Chapter 13
Sex Determination Cascade in Insects: A Great Treasure House of Alternative Splicing

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Abstract Cytological and genetic studies using insects, performed in the first decades of the twentieth century, greatly contributed to establishing the notion that genotypic factors determine sexual fate. Since then, excellent studies of *Drosophila* have provided important clues to answering the question of how sex is determined. In *Drosophila melanogaster*, somatic sexual differentiation is regulated by a well-characterized genetic hierarchy composed of a primary genetic signal (X:A ratio), master regulator (*Sex-lethal*), subordinate regulator (*transformer*/*transformer-2*), and double-switch (*dsx* and *fru*). On the basis of the knowledge obtained from studies with *Drosophila*, scientists have gained understanding of molecular mechanisms of sex determination in a variety of insect species. Recent studies have revealed that several insect species, such as the silkworm and the mosquito, have a unique sex determination cascade, which is surprisingly different from that in *Drosophila*. The most characteristic feature of the sex-determining genes in insects so far identified is that their sex-specific expressions are controlled by alternative splicing. In this chapter, we give an overview of the sex-determining genes revealed thorough the studies of *D. melanogaster* and those homologues identified in either nondrosophilid insects or animal species other than insects. In particular, we provide a detailed description of the novel sex-determining genes identified in the silkworm on the basis of our recent studies.

Keywords Sex determination · Sexual differentiation · Sex-determining gene · Alternative splicing · Insects · Sex determination cascade · Silkworm

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13.1 Introduction

How is sex determined? Aristotle once answered this question as follows: "The heat of semen at the time of copulation determines the sexual fate." According to his hypothesis, hot semen produces males, whereas cold semen yields females (Jacqart and Thomasset 1988). Similar hypotheses, where environmental conditions such as temperature and nutritional status are major factors in determining sexual fate, were believed until the end of nineteenth century. The first indication relevant to a sex chromosome came from cytological studies conducted by Hermann Henking in 1891. In the course of studying sperm formation in *Pyrrhocoris* fire wasps, Henking noticed that one chromosome did not have a counterpart, unlike all other chromosomes, during the stages of meiosis in sperm cells. He designated this curious chromosome as the "X element" because of its strange nature (Henking 1891). The X element could be found in males of a number of different species. On the basis of his findings, Henking speculated that the X element played a crucial role in sex determination, but he could not provide direct evidence to support his idea. Later the X element became known as the X chromosome after it was established to indeed be a chromosome. In 1902, as a consequence of his cytological studies using the long-horned grasshopper, *Xiphidium fasciatum*, Walter Sutton proposed the hypothesis that the Mendelian laws of inheritance could be applied to chromosomes at the cellular level of living organisms (Sutton 1903). More than a decade after Henking's work, Nettie Stevens set out to investigate gamete formation in many insects—from termites to sand crickets to aphids and mealworms. In this last species, *Tenebrio molitor*, she noticed that fully formed spermatids showed something odd in the chromosome number and its size: half of them had ten large chromosomes, all of a similar size, while the other half had nine large chromosomes and one small one. On the other hand, the eggs had ten large chromosomes, without exception. She also found that somatic cells from the male always had a total of 19 large chromosomes and always one small "accessory" chromosome, while somatic cells from female mealworms always contained 20 large chromosomes. In 1905, she correctly concluded that the accessory chromosome determines male sex (Stevens 1905). The accessory chromosome is now known as the Y chromosome. In contrast to the male-specific Y chromosome, which determines male sex, Yoshimaro Tanaka identified a female-specific chromosome in the silkworm, *Bombyx mori*, and demonstrated by genetic analysis that the presence of the female-specific chromosome determines female sex (Tanaka 1916). This female-specific chromosome was designated as the W chromosome, which has also been found in frogs, snakes, and birds. In 1921, Calvin Bridges found that the primary genetic signal for sex determination in *Drosophila melanogaster* was the ratio of X chromosomes to sets of autosomes (Bridges 1921). A ratio of 1.0 leads to female development, while a ratio of 0.5 leads to male development. Thus, cytological and genetic studies using insects greatly contributed to establishing the notion that genotypic factors determine the sexual fate.

The primary genetic signal for sex determination observed in insects is manifested in a wide variety of ways. In *Megaselia scalaris* (Traut 1994; Sievert et al. 2000), *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 Y chromosomes. The mosquito *Culex tritaeniorhynchus* has no sex chromosome, and its 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, *Lymantria disper* has a Z-linked male determinant (M) and a maternally inherited female determinant factor (F) (Goldschmidt 1955). Thus, insects have continued to provide a deep understanding of the genotypic sex determination system. In particular, much has been learned about the sex determination cascade from molecular and genetic analyses of *D. melanogaster*. On the basis of the knowledge obtained from studies of *Drosophila*, scientists have gained understanding of molecular mechanisms of sex determination in a variety of insect species such as the Mediterranean fruit fly (medfly), *C. capitata*; the yellow fever mosquito, *Aedes aegypti*; the silkmoth, *B. mori*; the honeybee, *Apis mellifera*; and the jewel wasp *Nasonia vitripennis*. These insects are highly diverged from *Drosophila*; for example, *A. aegypti* and *N. vitripennis* are separated from *D. melanogaster* by about 250 and 300 million years, respectively (Gailey et al. 2006; Hasselmann et al. 2008). Therefore, comparisons of genes constituting the sex determination cascade in insects will be very helpful to gain insight into the evolutionary dynamics of the regulatory mechanisms that give rise to sexual dimorphisms. In this chapter, we give an overview of sex-determining genes revealed through studies of *D. melanogaster* and those homologues identified in either non-drosophilid insects or animal species other than insects. In particular, we provide a detailed description of the novel sex-determining genes identified in the silkworm on the basis of our recent studies.

13.2 Sex Determination Cascade in *Drosophila melanogaster*

Sex determination in *D. melanogaster* is organized as a hierarchical order of genes designated as a sex determination cascade (Fig. 13.1). As described above, the first signal of sex determination is the ratio of X chromosomes to sets of autosomes (A) (Bridges 1921). The X:A ratio, a balance mechanism in which X chromosomal gene products are titrated against autosomal gene products, governs sex determination. Recent findings have indicated that the X:A ratio predicts the 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). The concentration of XES proteins determines the activity state of the *Sex-lethal* (*Sxl*) gene, which sits at the top of the sex determination cascade. A sufficient amount of XSE is supplied only when the animal has two X chromosomes (namely, XX female), leading

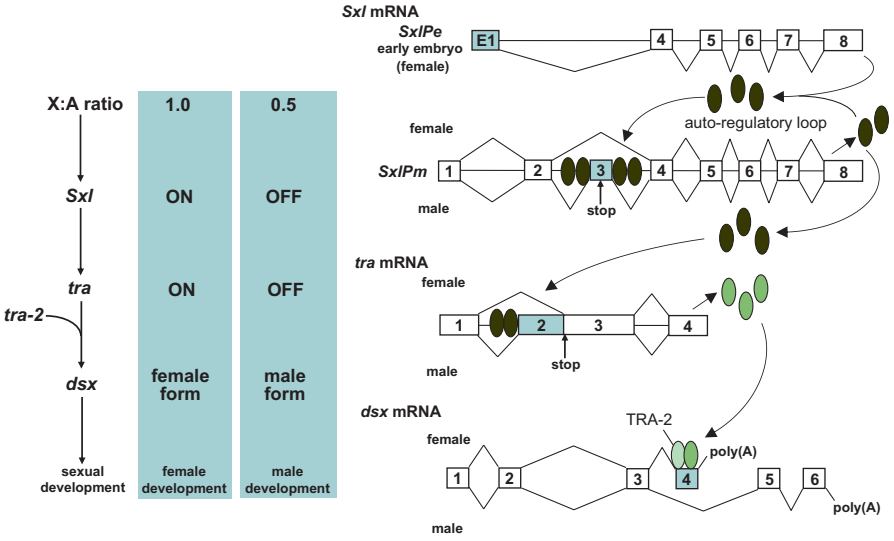

Fig. 13.1 Sex determination cascade and sex-determining genes in *Drosophila melanogaster*. The left panel shows the sex determination cascade, composed of *Sxl*, *tra*/*tra-2*, and *dsx* genes. The right panel shows sex-specific alternative splicing patterns in each gene. Transcripts from *tra-2* do not undergo sex-specific splicing. The primary genetic signal for sex determination is the ratio of X chromosomes to sets of autosomes (X:A ratio). Transcription from the *Sxl* establishment promoter (SxlPe) in the early embryonic stage is activated when the animal has two X chromosomes (namely, XX female). The protein encoded by transcripts from SxlPe induces female-specific splicing of *SxlPm* precursor messenger RNA (pre-mRNA), yielding the functional SXL protein. SXL protein binds to its own pre-mRNA to maintain the female-specific mode of splicing (an autoregulatory loop). SXL directs the female-specific splicing of *tra*, giving rise to functional TRA protein. TRA, together with TRA-2, binds to an exonic splicing enhancer (ESE) element located within the female-specific exon of *dsx*. The TRA and TRA-2 complex activates the weak 3′ splicing site preceding the female-specific exon to generate the female-type DSX protein (DSX^F). SxlPe is inactive when the animal has only one X chromosome (namely, XY male). In the absence of the protein products from SxlPe, *SxlPm* pre-mRNA is spliced by default, yielding transcripts including an intervening exon that contains a stop codon. A lack of functional SXL protein causes male-specific splicing of *tra*, making its encoded product nonfunctional because of a premature stop codon. In the absence of functional TRA protein, *dsx* pre-mRNA is spliced by default to generate the male-type DSX protein (DSX^M). The DSX^F and DSX^M proteins regulate the sex-specific transcription of target genes that encode the sexual phenotype of the body

to activation of transcription from the *Sxl* establishment promoter (SxlPe) at the early embryonic stage (Cline 1988; Keys et al. 1992; Estes et al. 1995; Erickson and Quintero 2007). The protein encoded by transcripts from SxlPe induces female-specific splicing of precursor messenger RNA (pre-mRNA) transcribed from the maintenance promoter, SxlPm, which is active in both sexes (Fig. 13.1). The female-specific splicing product from SxlPm encodes a functional SXL protein. The resulting SXL protein binds to its own pre-mRNA to maintain the female-specific mode of splicing (Fig. 13.1). The concentration of XSE is insufficient to activate SxlPe when the animal has only one X chromosome (namely, XY male).

In the absence of protein products from SxlPe, pre-mRNA from SxlPm is spliced by default, yielding transcripts including an intervening exon that contains a stop codon (Fig. 13.1). As a result, SxlPm transcripts in males do not encode a full-length protein. Thus, *Sxl* acts as the memory device for female sexual development via its autoregulatory function (Cline 1984; Bell et al. 1991). SXL directs female-specific splicing of its downstream gene *transformer* (*tra*), giving rise to functional TRA protein (Boggs et al. 1987) (Fig. 13.1). TRA, together with the protein product of *transformer-2* (*tra-2*), binds to an exonic splicing enhancer (ESE) element located within the female-specific exon of its downstream gene *doublesex* (*dsx*). The *dsx* ESE contains six copies of a 13-nucleotide (R1–R6) repeat sequence and is designated as dsxRE (Lynch and Maniatis 1995, 1996). TRA and TRA-2 are splicing factors belonging to the family of SR-type proteins whose amino acid sequences contain high frequencies of arginine (R) and serine (S) residues. The arginine- and serine-rich domain, which is known as the RS domain, mediates protein–protein interactions and regulates the recognition of specific splice sites (Manley and Tacke 1996). Proteins harboring the RS domain constitute a well-studied family of splicing regulators. An RS tetrapeptide (RSRS or SRSR) represents a functional unit in the RS domain of splicing activators (Graveley et al. 1998). Unlike other SR-type splicing factors, TRA lacks an RNA recognition motif (RRM) required for RNA binding. Therefore, TRA functions cooperatively with a factor such as TRA-2, which can bind to the cis-element in target pre-mRNAs. The TRA and TRA-2 (and one or more SR proteins) (Lynch and Maniatis 1995, 1996) complex binds to dsxRE functions to activate a weak 3′ splicing site preceding the female-specific exon, most likely by facilitating interactions of other general splicing factors with the RNA, to generate the female-type DSX protein (DSX^F) (Kan and Green 1999; Li and Blencowe 1999). A lack of functional SXL protein causes male-specific splicing of *tra*, making its encoded product nonfunctional because of a premature stop codon (Fig. 13.1). In the absence of functional TRA protein, *dsx* pre-mRNA is spliced by default to generate the male-type DSX protein (DSX^M). DSX^F and DSX^M regulate the sex-specific transcription of target genes that encode the sexual phenotype of the body (Baker and Wolfner 1988; Burtis and Baker 1989; Cline and Meyer 1996). To date, there are only a few validated direct gene targets for *dsx*: *Yolk Proteins* (*yp-1* and *yp-2*), *bric-à-brac 1*, *desatF* (also known as *Fad2*), and *Flavin-containing monooxygenase-2* (*Fmos-2*) (Burtis et al. 1991; Coschigano and Wensink 1993; Williams et al. 2008; Shirangi et al. 2009; Luo and Baker 2015). The regulation of yolk protein gene expression is by far the best characterized DSX function at the molecular level. DSX^F and DSX^M bind directly to several specific sites in an enhancer sequence designated as fat body enhancer (Burtis et al. 1991; Coschigano and Wensink 1993), which plays a role in directing the adult female fat body–specific transcription characteristic of the adjacent yolk protein genes, *yp-1* and *yp-2* (Garabedian et al. 1986). Recent genome-wide analyses by chromatin immunoprecipitation followed by sequencing (ChIP-seq) have revealed that DSX^F and DSX^M bind thousands of the same targets in multiple tissues in males and females, indicating that DSX action is regulated downstream from DSX binding (Clough et al. 2014).

Another known downstream target of *tra* is *fruitless* (*fru*). In females, transcripts from the *fru* gene undergo female-specific splicing by TRA and TRA-2; in addition, these two factors prevent translation of the female-specific *fru* transcripts (Ryner et al. 1996; Heinrichs et al. 1998; Usui-Aoki et al. 2000). In males, because of the absence of TRA, default splicing produces male-specific *fru* transcripts, yielding male-specific protein isoforms (FRU^M). Mutations in sex-specific *fru* transcripts disrupt both male courtship behavior and sexual orientation but have no effect on female behavior (Ito et al. 1996; Ryner et al. 1996; Anand et al. 2001; Lee and Hall 2001; Lee et al. 2001; Demir and Dickson 2005). The FRU^M proteins play central roles in producing sexual differences in the mAL interneuron cluster containing a male-specific ipsilateral neurite in the central nervous system (CNS) (Kimura et al. 2005). In females, a subset of mAL neurons dies by programmed cell death. In males, FRU^M proteins prevent the male counterparts from dying, allowing the development of mAL neurons. FRU^M proteins are also necessary for the development of the muscle of Lawrence (MOL), an abdominal muscle present only in males (Usui-Aoki et al. 2000; Anand et al. 2001). All FRU isoforms contain a BTB (Broad-complex, Tramtrack, and Bric-a-brac) domain and a C-terminal C2H2 zinc finger domain for the DNA-binding function (Zollman et al. 1994). Recently, the binding sequences for different FRU isoforms have been identified (Dalton et al. 2013; Neville et al. 2014). However, to date, no direct target gene has been validated for *fru*.

13.3 *Sxl* Orthologues and Their Sex Determination Role in Nondrosophilid Species

Is *Sxl*, the master regulatory switch for sex determination in *D. melanogaster*, conserved among insects? Several *Sxl* orthologues thus far characterized in dipteran species other than *D. melanogaster* have no sex-determining role. For example, the *Sxl* orthologues in the medfly (*C. capitata*), housefly (*Musca domestica*), and scuttlefly *M. scalaris* encode proteins similar to the SXL of *D. melanogaster* but do not show sexual differences in those expression patterns (Saccone et al. 1998; Meise et al. 1998; Sievert et al. 2000). Furthermore, transgenic expressions of these orthologues in *D. melanogaster* failed to induce feminization in males (Saccone et al. 1998; Meise et al. 1998), suggesting an alteration of protein functions. In nondipteran insects, *Sxl* orthologues identified from the silkworm, *B. mori*, were expressed in both sexes equally (Niimi et al. 2006). Taken together with these findings, sex-specific expression and the protein role of *Sxl* in *Drosophila* are not conserved among insects.

Hu antigen B (HuB), HuC, HuD, and HuR proteins belong to the ELAV/Hu protein family (Antic and Keene 1997; Wakamatsu and Weston 1997). HuR is expressed ubiquitously in various tissues, while the other ELAV/Hu proteins are expressed predominantly in neuronal cells and are involved in neuronal differentiation (Good

1995; Ma et al. 1996; Antic et al. 1999). The ELAV/Hu proteins have three copies of RNA-binding domains (RBDs). Interestingly, the amino acid sequences of the N-terminal two RBDs (RBD1 and RBD2) of ELAV/Hu proteins are significantly homologous (53% identity) to those of SXL (Szabo et al. 1991). NMR studies on RBDs of mouse HuC demonstrated that the two N-terminal RBD (RBD1 and RBD2) structures are quite similar to those of SXL (Inoue et al. 2000). In addition, RBD1-RBD2 binds specifically to a longer ARE RNA, UAUUUAUUUU, which is highly similar to the RNA sequence UGUUUUUUUU, which is preferentially bound by SXL RBD1-RBD2 (Handa et al. 1999). These findings indicate that the RNA-binding properties of the vertebrate HuC and SXL are similar, even though the target genes and the biological functions of the proteins are different. With regard to *Sxl* in *Drosophila*, it is postulated that not only the gene expression but also an altered protein function may have contributed to the gain of the sex determination function in *Drosophila*.

13.4 *tra* Orthologues and Their Sex Determination Role in Nondrosophilid Species

tra was considered to be a *Drosophila* genome–specific gene because *tra* orthologues showed an unusually high degree of evolutionary divergence when compared within *Drosophila* subgenus species (O'Neil and Belote 1992). The identification of *tra* orthologues from the medfly (*C. capitata*) and the olive fruit fly (*Bactrocera oleae*) changed this notion (Pane et al. 2002; Lagos et al. 2007). These *tra* homologues (*Botra* and *Cctra*) have a female-determining master function (Pane et al. 2002; Lagos et al. 2007). To date, the *tra* gene has been identified not only in dipteran and coleopteran species but also in more than a dozen hymenopteran species (ants and bees), which are the most basal order of the holometabolous insects (Schmieder et al. 2012; Geuverink and Beukeboom 2014). Six of seven sequenced ants have two copies of *tra*, resulting from an ancestral duplication rather than independent duplications in each of the six species (Privman et al. 2013). In three bee species where whole-genome information is available, the *tra* gene is also duplicated (Privman et al. 2013). One copy is named *complementary sex-determiner* (*csd*), which is the primary signal for sex determination in the honeybee, *A. mellifera* (Beye et al. 2003). It activates the second copy, designated as *feminizer* (*fem*), which is more conserved and retains the ancestral function to regulate its downstream target, *dsx* (Hasselmann et al. 2008). *csd* is considered to have arisen from duplication of the *fem* gene (Schmieder et al. 2012).

RNA interference (RNAi)–mediated knockdown of *tra* in *Musca* (Hediger et al. 2010), *Ceratitis* (Pane et al. 2002), *Anastrepha* (Ruiz et al. 2007), *Lucilia* (Concha and Scott 2009), *Apis csd* (Beye et al. 2003), *Apis fem* (Hasselmann et al. 2008), *Nasonia* (Verhulst et al. 2010), and *Tribolium castaneum* (Shukla and Palli 2012) caused disruption of endogenous *tra* function in these species and subsequent

male-specific splicing of the endogenous *dsx* pre-mRNAs, leading to the transformation of chromosomally female embryos into adult pseudomales. On the other hand, several previous studies and recent genome-wide analysis in a wide range of insect species revealed that *tra* shows a distinctly patchy distribution among insects (Geuverink and Beukeboom 2014). For example, no *tra* homologues have been found in lepidopteran insects, including *B. mori*, *Danaus plexippus*, and *Heliconius melpomene* (Mita et al. 2004; Geuverink and Beukeboom 2014) (Fig. 13.2). The most interesting pattern of *tra* distribution is seen in the Diptera genus. The *tra* gene is found in brachycera species, including *D. melanogaster*, while several mosquito species, which belong to a basal dipteran lineage, do not possess *tra* (Geuverink and Beukeboom 2014) (Fig. 13.2). The situation is the same in Coleoptera. One of the coleopteran species, *T. castaneum*, carries a *tra* orthologue (Shukla and Palli 2012), while the *tra* gene has not to date been found in the genome of the coleopteran *Dendroctonus ponderosae* (Geuverink and Beukeboom 2014). *Mengenilla moldrzyki*, which belongs to Strepsiptera—a sister order to Coleoptera—appears to lack a *tra* homologue (Geuverink and Beukeboom 2014) (Fig. 13.2).

TRA protein contains several conserved domains; one is shared only among Diptera (the "dipteran domain"), and another domain is present only in Hymenoptera (the "hymenopteran domain"). The most conserved part of the TRA protein is the TRA-CAM (C, *Ceratitis*; A, *Apis*; and M, *Musca*) domain (Hediger et al. 2010). This domain is found in all TRA proteins, except for those in *D. melanogaster*. Another conserved feature of TRA is a proline-rich region located near the C-terminus. This region is also present in all *tra* genes so far characterized in insects (Geuverink and Beukeboom 2014). However, the functional importance of the TRA-CAM domain and the proline-rich region remains unknown. The splicing pattern of all insect *tra* transcripts shows sexual dimorphism, and the female-specific splice variant only encodes a functional protein (Bopp et al. 2014).

The *tra* orthologue has also been isolated from two noninsect species, *Daphnia magna* and *Daphnia pulex*. They belong to the subphylum Crustacea, which is regarded as a sister group to insects (Kato et al. 2010; Chen et al. 2014) (Fig. 13.2). Recently, we have isolated a *tra* homologue in the acorn worm *Saccoglossus kowalevskii*, which is a hemichordate belonging to the superphylum Deuterostomia (Suzuki et al. 2015). *D. magna transformer* (*DmagTra*), *D. pulex transformer* (*Dptra*), and *S. kowalevskii tra* (*Sktra*) also contain the highly conserved TRA-CAM domain and the arginine/serine-rich region near the N-terminus. Unlike insect *tra* genes, all of these *tra* orthologues show no sexual dimorphism in their splicing patterns (Kato et al. 2010; Chen et al. 2014; Suzuki et al. 2015). However, the expression level of *Dptra* has been shown to be significantly higher in sexually mature males than in ephippial females, implying that *Dptra* might play an important role in switching between reproduction modes and sexual differentiation in *D. pulex*, but this requires further confirmation (Chen et al. 2014). Similarly, the mRNA level of *Sktra* was approximately 7.5-fold greater in the testes than in the ovaries, suggesting that *Sktra* might be involved in sexual differentiation of *S. kowalevskii* (Suzuki et al. 2015).

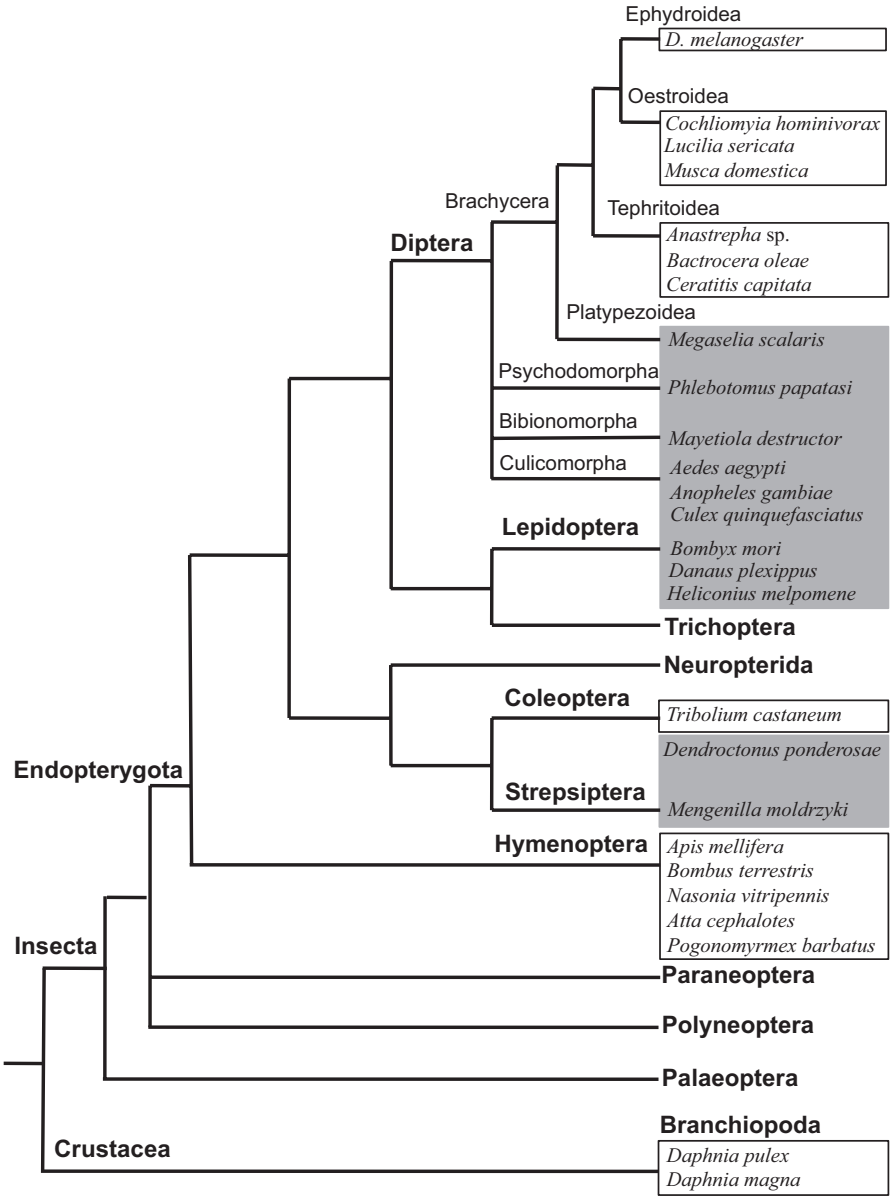

Fig. 13.2 Schematic diagram of the phylogenetic distribution of *tra*. The name of each species is noted on the *right side*. Species with *tra* are *squared*, while species lacking *tra* are in the *gray box*. The phylogenetic tree simply indicates the comparative relationship between each species, thus the branch length in the tree is not precisely indicated

These findings support the idea that the *tra* gene evolved independently in insect species and acquired a sex-determining gene function, especially as a *dsx*-splicing regulator. However, as described above, *tra* is present in the orders Hymenoptera, Coleotpera, and Diptera but, thus far, has not been found in Lepidoptera or in the basal lineages of Diptera (Geuverink and Beukeboom 2014), implying multiple independent losses or recruitment of *tra* into the sex determination cascade. Vertebrates and multiple metazoan phyla, including arthropods and nematodes, lack *tra*, which may be dispensable for embryogenesis, developmental processes, and some other biological processes. Interestingly, *dsx* genes in five Cladocera species (including *D. magna* and *D. pulex*), which are the closest relatives to the insects, are also expressed dominantly in males and do not exhibit the sex-specific splicing typical of insect *dsx* (Toyota et al. 2013). Unlike insect *tra* genes, both *D. magna tra* and *D. pulex tra* show no sexual dimorphism in their splicing patterns (Kato et al. 2010; Chen et al. 2014). These findings have led to the inference that the *tra–dsx* regulatory axis evolved independently in insect species.

13.5 *tra* as a Memory Device for Female Sexual Development

In tephritid fruit flies—such as *C. capitata*, *B. oleae*, and 12 *Anastrepha* species—the *Sxl* gene is not regulated in a sex-specific manner and does not appear to play the key discriminating role (memory device) in sex determination that it plays in *Drosophila* (Saccone et al. 1998; Lagos et al. 2007). As in the drosophilids, the tephritid *tra* gene is constitutively expressed in both sexes and its pre-mRNA undergoes sex-specific alternative splicing. In contrast to the *Drosophila* situation in which *Sxl* regulates *tra*, in the tephritids the gene *tra* acts as the memory device for sex determination via its autoregulatory function, i.e., through the contribution of the TRA protein to the female-specific splicing of its own pre-mRNA. The *tra* gene in the tephritids has male-specific exons that contain translation stop codons. The inclusion of these exons in mature *tra* mRNA in males results in the production of a truncated, nonfunctional TRA protein. In females, the male-specific exons are spliced out because of the presence of TRA protein (Pane et al. 2002; Lagos et al. 2007; Ruiz et al. 2007). The presence of putative TRA-TRA2 binding sites in the male-specific exons and in the surrounding introns may suggest that the TRA interacts with its own pre-mRNA through TRA-2, resulting in skipping of the male-specific exons. It has been found that TRA-2 proteins in *M. domestica* and *C. capitata* are required for female-specific splicing of *tra* pre-mRNAs (Burghardt et al. 2005; Salvemini et al. 2009; Hediger et al. 2010). In *Ceratitis* and *Musca*, it is postulated that maternal *tra* mRNAs trigger the autoregulatory loop in XX (female) embryos. The zygotically produced TRA protein controls the maintenance of *tra* autoregulation and female-specific splicing of *dsx* pre-mRNA. The resulting DSXF protein induces female development. In XY (male) embryos, the male-determining *M* factor located on the Y chromosome prevents *tra* autoregulation through unknown mechanisms. Therefore, TRA protein is not produced in XY embryos, and thus the

autoregulatory loop cannot initiate. Because of the absence of TRA protein, *dsx* pre-mRNA is spliced by default to yield DSXM protein, which induces male development (Pane et al. 2002; Hediger et al. 2010). A similar autoregulatory function is also thought to occur in the beetle *T. castaneum* (Shukla and Palli 2012).

The *fem* gene—a *tra* homologue in the honeybee, *A. mellifera*—needs its protein product to splice its own pre-mRNA into the productive female form, which establishes an autoregulatory feedback loop to maintain the female state throughout the bee's life (Gempe et al. 2009). The *csd* gene, which is a paralogue of *fem*, initiates the autoregulatory loop (Hasselmann et al. 2008). The female mode of *fem* splicing occurs only when the *csd* presents in the heteroallelic condition. The female form of Fem protein is active and induces female splicing of *A. mellifera dsx* (*Am-dsx*) pre-mRNA, resulting in the production of Am-DSXF. In the absence of Csd protein activity in males, where the *csd* gene is homo- or hemiallelic, *fem* pre-mRNA is spliced into the male form, which includes the male-specific exons containing an intervening stop codon. The male form of Fem protein is inactive; therefore, *Am-dsx* pre-mRNA is spliced by default to produce Am-DSXM protein. As in the dipteran insects *M. domestica* and *C. capitata*, Am-TRA2 protein acts together with heteroallelic Csd proteins and/or Fem proteins to mediate female *fem* splicing by binding to *fem* pre-mRNAs (Nissen et al. 2012).

The jewel wasp *N. vitripennis* employs a similar but slightly different autoregulatory function in the regulation of *tra* gene (*Nvtra*) expression. In this species, sex is determined on the basis of the ploidy of embryos (Heimpel and de Boer 2008); males are haploid, developing from unfertilized eggs, whereas diploid females develop from fertilized eggs. In diploid eggs, maternally provided *Nvtra* mRNA initiates the female-specific autoregulatory loop required to maintain the female state throughout the wasp's life (Verhulst et al. 2010). In haploid (unfertilized) eggs, it is postulated that zygotic transcription from the maternal *Nvtra* allele is prevented as a result of maternal imprinting, leading to male development.

13.6 *dsx*, the Most Conserved Regulatory Switch in Sex Determination

Orthologues of *dsx* have been found in each insect species examined, including Diptera, Lepidoptera, Coleoptera, and Hymenoptera species (Bopp et al. 2014). Primary transcripts from *dsx* genes thus far identified undergo sex-specific alternative splicing, producing either a male-specific (DSXM) or female-specific (DSXF) isoform (Schütt and Nöthiger 2000; Geuverink and Beukeboom 2014). The female-specific splicing of *dsx* transcripts in Diptera and Coleoptera is induced by TRA together with TRA-2 through canonical TRA-TRA2 binding sites, which are consistently present in the female-specific exon (Burghardt et al. 2005; Salvemini et al. 2009; Sarno et al. 2010; Shukla and Palli 2012). Interestingly, *dsx* pre-mRNAs in the honeybee, *A. mellifera*, lack the canonical binding sites of TRA-TRA2 proteins, even though its female-specific splicing needs Am-TRA2 proteins (Nissen et al.

2012). This finding suggests that the TRA-TRA2 protein-binding sites have evolved independently in the hymenopteran species. Consistent with this hypothesis, Am-TRA2 appears to have several amino acid replacements in the RNA-binding domain (RBD), one of which is a critical amino acid residue for female processing of *dsx* pre-mRNAs in *D. melanogaster* (Amrein et al. 1994). The *dsx* gene in the silkworm, *B. mori*—designated as *Bmdsx*—also lacks the canonical TRA-TRA2 binding sites (Suzuki et al. 2001). In contrast to *dsx* in other insect species, female-specific *Bmdsx* transcripts are produced by default splicing (Suzuki et al. 2001). TRA-2 proteins in this species are not relevant to sex-specific splicing of *Bmdsx* (Suzuki et al. 2012).

Geuverink and Beukeboom (2014) recently identified putative *dsx* orthologues in a number of primitive insect groups. For example, the human body louse, *Pediculus humanus corporis*, has a hypothetical protein composed of both a DM (Dsx/Mab-3 DNA-binding motif) domain and a dimerization domain (OD2). The DM domain is found in multiple genes of the DM superfamily group consisting of *dsx*, *mab3*, and related transcription factors (*DMRT*), while the dimerization domain is exclusively found in *dsx*. Five Cladocera species (*Daphnia magna*, *Daphnia pulex*, *Daphnia galeata*, *Ceriodaphnia dubia*, and *Moina macrocopa*), which are the closest relatives to the insects examined, also possess *dsx* genes (Kato et al. 2011; Toyota et al. 2013).

The *DMRT* genes appear to play a role in sex determination or sexual differentiation in all Metazoa; therefore, this gene family may represent the first example of sexual regulatory genes conserved across phyla (Hodgkin 2002; Kopp 2012). In vertebrates, orthologues of *DMRT1* are expressed dominantly in males and are required only to guide male sex determination (Matson and Zarkower 2012). Therefore, it is conceivable that animal species in which *DMRT1* is responsible for maleness do not necessarily need a *dsx*-splicing regulator such as an insect TRA. Interestingly, *dsx* genes in five Cladocera species (including *D. magna* and *D. pulex*), which are the closest relatives to the insects, are also expressed dominantly in males and do not exhibit the sex-specific splicing typical of insect *dsx* (Toyota et al. 2013). Unlike insect *tra* genes, both *D. magna* and *D. pulex tra* show no sexual dimorphism in their splicing patterns (Kato et al. 2010; Chen et al. 2014). These findings lead to the inference that the sex-specific splicing feature in *dsx* was acquired early in the evolution of insects and that the *tra–dsx* regulatory axis evolved independently in insect species.

13.7 *fru*, Another Downstream Regulator in the Sex Determination Cascade

In *D. melanogaster*, the male-specific splice isoform of the *fru* gene (FruM) encodes a set of transcription factors involved in the regulation of male courtship and copulation, as described above. Recent insights from nondrosophilid insects suggest a conserved evolutionary role for the transcription factor Fruitless. In the housefly, *M. domestica*, male-specific transcripts from the *fru* orthologue *Md-tra* are generated

by a conserved mechanism of sex-specific splicing (Meier et al. 2013). As in *Drosophila*, *Md-fru* is similarly involved in controlling male courtship behavior. A male courtship behavioral function for *Md-fru* was revealed by behavioral and neuroanatomic analyses with a hypomorphic allele of *Md-tra*, which specifically disrupts the expression of *Md-fru* in males, leading to severely impaired male courtship behavior. In addition, expression of *Md-fru* is confined to neural tissues in the brain, most prominently in the optic neuropil and in peripheral sensory organs (Meier et al. 2013). In the mosquitoes *Anopheles gambieae* and *A. aegypti*, *fru* orthologues show conservation of sex-specific alternative splicing and male-specific protein expression in neural tissues (Gailey et al. 2006; Salvemini et al. 2013). The female-specific exons of both genes each contain short sequences resembling the TRA-TRA2 binding sites but showing degeneration and lack of a consensus. Therefore, it is postulated that sex-specific splicing of *fru* transcripts in the mosquitoes is most likely under the control of splicing regulatory factors, which are different from TRA and TRA-2 found in other dipteran insects. This is consistent with the fact that sex determination in *A. aegypti* is different from that in *Drosophila*, where the key male determiner *M*, located on one of a pair of homomorphic sex chromosomes, controls sex-specific splicing of the *dsx* and *fru* orthologue (Hall et al. 2015). Moreover, a *tra* orthologue seems to be absent from both *Anopheles* and *Aedes* genomes (Gailey et al. 2006; Nene et al. 2007). In the hymenopteran *N. vitripennis*, the *fru* architecture is essentially identical to that in *Drosophila*, and the P1 transcripts, derived from the most distal *fru* promoter, undergo a conserved sex-specific splicing regulation (Bertossa et al. 2009). These findings suggest that conserved *fru* sex-specific splicing evolved prior to the divergence between Hymenoptera and Diptera (250–300 million years ago) rather than being acquired independently in both lineages.

In Orthoptera, which belongs to a phylogenetically more basal insect lineage, *fru* orthologues have also been isolated, but the situation is different from that of the *fru* genes in Diptera (Salvemini et al. 2010). For example, *fru* orthologues thus far identified in several *Chorthippus* grasshopper species, the desert locust *Schistocerca gregaria*, and the cockroach *Blatella germanica* show no sexual dimorphism in those splicing patterns (Ustinova and Mayer 2006; Boerjan et al. 2011; Clynen et al. 2011). In spite of this, RNAi knockdown experiments have demonstrated that *fru* orthologues in *S. gregaria* and *B. germanica* play important roles in regulation of successful copulation in the adult male and in male sexual behavior, respectively (Boerjan et al. 2011; Clynen et al. 2011). In addition, the spermatheca of female copulation partners of *fru* knockdown males in both species contained fewer or no spermatozoids, resulting in a reduction in progeny numbers in their naïve female copulation partners. This seemed likely due to smaller numbers of spermatozoa in the male seminal vesicles, caused by *fru* knockdown (Boerjan et al. 2012). These findings suggest that the role of fruitless as a master regulator of male sexual behavior has been conserved in insect evolution, at least from cockroaches to flies. This raises the question of whether the *fru* gene exists not only in insect genomes but also in vertebrate genomes, like its counterpart *dsx*, which is at the bottom of the sex determination cascade. Homology searches of reported sequences using *fru* BTB and ZnF domains as virtual probes resulted in no hits outside insects (Gailey et al. 2006).

13.8 Sex Determination Mechanisms in Insects Lacking the *tra* Gene

As described above, the *tra* gene acts as a memory device for female sexual development and thus plays a central role in the sex determination cascade. Conservation of the *tra* orthologue in more than a dozen hymenopteran species, which are the most basal order of the holometabolous insects, strengthens the importance of this gene in insect sex determination. On the other hand, considerable numbers of insect species appear to lack a *tra* orthologue, even though these species have sex-specifically spliced *dsx* and *fru* orthologues, both of which are well known as *tra*-downstream targets (Suzuki et al. 2001; Gailey et al. 2006; Nene et al. 2007; Salvemini et al. 2013). How do they control sex-specific splicing of *dsx* and *fru*, and determine their sexual fate, without a *tra* gene?

Maleness in the mosquito *A. aegypti* is determined by an *M* factor located on the homomorphic sex-determining chromosome within a Y chromosome–like region called the M locus (Newton et al. 1974). To date, no *tra* orthologues have been found in the *A. aegypti* genome. Consistent with this, there are no well-conserved TRA-TRA2 binding sites in both *fru* and *dsx* pre-mRNAs (Salvemini et al. 2011, 2013). *A. aegypti* has four *tra-2* paralogues, which phylogenetically form a single clade, apart from the other known dipteran and even nondipteran orthologues (Salvemini et al. 2013). It is therefore postulated that these *tra-2* paralogues evolved to have different sequence binding specificity and novel functions under selective pressure relaxation after gene duplication. Recently, Hall et al. (2015) reported that an M locus gene, *Nix*, which is a distant homologue of *tra-2*, functions as an *M* factor in *A. aegypti*. *Nix* knockout resulted in largely feminized genetic males and the production of female forms of *dsx* and *fru*. These findings suggest the possibility that *tra-2* paralogues may have acquired the function of a primary sex-determining gene during evolution in insects that lack a *tra* orthologue.

The silkworm, *B. mori*, also lacks a *tra* gene (Mita et al. 2004; Geuverink and Beukeboom 2014). Moreover, TRA-2 proteins in this species are not relevant to the sex-specific splicing of *Bmdsx* (Suzuki et al. 2012). In contrast to *dsx* in other insect species, female-specific *Bmdsx* transcripts are produced by default splicing (Suzuki et al. 2001). In accordance with these findings, *Bmdsx* lacks the canonical TRA-TRA2 binding sites (Suzuki et al. 2001). In this species, male splicing of *Bmdsx* transcripts requires the splicing inhibitor (BmPSI) and the male-specific isoform of IMP (IMP^M) proteins. These proteins form a complex that binds to a cis-regulatory element called CE1, located in the female-specific exon, and inhibits the female mode of splicing in males (Suzuki et al. 2008, 2010). Our previous data suggested that Imp^M maintains its male-specific mode of expression by an autoregulatory function and thus may function as a memory device for male development (Suzuki et al. 2014). Alternative splicing regulation by an autoregulatory function is not restricted to the female-specific splicing of *tra* and is also seen in common splicing regulators. For example, SRp20 and ASF/SF2, both of which belong to a highly conserved SR family of splicing factors, autoregulate the alternative splicing of

their own pre-mRNAs (Ge et al. 1991; Jumaa and Nielsen 1997). It could therefore be expected that the autoregulatory function originally present in the *Imp* gene may have been recruited to the male-determining pathway and utilized as a memory device for male development during evolution. *Imp* is a Z chromosome–linked gene whose dosage is twice as high in males as in females (Suzuki et al. 2010). The *Imp* orthologue is located on the Z chromosome in another lepidopteran species, *Samia cynthia ricini*, which lacks a W chromosome (Yoshido et al. 2011). If the *Imp* orthologue in this species also produces a male-specific splice isoform, then it is highly possible that such a male isoform of *Imp* may function as a memory device for male sexual development.

It has been shown genetically that female sex in *B. mori* is determined by the presence of a dominant feminizing factor (*Fem*) on the W chromosome (Hashimoto 1933). The W chromosome in *B. mori* lacks protein-coding genes and is almost completely occupied by selfish repetitive elements such as transposons (Abe et al. 2005). The only transcripts produced from the W chromosome are PIWI-interacting RNAs (piRNAs) (Kawaoka et al. 2011). piRNAs are 23–30 nucleotides of small RNAs acting as sequence-specific guides for PIWI proteins that cleave target RNAs mainly to disrupt the activity of transposons in the gonads (Garabedian et al. 1986; Malone and Hannon 2009). Recently, one of the piRNAs originating from the sex-determining region of the W chromosome appeared to function as *Fem* (Kiuchi et al. 2014). Inhibiting the expression of *Fem* piRNA in female embryos not only induced Imp^M expression (Sakai et al. 2015) but also shifted the splicing pattern of *Bmdsx* from the female to the male forms, suggesting that this piRNA is required for femaleness (Kiuchi et al. 2014; Sakai et al. 2015). *Fem* piRNA targets and cleaves mRNAs transcribed from the Z chromosome–linked gene that encodes a CCCH-tandem zinc finger protein. Knockdown of this gene expression in male embryos decreased the Imp^M expression (Sakai et al. 2015) and led to the production of the female-type *Bmdsx* transcripts (Kiuchi et al. 2014; Sakai et al. 2015), indicating that this gene is essential for silkworm masculinization, and it was therefore named *Masculinizer* (*Masc*). Recently our studies using transgenic silkworms have demonstrated that forced expression of *Masc* in females inhibits the normal development of ovaries and induces the formation of spermatids in the abnormal tissues [manuscript in preparation].

On the basis of these findings, we have proposed a genetic cascade regulating sex determination in *B. mori*, as follows (Fig. 13.3). In females (ZW), *Fem* piRNA disrupts the expression of *Masc*. Lower expression of *Masc* fails to induce expression of Imp^M. In the absence of Imp^M, *Bmdsx* pre-mRNA is spliced by default to generate the female-type BmDSX protein, which promotes female development. In males (ZZ), *Masc* expression remains at a higher level than in ZW individuals. This boosts the expression of *Imp* to a level sufficient to impose the male splicing mode on its own RNA. Once IMP^M protein is produced, its male-specific expression is maintained by an autoregulatory function. IMP^M protein, together with BmPSI, is altered to produce the male-specific splicing of *Bmdsx*. The male-type BmDSX protein promotes male development.

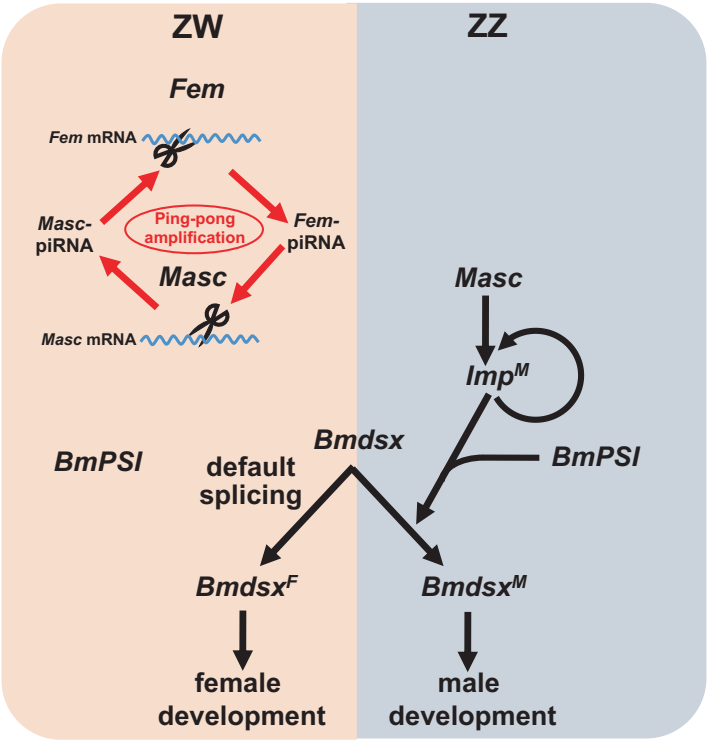

Fig. 13.3 Proposed genetic cascade regulating sex determination in *Bombyx mori*. In females (ZW), transcripts from the *Fem* locus are cleaved by the maternally transmitted *Masc* PIWI-interacting RNA (piRNA)–BmAgo3 complex. The *Fem* piRNA–Siwi complex cleaves *Masc* mRNA, resulting in the accumulation of *Masc* piRNA and further enhancement of feminization (for more details, refer to the review described by Katsuma et al. in 2015). Lower expression of *Masc* fails to induce expression of Imp^M. In the absence of Imp^M, *Bmdsx* precursor messenger RNA is spliced by default to generate the female-type BmDSX protein ($BmDSX^F$), which promotes female development. In males (ZZ), *Masc* expression remains at a higher level than in ZW individuals. This initiates the expression of Imp^M. Once the IMP^M protein is produced, its male-specific expression is maintained by an autoregulatory function. IMP^M protein, together with BmPSI, is altered to produce the male-specific splicing of *Bmdsx*. The male-type BmDSX protein ($BmDSX^M$) promotes male development

To date, *Fem* piRNA has been found only in *Bombyx* genomes, while its downstream target *Masc* is conserved among several lepidopteran species, some of which lack a W chromosome (Kiuchi et al. 2014). In such species, a Z chromosome–counting mechanism, such as XES factors in *Drosophila*, might regulate *Masc* expression, or a difference in the gene dosage of *Masc* itself may determine the sexual fate: ZZ animals with two genes develop into males, while ZO animals with only one *Masc* gene develop into females. A similar sex-determining mechanism has been reported in the chicken, which has a Z-linked *dmrt1* gene. In this species, where the homogametic sex is male (ZZ) and the heterogametic sex is female (ZW),

it is considered that two copies of the *dmrt1* gene promote male differentiation, while a single copy of the *dmrt1* gene promotes female differentiation (Smith et al. 2009).

B. mori presents the first known example of a sex-determining pathway controlled by the presence or absence of a piRNA. This *tra*-lacking insect also provides the first known example of a sex-specific splicing regulatory mechanism of *dsx* controlled by PSI and IMP orthologues. Whether this pathway is evolutionarily conserved among lepidopteran species will certainly be the subject of future research.

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