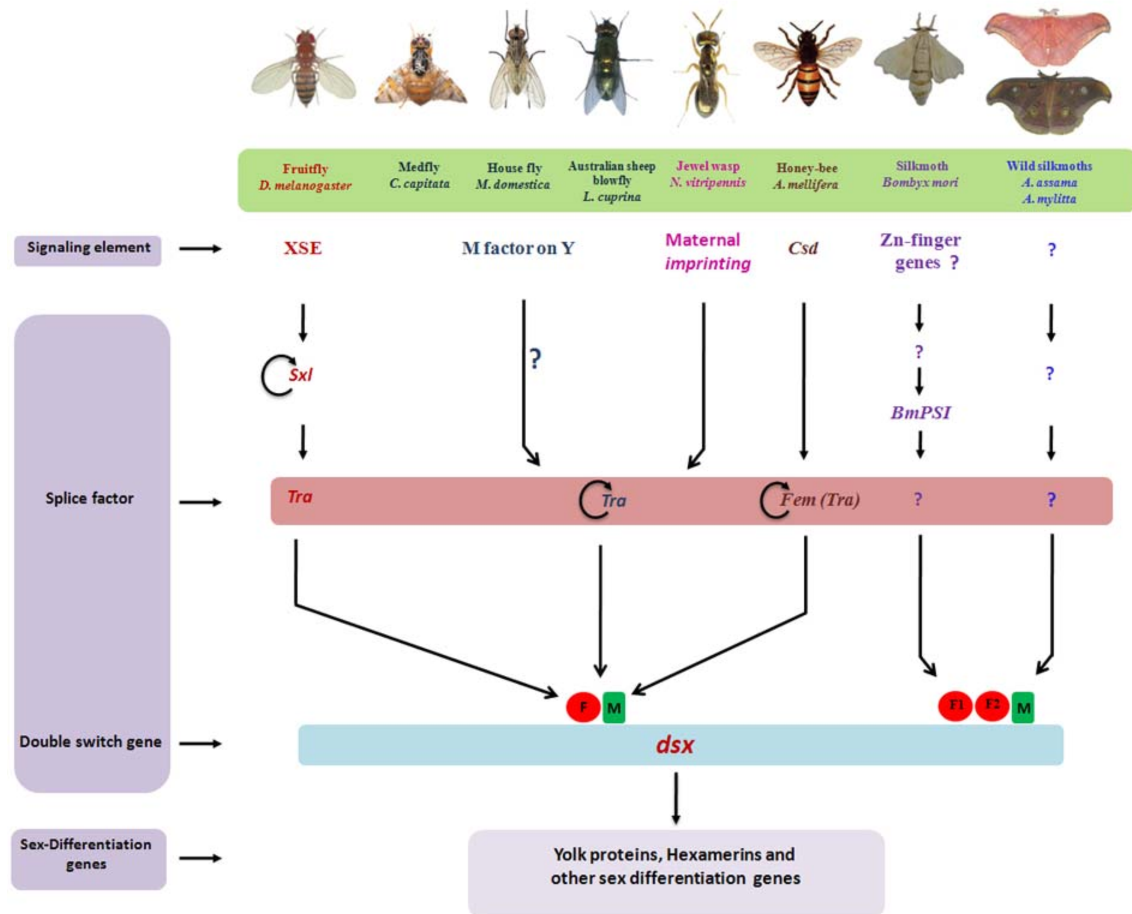

Figure 10. Schematic diagram representing the players of the sex-determination cascade in the model insect species of Diptera (*D. melanogaster*, *M. domestica*, *C. capitata* and *L. cuprina*), Hymenoptera (*A. mellifera*, *N. vitripennis*) and Lepidoptera (*B. mori*, *A. assama* and *A. mylitta*). Wild silkmoths include *A. assama* (upper) and *A. mylitta* (lower). Upstream genes of the sex-determination cascade vary whereas *dsx*, the bottom most gene is conserved in all the insect species of different orders. In case of dipterans and hymenopterans, one male and one female-specific Dsx protein are produced. Recent studies on wild silkmoths (Shukla and Nagaraju 2010) and *B. mori* (Shukla *et al*. 2010) reveal that silkmoths produce two female-specific (DsxF1 and DsxF2) and one male-specific (DsxM) Dsx proteins. Both the female Dsx proteins are shown to be essential for the regulation of downstream sex-differentiation genes. Question marks (?) represent the unknown factors or the unknown mechanism of action.

Special features have also been added by the *Am-dsx* where a non-sex-specific transcript, *Am-dsxb* is produced besides sex-specific transcripts and there are two female-specific transcripts having the same ORF. Further, complexities have been added by the *dsx* homologues in silkmoths where two female-specific ORFs are found. Future studies of *dsx* homologs in different unexplored insect species may ignite the field with more ingredients which will help us to understand the evolution of *dsx* in insect kingdom.

Acknowledgments

J. Nagaraju acknowledges Centre of Excellence (COE) grant from the Department of Biotechnology, Government of India, and IAEA-FAO grant. J. N. Shukla is recipient of a fellowship from Council of Scientific and Industrial Research (CSIR), Government of India.

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Received 15 May 2010, in revised form 20 June 2010; accepted 1 July 2010
Published on the Web: 6 September 2010