REVIEW ARTICLE

Sex determination: insights from the silkworm

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Abstract

The sex-determining system differs considerably among organisms. Even among insect species, the genetic system for sex-determination is highly diversified. In *Drosophila melanogaster*, somatic sexual differentiation is regulated by a well characterized genetic hierarchy X : A > *Sxl* > *tra/tra2* > *dsx* and *fru*. This cascade seems to control sex determination in all *Drosophila* species and is partially conserved in another dipteran species, *Ceratitis capitata: Cctra/Cctra-2* > *Ccdsx* and *Ccfru*. However, in the silkworm, *Bombyx mori*, femaleness is determined by the presence of a dominant feminizing factor on the W chromosome. Moreover, no sex-specific regulatory *Sxl* homolog has been isolated from *B. mori*. Also, no *tra* homolog has yet been found in the *Bombyx* genome. Despite such differences, *dsx* homolog of *B. mori* (*Bmdsx*) is implicated in the sex determination. *Bmdsx* produces alternatively spliced mRNA isoforms that encode sex specific transcription factors as observed in *dsx*. While the female-specific splicing of *dsx* is activated by splicing activators, Tra and Tra2, the female splicing of *Bmdsx* represents the default mode. Instead, a splicing inhibitor, BmPSI is involved in the regulation of male-specific splicing of *Bmdsx*. Since BmPSI does not exhibit any sequence relationship to known SR proteins, such as Tra and Tra2, the regulatory mechanism of sex-specific alternative splicing of *Bmdsx* is distinct from that of *dsx*.

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Introduction

In most cases, key developmental and regulatory genes show high homology and are functionally conserved among diverse model organisms (Carroll *et al*. 2001). However, molecular studies on the evolution of the regulatory genes that control sex determination in the insect *Drosophila melanogaster*, the nematode *Caenorhabditis elegans* and mammals suggest that certain sex-determination-regulatory genes have evolved relatively rapidly (Marin and Baker 1998). Even closely related species differ in their primary chromosomal sex-determination signals. One of the best studied animal lineages in this regard is the insect order Diptera. In *D. melanogaster*, the first signal of sex determination is the ratio of X chromosomes to sets of autosomes (A). The X : A ratio, a balance mechanism in which X chromosomal gene products are titrated against autosomal gene products, governs sex determination. However, recent findings revealed that the sex-determination signal is more accurately viewed as a function of the number of X chromosomes rather than as a value of the X : A ratio (Erickson and Quintero 2007). The primary chromosomal sex-determination signal observed in *Drosophila* is not common in any other previously examined insect. In *Megaselia scalaris* (Traut 1994), *Ceratitis capitata* (Willhoeft and Franz 1996), *Bactorocera tryoni* (Shearman and Formmer 1998), *Lucilia cuprina* (Bedo and Foster 1985) and *Chironomus thummi* (Hägele 1985), an epistatic maleness factor is found on the Y chromosomes. The mosquito *Culex tritaeniorhynchus* has no sex chromosome, and male sex is determined by a dominant gene on an autosome (Baker and Sakai 1976). The diploid/haploid sex-determination system is well known in Hymenoptera (Beukeboom 1995). In Lepidoptera, *B. mori* has epistatic female determinant on the W chromosome (Hashimoto 1933), while *Lymantria disper* has Z-linked male determinant (M) and maternally inherited female determinant factor (F) (Goldschmidt 1955). Thus, the existence of a variety of sex-determining mechanisms indicates that the genes regulating this mechanism have been highly diversified and may evolve particularly rapidly by en-

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vironmental selection or genetic factors depending on the organism concerned (Kuwabara 1996; Marin and Baker 1998). The unusual rapid divergence of sex-determination mechanisms presents both a fascinating puzzle and unique opportunity to study the evolution of a key developmental process.

To further our understanding of sex-determination systems, a direct approach using comparative molecular genetics and inverse genetics is required. Indeed PCR technology, in combination with an RNAi approach and gene transfer techniques, have already proven to be very useful in isolating orthologues and in studying their *in vivo* functions in new species (Berghammer *et al*. 1999; Christophides *et al*. 2000). All these techniques as well as EST and WGS database are now available for *B. mori* (Mita *et al*. 2003, 2004). Therefore, *Bombyx* should be a suitable material for studies on sex determination. Here, we describe the results of recent studies of sex determination in *Bombyx* in the context of understanding the evolution of sex determination among insects.

Female determinants on the W chromosome

In *B. mori*, females are the heterogametic sex, carrying one copy each of the Z and W sex chromosomes. Males are homogametic (ZZ). However, in some lepidopteran insects, females are monosomic (ZO) and males are ZZ. The W chromosome has been found only in Ditrysia, one of the taxonomic suborders in Lepidoptera. In Trichoptera, the taxonomic order most closely related to Lepidoptera, all species examined to date adopt the ZO/ZZ sex chromosome system (Traut and Marec 1996). Therefore, it is reasonable to consider that the W chromosome emerged after the divergence between Ditrysia and other suborders in Lepidoptera.

In 1933, Hashimoto began to understand the principles underlying sex determination from studies of the phenotypes of sex chromosome aneuploids, individuals with unusual combination of sex chromosomes (Hashimoto 1933). For example, the observations that 3A; ZZ and 3A; ZZW triploids develop into phenotypic males and females, respectively, conclusively demonstrate a role of the W chromosome in triggering femaleness in the silkworm. He proposed that femaleness of the silkworm is simply determined by the presence of the feminizing factor (*Fem*) on the W chromosome. Tazima and his colleagues established the W chromosome flanked on both ends by translocated chromosomes carrying phenotypic marker genes (Tazima 1954). After inducing breakage of this marked W chromosome by X-ray irradiation, they investigated relation between femaleness and the marker genes translocated to the W chromosome. Finally, they concluded that *Fem* is located in a restricted area on the W chromosome (Tazima 1954). To further narrow the *Fem* localizing region, deletion mapping of W-specific RAPD markers was carried out. Based on the presence or absence of W-specific RAPD markers in the six deleted W chromosomes, the relative positions of the W-RAPD markers and the *Fem* gene were determined

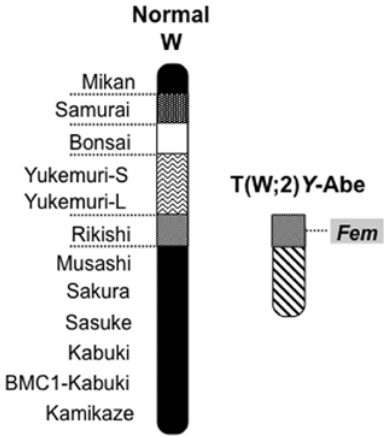

Figure 1. Relative positions of the W-specific RAPD markers and the *Fem* gene. To date, the presence or absence of 12 W-specific RAPD markers have been determined using unique mutants for the W chromosome generated by X-ray irradiation. T(W;2)*Y*-Abe chromosome was generated by translocation between the W and the chromosome 2. This mutant W chromosome has a normal feminizing activity. A hatched region indicates a fragment of the chromosome 2 translocated on to the W chromosome.

(figure 1). As shown in figure 1, T(W;2)*Y*-Abe chromosome contains only one W-RAPD marker, Rikishi. T(W;2)*Y*-Abe chromosome has normal feminizing activity (Kimura *et al*. 1971). These findings indicate that *Fem* is located in the Rikishi mapped region. Recently, Ajimura (2006) carried out genome subtraction between females and males in an attempt to isolate *Fem* gene. They identified a 380-bp DNA fragment located on the W chromosome. Further, they found two genes encoding a putative zinc-finger protein from a BAC clone isolated using the 380-bp DNA fragment. Interestingly, these two genes are present on the T(W;2)*Y*-Abe chromosome. The other group identified a gene identical to one of the two genes by differential display experiments using cDNAs separately extracted from female and male eggs at around the same time (Satish *et al*. 2006). Efforts continue to reveal the function of these two genes using RNAi and transgenic approaches.

Z chromosome–its role in sexual dimorphism

In *B. mori*, the Z chromosome is cytogenetically a relatively large chromosome. Whole-genome sequence analysis revealed that the Z chromosome is the third largest chromosome, accounting for about 4.6% of the haploid genome, and predicted that more than 700 genes are located on the Z chromosome. On the other hand, no gene except for *Fem* has been mapped to the W chromosome. Although no syntenic similarity has been found between the Z and W chromosomes, they pair with synaptonemal complexes throughout their length during female meiosis (Traut *et al*. 1999).

In metazoan organisms in which the male is heterogametic (XY) or hemizygous (XO), the dosage compensation mechanism equalizes the doses of X-linked genes between males and females. Mutations defective for the dosage com-

pensation lead to lethality in *D. melanogaster*. However, several reports have demonstrated that Z-linked genes are not dosage compensated in *B. mori* and other lepidopteran insects (Suzuki *et al*. 1998, 1999; Koike *et al*. 2003). Since the Z chromosome makes up nearly 5% of the haploid genome, the viability of heterogametic females in spite of an absence of dosage compensation raises questions about the role of this process.

Several studies on butterflies suggest that the absence of dosage compensation may be the result of a precise adaptation (Stehr 1959; Grula and Taylor 1980; Sperling 1994). Johnson and Turner (1979) suggested that the absence of dosage compensation may provide the metabolic basis for the limitation of expression to the female sex, which is a common feature of mimicry, and of some nonmimetic polymorphisms in butterflies. They also pointed out that the lack of dosage compensation would explain that female-limited characters will be predominantly autosomal, whereas male-limited sexual characters can be both sex-linked and autosomal (Johnson and Turner 1979). Among butterflies, a large proportion of genes controlling female mate-selection behaviour and male-courtship signals are located on the Z chromosome (Grula and Taylor 1980), and genetic data on these phenotypes are consistent with the view that most, if not all, of the Z chromosome lacks dosage compensation (Johnson and Turner 1979; Grula and Taylor 1980). In *B. mori*, four genes encoding muscle protein, such as kettin, titin and projectin, are mapped to the Z chromosome. The expression levels of these genes in males are more than twice as compared with those in females (Suzuki *et al*. 1999; Koike *et al*. 2003). Such difference in the expression levels of muscle proteins between males and females may be the result of an adaptation to the sexual difference in the demand for wing flapping activity. Thus, in lepidopteran insects, it appears that there is a close relationship between an absence of dosage compensation of Z-linked genes and the sexual dimorphism in phenotype. To date, however, genes implicated in the sex determination or sex differentiation have not been found on the Z chromosome of *B. mori*.

Bombyx homologs of the *Drosophila* sex-determining genes

In *Drosophila*, the ratio between the number of X chromosomes and autosomal sets (X : A) serves as the initial determinant of sex. On the other hand, recent findings indicated that the X : A ratio predicts sexual fate, but does not actively specify it. Instead, the instructive X chromosome signal is more appropriately seen as collective concentrations of several X-encoded signal element (XES) proteins in the early embryo (Erickson and Quintero 2007). Proper concentration of XES proteins induces female-specific activation of the *Sex lethal* (*Sxl*) gene that encodes a splicing regulator. Sxl protein then activates the female-specific splicing of its downstream gene *transformer* (*tra*), giving rise to functional Tra protein. Tra, together with Tra2, binds to the *cis*-regulatory element (dsxRE) within the female-specific exon

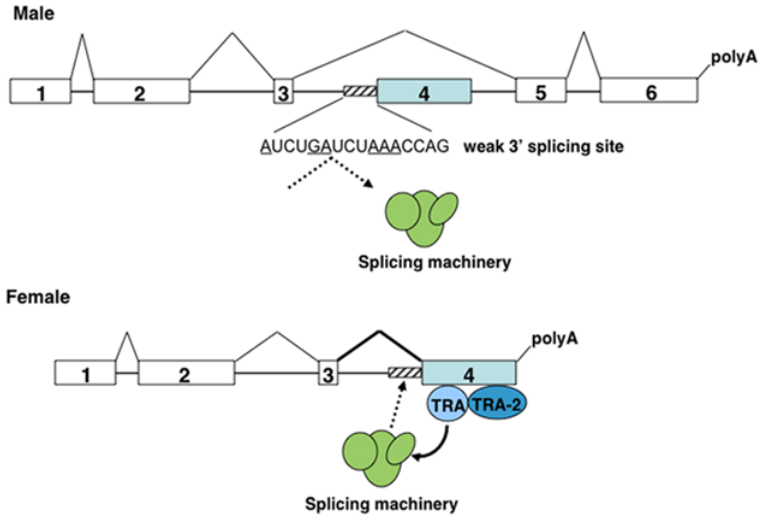

Figure 2. Sex-specific splicing regulation of *dsx* by Tra and Tra2. Tra, together with Tra2, binds to the *cis*-regulatory element (dsxRE) within the female-specific exon (exon 4) of *dsx*, activating the weak 3′ splicing site preceding the exon to generate the female-type Dsx protein (Dsx^F). Both Tra and Tra2 contain Arg/Ser-rich (RS) domains, protein interaction domains characteristic of the Ser/Arg (SR) family of essential splicing factors. Tra and Tra2 promote recruiting the splicing machinery to the weak 3′ splicing site. In the absence of Tra, the transcripts of *dsx* undergo default processing to generate the male-type DSX protein (Dsx^M).

of its downstream gene *doublesex* (*dsx*), activating the weak 3′ splicing site preceding the exon to generate the female-type Dsx protein (Dsx^F), which regulates its downstream genes for the female development to proceed (figure 2). In males, the X : A ratio of 0.5 prevents *Sxl* from being produced, leading to the male-specific splicing of *tra*, rendering its encoded product nonfunctional due to a premature stop codon. In the absence of Tra activity, Dsx^M is produced by the default male-type splicing of *dsx*, which regulates its downstream genes for male development (Cline and Meyer 1996).

Many homologs of *Drosophila* sex-determining genes have been already found in *B. mori*. First, *BmSxl*, a *Bombyx* homolog of *Sxl*, was found by Niimi *et al*. (2006). It is, however, expressed in both sexes equally, suggesting that it plays a sex-independent role in *B. mori*. The same situation is observed in the other non-*Drosophila* insect species, such as *Megaselia scalaris, Musca domestica*, and *Ceratitis capitata* (Meise *et al*. 1998; Saccone *et al*. 1998; Sievert *et al*. 2000; Lagos *et al*. 2005).

Given the unusually high degree of sequence divergence among *tra* homologs in *Drosophila* (O'Neil and Belote 1992), it would be very difficult to identify a *tra* homolog from non-*Drosophila* insect species. However, recent studies demonstrate that a homolog of *tra*, although highly diverged in sequence, is indeed present in the genome of the olive fruit fly (*Bactrocera oleae*) and Mediterranean fruit fly (*Ceratitis capitata*), two non-*Drosophila* flies. These *tra* homologs (*Botra, Cctra*) have a female-determining-master function (Pane *et al*. 2002; Lagos *et al*. 2007). In addition, the gene *feminizer* (*fem*), a sex-determining gene of honeybee (*Apis mellifera*), is also found to be a homolog of *Cctra* (Hasselmann *et al*. 2008).

The genes *fem* and *Cctra* seem to have equivalent functions in sex determination, belong to the same family of SR-type proteins, share the same arrangement of regions enriched with arginine and serine (Arg/Ser domain) and proline (Pro-rich region), but harbour no significant identity in sequence motifs. Hasselmann *et al*. (2008) identified a 30-amino-acid motif in which 15 residues are identical when they compared Fem with CcTra. These homologies establish a common ancestral pathway of sexual regulation at the level of the *tra* gene across insect orders and ~300 million years of independent evolution. If this is the case, then it is expected that *B. mori* contains a *tra* homolog. However, whole-genome sequence analysis has not found a *tra* homolog in the *Bombyx* genome (Mita *et al*. 2004).

DSX, the most downstream component of the *Drosophila* sex-determination pathway that controls most sex-specific phenotypes, is a transcriptional factor with a zinc-finger DNA-binding domain known as the DM domain. Several DM-domain-containing proteins are known to participate in sex determination in a diverse array of animals. Orthologs of *D. melanogaster dsx* have been identified and studied in a number of dipterans (Shearman and Formmer 1998; Kuhn *et al*. 2000; Hediger *et al*. 2004; Lagos *et al*. 2005; Ruiz *et al*. 2005; Scali *et al*. 2005). In all these species, the gene structure and the sex-specific splicing pattern of *dsx* are generally conserved. Outside the order Diptera, several studies of *dsx* have been conducted in the honeybee,

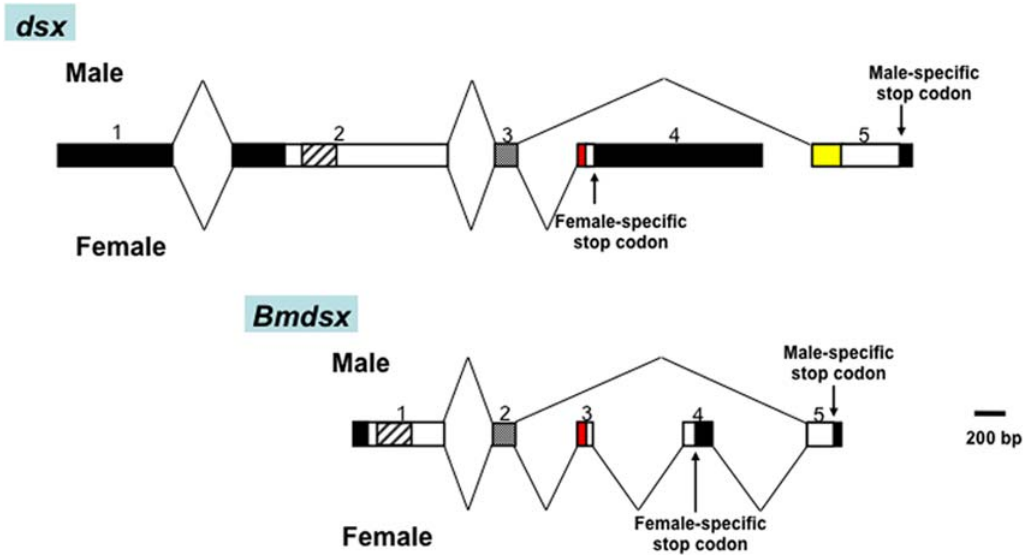

Figure 3. Comparative analysis of the exon/intron layout of the *dsx* and *Bmdsx* genes. The exon/intron boundaries of *Bmdsx* are compared with that of the *dsx* gene. Exons are shown as boxes. Untranslated regions are shown as black boxes and coding regions as open boxes. OD1 domains are indicated as diagonally hatched boxes. Sex-independent regions of the OD2 domain are depicted by stippled boxes. Female-specific and male-specific OD2 domains are represented by red and yellow boxes, respectively.

Apis mellifera (Cristino *et al*. 2006; Cho *et al*. 2007). We have identified a *dsx* homolog in *B. mori* (Ohbayashi *et al*. 2001). Like *dsx*, the primary transcript of the *Bmdsx* gene is alternatively spliced in males and females to yield sex-specific mRNAs that encode male-specific (BmDSXM) and female-specific (BmDSXF) polypeptides (Ohbayashi *et al*. 2001; Suzuki *et al*. 2001) (figure 3). Transgenic male silkworms carrying the female-type *Bmdsx* mRNA express vitellogenin, which is barely detectable in normal males (Suzuki *et al*. 2003). In contrast, transgenic expression of the male-type *Bmdsx* mRNA in females caused repression of the vitellogenin gene, and also resulted in activation of the pheromone-binding protein gene that is dominantly expressed in males (Suzuki *et al*. 2005). Transgenic analysis also revealed that the expression of BmDSXM in females resulted in abnormal differentiation of certain female-specific-genital organs and caused partial male differentiation in female genitalia (Suzuki *et al*. 2005). These data strongly suggest that *Bmdsx* functions as a double-switch gene at the final step in the *B. mori* sex-determination cascade.

Despite these similarities between dipteran *dsx* orthologues and *Bmdsx*, the underlying mechanism for sex-specific splicing is clearly different. We have demonstrated that female splicing of *Bmdsx* pre-mRNA represents the default mode when tested in HeLa nuclear extracts and also that the female exon is devoid of putative Tra/Tra2-binding sites (Suzuki *et al*. 2001). These findings indicate that the female exon is selectively repressed in male silk moths by a yet-unknown mechanism. We have identified a distinct sequence (CE1) in the exon 4 as a splicing silencer element responsible for exclusion of female specific exons (Suzuki *et al*. 2008). BmPSI, a *Bombyx* homolog of PSI, binds specifically to CE1 and regulates male-specific splicing of *Bmdsx* pre-mRNA. PSI was originally identified in *D. melanogaster* as a splicing inhibitor that represses splicing of the *P*-element third intron by binding to a pseudo-splice site using four KH-type RNA-binding motifs (Siebel and Rio 1990; Adams *et al*. 1997). Northern and Western blot analyses showed that no differences in expression patterns of BmPSI mRNAs were seen between male and female cells. One possible explanation for this is that the activity of BmPSI is different between males and females. For several KH-domain RNA-binding proteins, their ability to bind RNA is negatively regulated by tyrosine phosphorylation (Wang *et al*. 1995; Haegebarth *et al*. 2004; Messias *et al*. 2006). The KH-domain protein Sam68 is acetylated *in vivo*, and its acetylation correlates with enhanced RNA-binding activity (Babic *et al*. 2004). Likewise, the RNA-binding activity of BmPSI may be sex specifically regulated by some posttranslational modifications.

Since BmPSI does not exhibit any sequence relationship to known SR proteins, such as Tra and Tra2, the regulatory mechanism of sex-specific alternative splicing of *Bmdsx* pre-mRNA is distinct from that of *dsx*. Further, to the best of our knowledge there are no reports describing PSI involvement in regulating sex-specific splicing of *dsx* pre-mRNA or the sex-determination cascade. Further work is needed to elucidate how BmPSI represses inclusion of female-specific exons in a male-specific manner.

An ectopic expression of the female *Bmdsx* transcript is insufficient to induce full sex reversal in transgenic animals. Therefore, it is possible that another molecule cooperates with BmDSX to bring about the complete set of events associated with the female mode of development. In *Drosophila*, the female-type DSX protein has been reported to interact with a partner protein encoded by the *intersex* (*ix*) gene, which activates transcription of the yolk protein gene (*Yp*). Although the whole-genome sequence analysis found a homolog of *ix* (Mita *et al*. 2004), its protein did not interact with BmDSX in the yeast two hybrid system (H. Fujita, M. G. Suzuki and T. Shimada, unpublished data). Thus, it is possible that BmDSX interacts with an as yet unidentified molecule in order to bring about female-specific regulation.

Germ line sex determination

In *Drosophila*, control of germ line sex determination is distinct from that of somatic cells. Although the mechanisms of germ line sex determination in *Drosophila* are not yet fully understood, it is known that *Sxl, bam, otu, ovo*, and *snf* regulate sex determination of the germ line (Casper and Van Doren 2006). Although whole-genome sequence analysis identified orthologs of *Sxl, otu, ovo* and *snf* in *Bombyx* genome (Mita *et al*. 2004), a recognizable ortholog of *bam*, critically important for determination of female germ cells in *Drosophila*, is absent in *Bombyx* genome. Therefore, the sexual differentiation pathway in *Bombyx* germ cells may be different from that in *Drosophila*.

To elucidate the mechanism of germ cell differentiation in *Bombyx*, a serial analysis of gene expression (SAGE) analysis was performed in an attempt to trace gene expression profiles during cystocyte differentiation. As a result, a novel non-coding RNA appeared to be abundantly expressed in cystocytes and later in female germ cells (Funaguma *et al*. 2007). Knock-down analysis of this non-coding RNA by RNAi induced an interesting phenotype; i.e., oocyte-nurse cell differentiation was frequently disrupted in RNAi-treated individuals and the genitalia of females were transformed into male-like structures, similar to what is observed for transgenic moths expressing the male form of *Bmdsx* (S. Funaguma and T. Shimada, unpublished data).

Conclusions and future directions

Comparative studies on sex-determining mechanisms between *D. melanogaster* and *B. mori* provide distinct evidence that genes forming the sex-determining cascade are likely to show differences at levels upstream of *dsx* between *D. melanogaster* and *B. mori*. That is, structural features of the sex-specific splicing patterns of *Bmdsx* pre-mRNA are similar to those of *Drosophila dsx* but the regulation of sex-specific alternative splicing of *Bmdsx* pre-mRNA is differ-

ent. Which is more ancestral a *Drosophila*-type *dsx* splicing mechanism or a *Bombyx*-type mechanism? In this regard, Cho *et al*. (2007) propose an interesting hypothesis that the mechanism underlying the honeybee (*A. mellifera*) sex-specific splicing of *dsx* is more similar to that of *B. mori* due to the following three reasons. First, as is the case of *Bmdsx*, no Tra/Tra2-binding site is found in the female-specific exon. Second, the 3′ splice site preceding the female-specific exon (5′-...ctttattctctag-3′) has only a few purine nucleotides and thus does not appear to be weakened. Third, RT-PCR experiments detected a low level of female-type *Amdsx* expression in male samples but no male type in any female samples, suggesting that the female-type splicing is more likely to be the default. If their hypothesis is true, it would be most parsimonious to hypothesize that the common ancestor of all holometabolous insects used a *Bombyx*-type system and the switch to a *Drosophila*-type system occurred during dipteran evolution in the common ancestor of fruit flies and mosquitoes. Studies on *dsx* in other holometabolous insects, such as *Tribolium castaneum* (order Coleoptera), could further test this idea.

Until recently, genome research in *B. mori* has lagged behind that in *D. melanogaster*. However, this situation is being rectified, with the silkworm WGS database (http://sgp.dna.affrc.go.jp/KAIKObase/) and growing EST resources available (http://kaikocdna.dna.affrc.go.jp/). Integration of these genomic data with the wealth of germ line transgenesis and RNAi technique will strengthen the position of the silkworm as a useful experimental model in the field of sex determination.

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