

Lepidopteran Sex Determination: A Cascade of Surprises

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Abstract

Sex determination is a developmental pathway that fixes the sexual fate (male or female) of an individual at early stages of embryonic development. This pathway is ideally suited for evolutionary studies given the astoundingly diverse mechanisms found in the animal kingdom. In particular, insects use multiple different cues to specify the sexual fate of an individual. In this review, we focus on genes and genetic interactions involved in the sex determination of insect species belonging to the order Lepidoptera. Unique features of the lepidopteran sex determination system are discussed.

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Global crop losses by insects are estimated to be 13% per annum despite the usage of multiple pesticides. The insect order Lepidoptera (Lepido = scales, ptera = wing) includes a major fraction of crop pests and is divided into 4 suborders: Zeugloptera, Aglossata, Heterobathmiina, and Glossata, containing 15,578 genera and 157,424 spe-

cies [van Nieukerken et al., 2011]. Lepidopterans undergo complete metamorphosis; life cycles contain 3 distinct stages, i.e. caterpillar/larva, chrysalis/pupa, and moth/adult stages. Generally, larval stages are crop destroyers that include defoliators, shoot/root borers, and seed predators causing a significant agricultural loss. Use of pesticides is not eco-friendly, and risk of developing resistance is an alarming threat from insects of this order. Strategies utilizing the release of sterile males, e.g. sterile insect technique, is a promising approach for pest control. Sterile insect technique achieved success in controlling the New World screwworm fly, Mediterranean fruit fly, and melon fly [Lindquist et al., 1992; Hendrichs et al., 1995; Koyama et al., 2004]. The productive female sex of insects could be targeted using modern techniques of genetic engineering, e.g. release of insects carrying a dominant lethal gene or genetic system [Thomas et al., 2000; Alphey and Andreasen, 2002; Jin et al., 2013]. A major improvement in sterile insect technique and its application can be envisaged by an understanding of molecular mechanisms

This article is dedicated to Dr. Jawaregowda Nagaraju, who unexpectedly passed away during the preparation of this paper. G.G. and V.S. contributed equally to this paper.

of sex determination in insects [Saccone et al., 2002; Marec et al., 2005; Dafa'alla et al., 2010].

Sex determination is an essential and universal phenomenon among higher eukaryotes. Organisms show an astounding diversity in the mechanisms regulating sex determination [Gamble and Zarkower, 2012]. This is also true for insects that utilize different strategies for their sex determination [Sánchez, 2008]. In this review, we present the current understanding of genes involved in sex determination of lepidopteran insects, mainly based on studies in the domesticated silkworm, *Bombyx mori*.

Sex Chromosomes in Lepidopterans

Lepidopteran insects, which include all species of moths and butterflies, display female heterogamety [Traut and Marec, 1996]. Lepidopteran sex chromosomes (ZW) exhibit various degrees of structural differentiation similar to XY chromosomes in groups with male heterogamety. Variability in the pattern of Z and W chromosomes include (i) size differences (e.g. *Philosamia cynthia*); (ii) number differences, such as W1W2Z/ZZ (e.g. *Phragmatobia fuliginosa*) and WZ1Z2/Z1Z1Z2Z2 (e.g. *Yponomeuta* spp.) which result from fission or fusion events, or Z/ZZ (e.g. *Talaeporia tubulosa*) resulting from total loss of the W chromosome; (iii) differences in pachytene chromomere pattern (e.g. *Orgyia thyellina*); (iv) heterochromatinization of the W chromosome in interphase nuclei (e.g. *Choristoneura fumiferana*); and (v) absence or loss of most gene functions in the W chromosome (e.g. *B. mori* and *Ephestia kuehniella*) [Traut and Marec, 1996, 1997; Traut et al., 2013]. The W and Z chromosomes in *B. mori* and *B. mandarina* are recombinationally isolated and are highly enriched with repetitive sequences/transposons. W-chromosome-specific repetitive sequences of *B. mori* have been categorized as long terminal repeats, non-long terminal repeat retrotransposons, retrotransposons, and DNA transposons [Abe et al., 1998, 2000, 2010]. A recent study suggests the W chromosome of *B. mori* to be the source of multiple female-specific piRNAs with unknown function(s) [Kawaoka et al., 2011]. Genetic evidence suggests that femaleness in *B. mori* is determined by the presence of a dominant feminizing (*Fem*) factor present on the W chromosome, irrespective of the number of autosomes and Z chromosomes. The presence of a single W chromosome leads to female determination and development [Hashimoto, 1933]. Investigations dealing with the search for the *Fem* factor present on the W chromosome are still underway. Results obtained from

rearrangements of the sex chromosome in the Mediterranean flour moth, *E. kuehniella*, suggest the presence of a male killing factor on the W chromosome that might function as a feedback control of sex rather than as a factor contributing to the female sex differentiation pathway [Marec et al., 2001]; though, it has not been characterized molecularly. Existence of both kinds of mechanisms is possible since the absence of a W chromosome in primitive Lepidoptera and frequent secondary loss of the W chromosome in advanced Lepidoptera favor a balanced mechanism of sex determination with Z-linked male-promoting factors instead of W-linked female-promoting factors [Traut and Marec, 1996]. Extensive hybridization experiments between Japanese and European races of the gypsy moth, *Lymantria dispar*, suggest the possibility of Z chromosomes carrying male-promoting genes that might act against a zygotic or a maternal female-promoting effect [Goldschmidt, 1942].

Sex-Determining Genes in *B. mori* and Other Lepidopterans

Sex determination studies in the order Lepidoptera are still in their infancies. *B. mori* is an ideal lepidopteran model insect because of its economic importance, ease of handling, availability of a large number of genetic mutants, linkage maps, and whole genome sequencing [Mita et al., 2003, 2004]. Hence, it is not surprising that most of the information available on sex determination mechanism in lepidopteran insects comes from studies in *B. mori*.

Screening and characterization of homologs of *Drosophila* sex determination cascade genes *Sex lethal (Sxl)*, *transformer (tra)*, and *doublesex (dsx)* revealed that only a *dsx* homolog is involved in the sex determination process in *B. mori* [Fujii and Shimada, 2007]. Further, *Bombyx* homolog of *P-element somatic inhibitor (Bmpsi)* and *Bombyx* homolog of *IGF-II mRNA-binding protein (Bmimp)* (discussed later) have recently been reported to be involved in the *Bombyx* sex determination pathway [Suzuki et al., 2008, 2010].

Dsx Homologs in Lepidopterans

Dsx is the most downstream gene of the *Drosophila* sex determination cascade. Pre-mRNA of *dsx* is sex-specifically spliced to produce sex-specific transcript variants that encode sex-specific DSX protein isoforms

[Hoshijima et al., 1991; Tian and Maniatis, 1993]. These protein isoforms share a common Doublesex-Mab3 domain but contain different dimerization domains [Erdman and Burtis, 1993; Erdman et al., 1996]. As a result, female- and male-specific DSX proteins regulate a number of cytodifferentiation genes in an antagonistic manner [Burtis et al., 1991; Coschigano and Wensink, 1993; Shirangi et al., 2009; Chatterjee et al., 2011; Wang et al., 2011].

A *dsx* homolog (*Bmdsx*) in *B. mori* is present on chromosome 25, and its pre-mRNA is spliced in a sex-specific manner producing 2 female- and 1 male-specific isoforms [Ohbayashi et al., 2001; Shukla et al., 2011]. *Bmdsx* harbors 5 exons and 4 introns out of which exons 1, 2, and 5 are constitutively spliced, whereas exons 3 and 4 are female-specific [Suzuki et al., 2001]. Both female-specific isoforms of *Bmdsx* differ from each other by a 15-bp alternatively spliced (alternative 5' splice site selection) sequence present after exon 3 [Shukla et al., 2011]. Due to this difference, both BmDSX^F proteins differ from each other by 18 amino acids. Sex-specific BmDSX isoforms have been shown to regulate downstream genes in a sex-specific manner suggesting the role of BmDSX in the sex differentiation process in *B. mori* [Suzuki et al., 2003, 2005]. *Dsx* homologs (*Aadxs* and *Amdsx*) have also been characterized in the wild silkmoths *Antheraea assama* and *A. mylitta* [Shukla and Nagaraju, 2010]. *A. assama* contains fewer chromosomes ($n = 15$) compared to that in other known silkmoths, and females lack the W chromosome (Z0) [Deodikar et al., 1962; Arunkumar et al., 2009]. *A. mylitta*, another saturniid moth ($n = 31$), has a sex chromosome composition (ZW females and ZZ males) similar to *B. mori* [Mahendran et al., 2006a; Arunkumar et al., 2008]. Displaying functional conservation, the pre-mRNAs of *Aadxs* and *Amdsx* sex-specifically splice and regulate downstream genes in a sex-specific manner [Shukla and Nagaraju, 2010]. Among other lepidopterans, *dsx* has also been characterized in the adzuki bean borer, *Ostrinia scapularis* [Sugimoto et al., 2010]. Like *Bmdsx*, the pre-mRNA of *Osdsx* also produces 2 female- and 1 male-specific isoform. Both the female isoforms differ in their 3' UTR regions (presence or absence of exon 5). In spite of the functional conservation of *dsx* homologs in silkmoths, sex-specific splicing of the pre-mRNA of *dsx* and *Bmdsx* was found to be different, since unlike *dsx*, the female-specific splicing of *Bmdsx* pre-mRNA represents the default mode of splicing, as shown in HeLa nuclear extracts [Suzuki et al., 2001]. Based on this fact, a different mechanism was proposed for *Bmdsx* splicing regulation.

Tra/tra2

In *Drosophila*, functional TRA protein is produced only in females [Boggs et al., 1987; Belote et al., 1989; Valcárcel et al., 1993]. TRA, together with the product [Transformer 2 (TRA2)] of a constitutively expressed gene, *tra2*, is required for the splicing of *dsx* pre-mRNA in the female mode [Hoshijima et al., 1991].

A *tra* homolog thus far has not been identified in *B. mori* probably due to the rapid divergence of its sequence during evolution, as evidenced from studies on different *Drosophila* species and on other dipteran species [O'Neil and Belote, 1992; Kulathinal et al., 2003; Ruiz et al., 2007; Concha and Scott, 2009; Hediger et al., 2010]. BLAST analysis has revealed the presence of a *tra2* homolog (*Bmtra2*) in *B. mori* [Niu et al., 2005]. Due to the selection of 3 different 3' splice sites within the second intron and 2 different splice sites within the seventh intron, *Bmtra2* pre-mRNA is alternatively spliced to produce 6 different isoforms. All 6 deduced BmTRA2 proteins contain 1 RNA recognition motif domain flanked by RS (Arg/Ser) domains, a typical characteristic feature of the *Drosophila* TRA2 protein [Suzuki et al., 2012]. RNAi-mediated knockdown of all *Bmtra2* isoforms in *B. mori* cultured cell lines does not affect sex-specific splicing of a *Bmdsx* mini gene [Suzuki et al., 2012]. Also, RNAi of *Bmtra2* in *Bombyx* eggs does not affect female development but causes abnormal development of testis in males. Hence, *Bmtra2* has a role in early testis morphogenesis, but it does not play a role in *Bombyx* sex determination [Suzuki et al., 2012].

Sex-Specific Splicing Regulators of Bmdsx

Besides *B. mori* lacking a *tra* homolog and the finding that *Bmtra2* is not involved in the sex-specific splicing of *Bmdsx* pre-mRNA, *Bmdsx* also lacks cis-regulatory elements needed for TRA/TRA2-mediated splicing regulation, namely the *dsx* repeat element and purine-rich element sequences [Suzuki et al., 2001]; exon 4 of *Drosophila dsx* contains repeat sequences (*dsx* repeat elements) that act as an exonic splicing enhancer, whereas a purine-rich element in the fourth exon makes the preceding 3' splice site a weak splicing acceptor. Binding of TRA/TRA2 along with other proteins (splicing factors) to the *dsx* repeat elements makes the 3' splice site a strong splicing acceptor, and as a result, the fourth exon is spliced-in to produce *dsx^f* transcripts [Hedley and Maniatis, 1991; Hoshijima et al., 1991; Tian and Maniatis,

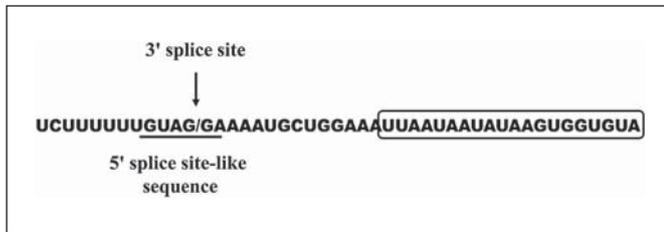


Fig. 1. Nucleotides surrounding the 3' splice site (= /; indicated by arrow) of the fourth exon of *Bmdsx* showing the presence of consensus 5' splice site-like sequence (underlined). Boxed nucleotides represent CE1 cis-regulatory element. Figure mimicked from Suzuki et al. [2008].

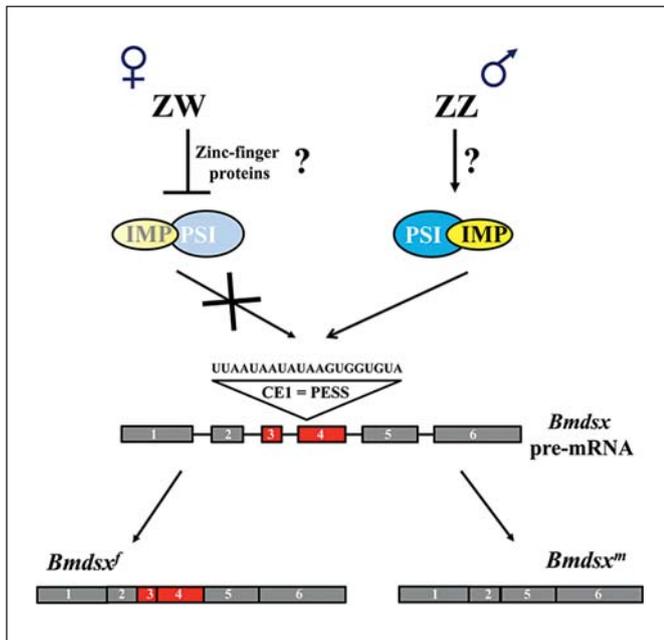


Fig. 2. Model for sex determination in *B. mori*. BmPSI and BmIMP proteins in ZZ individuals act together to direct splicing of *Bmdsx* pre-mRNA (in male mode) by binding to CE1 cis-regulatory element (PESS) present in the fourth exon of *Bmdsx*. Feminizing factor (protein products of zinc-finger genes present in the *Fem* region) from W chromosome inhibits the production of BmIMP proteins in females. This leads to the default splicing of *Bmdsx* pre-mRNA causing the inclusion of exons 3 and 4 in females.

1993; Lynch and Maniatis, 1996]. In order to search for the cis-regulatory elements present on *Bmdsx* and trans-acting factors regulating the sex-specific splicing of *Bmdsx* pre-mRNA, Matsumoto and colleagues designed an in vivo sex-specific splicing assay for a minigene of *Bmdsx* using male and female cell lines of *B. mori* [Suzuki et al., 2008]. Initially, 3 sequences (CE1, CE2, and

CE3) on exon 4 of *Bmdsx* were identified as putative exonic splicing silencers responsible for male-specific splicing of *Bmdsx* pre-mRNA. CE1 is a UA rich sequence (UUAUAAUAUAAGUGGUGUA) and harbors 3 UAA repeats.

BmPSI

Experimental evidence suggests that BmPSI protein, a *Bombyx* homolog of P-element somatic inhibitor (PSI), binds to the CE1 sequence, but only in males. RNAi-mediated knockdown of *Bmpsi* resulted in a significant increase of female-specific splicing of *Bmdsx* pre-mRNA in male cells [Suzuki et al., 2008]. These experiments unambiguously suggested that the cis-regulatory element CE1 and the trans-regulatory protein BmPSI are required for male-specific splicing of *Bmdsx* pre-mRNA [Suzuki et al., 2008]. These findings also confirmed that female-specific splicing of *Bmdsx* is the default mode, as proposed by splicing experiments of *Bmdsx* pre-mRNA in HeLa nuclear extracts [Suzuki et al., 2001].

BmPSI contains 4 KH (K Homology) domains and 2 tandem repeats of a 30-amino acid motif (homologous to PSI-A and PSI-B at its C-terminus) [Suzuki et al., 2008]. The KH domain is involved in RNA recognition and RNA interaction. *Bmdsx* harbors a sequence homologous to the consensus of the weak 5' splice site about 13 nt upstream to the CE1 element. This pseudo 5' splice site-like sequence is located within the actual 3' splice site just upstream of exon 4 (fig. 1). As mentioned earlier, exon 4 of *Bmdsx* is a female-specific exon (see fig. 2 for sex-specific splicing of *Bmdsx* pre-mRNA). Suzuki et al. [2008] proposed the recruitment of U1 snRNP to the 5' splice site-like sequence (not to the actual 5' splice site) driven by BmPSI binding to the CE1 element in males. Once U1 snRNP is recruited to this pseudo 5' splice site (actual 3' splice site region), other splicing factors (e.g. U2AF) are unable to assemble on the 3' splice site leading to the skipping of exon 4 [Suzuki et al., 2008]. Exon 3, which skips along with exon 4 in males, does not harbor any cis-regulatory sequence for the sex-specific splicing of *Bmdsx* pre-mRNA. Hence, the mechanism by which exon 3 is skipped along with exon 4 is yet to be explored.

The 20-nt CE1 sequence of *Bmdsx* is completely conserved and present in the female-specific regions of *Aadsx*, *Amdsx* [Shukla and Nagaraju, 2010], and *Osdsx* [Sugimoto et al., 2010]. *O. scapularis* belongs to Pyraloidea, *A. assama* and *A. mylitta* belong to Saturniidae, and *B. mori* belongs to Bombycoidea; all of these families are

phylogenetically distantly related [Mahendran et al., 2006b; Regier et al., 2009], suggesting the CE1 sequence to be the common cis-regulatory element present in lepidopteran *dsx*.

BmIMP

Though the BmPSI protein is present in both male and female cell lines, it functions only in males regarding the skipping of exon 3 and 4 of *Bmdsx* [Suzuki et al., 2008]. On this basis, Suzuki et al. [2010] hypothesized the existence of a sex-specific cofactor. Another splicing regulator (BmIMP) of *Bmdsx* pre-mRNA was identified in a search for male-specific binding partner(s) of BmPSI [Suzuki et al., 2010]. BmIMP is a *Bombyx* homolog of IGF-II mRNA-binding protein (IMP) [Suzuki et al., 2010]. IMPs are members of KH-domain containing RNA binding proteins with functions in cell polarity and migration, cell proliferation, and cancer [Yisraeli, 2005]. BmIMP was identified in pull down experiments using CE1 sequences as bait. BmIMP is male-specifically expressed in various tissues and contains a single N-terminal RNA recognition motif domain followed by 4 KH domains [Suzuki et al., 2010]. Experimental evidence suggests BmIMP to be the splice regulator of *Bmdsx* pre-mRNA, as evidenced from (1) specific binding of BmIMP to CE1 cis-regulatory sequences in gel mobility shift assays, (2) a shift to female-specific splicing of *Bmdsx* upon RNAi-mediated knock-down of BmIMP in differentiated male cells, and (3) a shift to male-specific splicing of *Bmdsx* pre-mRNA upon coexpression of BmIMP and BmPSI in differentiated female cells. It has been suggested that BmPSI and BmIMP act together in male-specific splicing of *Bmdsx* pre-mRNA, as expression of either BmPSI or BmIMP alone cannot induce male-specific splicing of *Bmdsx* in female cells [Suzuki et al., 2010]. BmIMP interacts with BmPSI through the KH domains and increases the binding affinity of BmPSI to the CE1 element by reducing its dissociation rate [Suzuki et al., 2010]. Coexpression of BmIMP and BmPSI induces male-specific splicing of *Bmdsx* pre-mRNA in differentiated female cells, however, the relative amount of male-specific isoforms is lower than in differentiated male control cells. The authors proposed different possibilities for this observation: (a) insufficient levels of expressed BmPSI and BmIMP proteins and/or (b) requirement of an additional male-specific factor and/or (c) presence of a female-specific factor that counteracts the activities of BmPSI and BmIMP proteins [Suzuki et al., 2010]. Ongoing studies of the *Fem* factor

present on the W chromosome of *B. mori* may shed some light on this aspect.

Based on X-ray irradiation-induced chromosomal breakage techniques, a small portion of the W chromosome (*Fem* locus) was translocated to autosomes that were capable of inducing feminization [Hashimoto, 1933; Tazima, 1954; Abe et al., 2008]. Upon further dissection of W chromosomes using W-specific randomly amplified polymorphic DNA markers and by deletion mapping, the feminizing factor was found to be linked to the Rikishi marker (randomly amplified polymorphic DNA sequence of the W chromosome required to determine femaleness) [Kimura et al., 1971]. Recently, 2 groups [Ajimura et al., 2007; Satish et al., 2007] independently attempted to identify the genes in the *Fem* locus. A 380-bp DNA fragment of the W chromosome was identified by Ajimura et al. [2007] in genome subtraction between females and males. Making use of this 380-bp sequence, a W-BAC clone harboring 2 putative zinc-finger protein encoding genes (C3H and C2H2 type) was isolated. Interestingly, these genes lie within the minimal *Fem* region on chromosome W. Satish et al. [2007] identified a C3H type zinc-finger protein gene (identical to the one identified by Ajimura et al. [2007]) by differential display of transcripts from male and female embryos. A functional analysis of these candidate genes is in progress.

W-Linked Zinc-Finger Genes and Their Possible Role in Sexual Development in *B. mori*

Four copies each of C3H type (*Bmz1-1a*, *Bmz1-1b*, *Bmz1-2*, and *Bmz1-3*) and C2H2 type (*Bmz20-1a*, *Bmz20-1b*, *Bmz20-2*, and *Bmz20-3*) zinc-finger genes, arranged in tandem repeats, are linked to the *Fem* region of the *Bombyx* W chromosome (unpubl. data). Homologs of these zinc-finger genes [2 C3H type (*z2* and *z3*) and 2 C2H2 type (*z21* and *z22*)] were also found on the 25th chromosome of *B. mori*. W-linked zinc-finger genes were found to be expressed very early during embryonic development prior to the expression of *Bmdsx*, whereas autosomal zinc-finger genes are constitutively expressed throughout embryogenesis [Ajimura et al., 2007; Satish et al., 2007].

Taken together, the location of these zinc-finger genes (*z1* and *z20*) in the *Fem* region of the W chromosome and their early embryonic expression make it plausible that these genes are the source of the initial signal for female development. Zinc-finger proteins can bind to DNA, RNA, protein, and /or lipid substrates [Klug, 1999; Laity

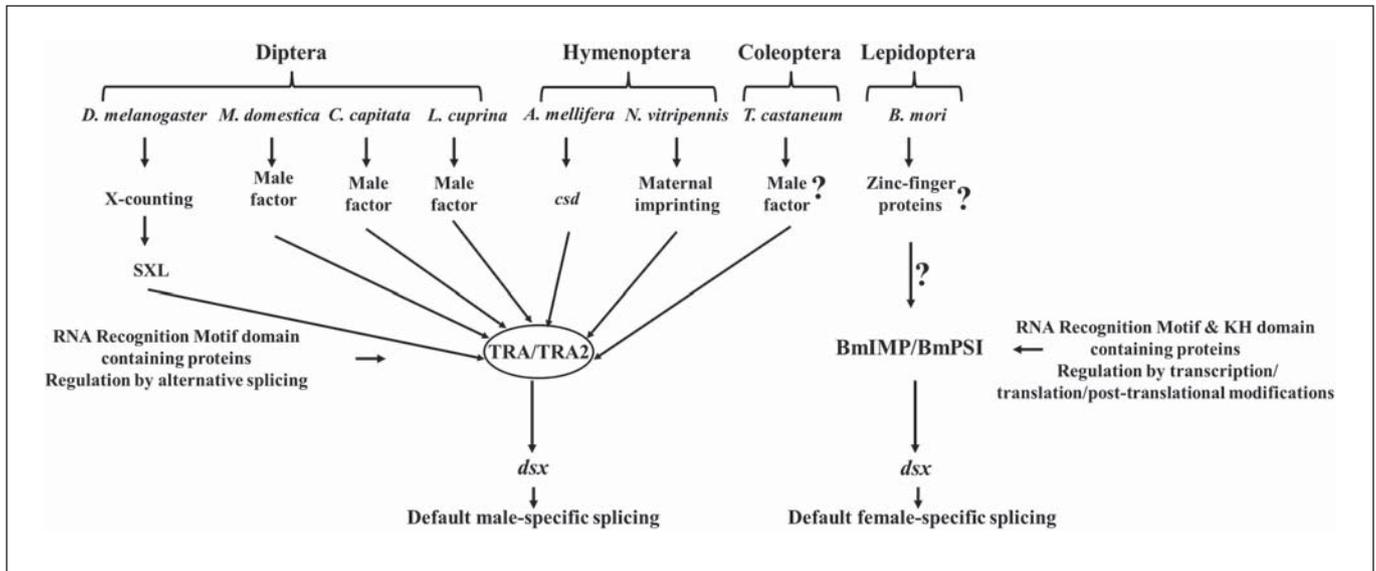


Fig. 3. Schematic diagram representing the sex determination cascade in the model insect species of Diptera (*D. melanogaster*, *Musca domestica*, *Ceratitis capitata*, and *Lucilia cuprina*), Hymenoptera (*Apis mellifera*, *Nasonia vitripennis*), Coleoptera (*Tribolium castaneum*), and Lepidoptera (*B. mori*). Dipteran, hymenopter and coleopteran insects share a common feature of regulation of *dsx* pre-mRNA by the TRA/TRA2 protein complex and differ only in the mechanism (upstream genes) by which *tra* is regulated [reviewed in Sánchez, 2008; Verhulst et al., 2010]. Lepidopterans have adopted different players for their sex determination (except *dsx*, no other sex-determining gene has been characterized in lepidopterans other than *B. mori*; hence, only the *B. mori* cascade has been shown here). Though *dsx* is functionally conserved in lepidopter-

ans (*B. mori*, *A. assama*, *A. mylitta*, and *O. scapularis*), they all harbor unique cis-regulatory elements required for male-specific splicing in *B. mori*. Also, BmPSI and BmIMP are required for male-specific splicing of *dsx* pre-mRNA in *B. mori*. Presence of the CE1 element is unique to lepidopteran *dsx*, and requirement of PSI and IMP proteins for the sex-specific splicing of *dsx* pre-mRNA is unique to *B. mori*, as yet. Future studies may lead to the discovery of PSI and IMP proteins as splice regulators of *dsx* pre-mRNA in other lepidopterans considering the conservation of the CE1 element in *dsx* of all the lepidopteran insects studied to date. Question marks represent the unknown factors or the unknown mechanism(s) of action.

et al., 2001; Matthews and Sunde, 2002; Brown, 2005; Hall, 2005; Gamsjaeger et al., 2007; Pomeranz et al., 2010]; hence, it is hard to predict the role and mode of action of these zinc-finger proteins in *Bombyx* sex determination. Future studies aimed at functional characterization of these zinc-finger genes (W-linked and autosomal) and their interaction with known genes (BmPSI and BmIMP) of the pathway may unravel the mechanism underlying sex determination in *B. mori*. Figure 2 represents the model of *B. mori* sex determination cascade based on available data.

Intersex and Fruitless

In *Drosophila*, DSX^F interacts with the gene product of *intersex* (*ix*) to execute its function [Waterbury et al., 1999]. Though IX is expressed in both sexes, it is known to function only with DSX^F to regulate female differen-

tiation [Garrett-Engle et al., 2002]. Among lepidopterans, *ix* homologs have been reported to be present in domesticated and wild silkmoths, including *B. mori*, *A. assama*, *A. mylitta*, and *Samia cynthia ricini* [Siegal and Baker, 2005; Arunkumar and Nagaraju, 2011]. Besides silkmoths, *ix* has also been characterized in *Maruca vitrata*, a Piralidae moth [Cavaliere et al., 2009]. Unlike *Drosophila*, *ix* in *B. mori*, *A. assama*, and *M. vitrata* produces sex-specific alternatively spliced isoforms [Cavaliere et al., 2009; Arunkumar and Nagaraju, 2011]. Functional significance of the different IX isoforms in lepidopteran sex determination has yet to be investigated.

In *Drosophila*, a specific set of Fruitless proteins is expressed in the male central nervous system and is responsible for innate male-specific behaviors such as courtship behavior [Lee et al., 2000; Kimura et al., 2008]. Sex-specific splicing regulation of *fru* pre-mRNA is governed by *tra/tra2* such that sex-specific *fru* splice variants are produced at late stages, of which only the male vari-

ants have an intact ORF and are functional [Heinrichs et al., 1998].

In *B. mori*, adult males and females show distinct sexual behaviors, where males perform a pheromone-triggered mating dance involving walking behavior, wing vibrations (30–40 Hz), and occasional abdominal movements [Ai et al., 1998; Sasaki et al., 2009]. In the *B. mori* genome, a well-conserved *fru* homolog (*Bmfru*) is present. Similar to *Drosophila*, *Bmfru* also produces multiple isoforms as a result of different transcription initiation sites and also by alternative splicing in its pre-mRNA [Ohbayashi, 2001; Fujii and Shimada, 2007]. The role of multiple *Bmfru* isoforms has not been functionally analyzed as yet.

Conclusion

Sex determination studies in insects belonging to different orders suggest the existence of a conserved central axis (*tra* gene) around which sex is determined [Verhulst et al., 2010]. *Dsx*, which is downstream to *tra* in the insect sex determination hierarchy, is conserved even in nematodes [*male abnormal-3 (mab-3)*] and vertebrates [*dsx* and *mab-3* related transcription factor (*Dmrt1*)] [Matson and Zarkower, 2012]. The variation of the sex determination mechanism among insect species (except lepidopterans) mainly represents the different modes by which *tra* is regulated. Silkworms, belonging to the order Lepidoptera, are the exception to this rule: in *B. mori*, only *dsx* is conserved (*tra* homolog is absent in the *Bombyx* genome, and *Bmtra2* has no role in the splicing of *Bmdsx* pre-

mRNA) [Mita et al., 2004; Nissen et al., 2012]. Splice regulators of *Bmdsx* pre-mRNA, BmPSI, and BmIMP, were reported for the first time to be involved in the sex determination cascade in any organism [Suzuki et al., 2008, 2010]. Absence of TRA/TRA2-binding sites together with the conservation of the fourth exon (which harbors BmPSI and BmIMP binding sequences) of *Bmdsx* in the *dsx* gene of wild silkworms provides a clue to the involvement of PSI and IMP proteins in sex-specific splicing of *dsx* pre-mRNA in silkworms. Future studies dealing with wild silkworms and other lepidopteran insects may show the extent to which PSI and IMP proteins are conserved splicing regulators of *dsx* pre-mRNA in the lepidopteran lineage. Besides the linear cascade of sex determination (? → BmPSI/BmIMP → *dsx*; rather than TRA/TRA2 → *dsx*), lepidopterans are also unique in *ix*, owing to the sex-specific splicing of its pre-mRNA. Also, the discovery of the *Fem* locus and W-linked zinc-finger genes in *B. mori* unveils a unique case involving the diversity of insect sex determination mechanisms. Figure 3 summarizes the various sex determination mechanisms found so far in different insect species. More surprises may be expected in future studies in lepidopteran species other than the silkworms.

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