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Sexually dimorphic traits in the silkworm, *Bombyx mori*, are regulated by *doublesex*

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ABSTRACT

The DM domain genes, *doublesex* (*dsx*) in insects, or their structural homologs, *male abnormal 3* (*mab-3*) in nematodes and *Dmrt1* (*doublesex* and *mab-3*-related transcription factor 1) in mammals, are downstream regulators of the sex determination pathway that control sexually dimorphic development. Despite the functional importance of *dsx* and its potential applications in sterile insect technologies (SITs), the mechanisms by which it controls sexually dimorphic traits and the subsequent developmental gene networks in insects are poorly understood. Phylogenetic analyses indicate that insect *dsx* genes have sex-specific alternative splicing isoforms, whereas other taxa do not. We exploited genome editing and transgenesis technologies to induce mutations in either the male-specific isoform (dsx^M) or common region (dsx^C) of *dsx* in the somatic tissues of the lepidopteran model insect *Bombyx mori*. Disruptions of gene function produced either male-specific sexually-dimorphic defects or intersexual phenotypes; these results differ from those observed in other insects, including *Drosophila melanogaster*. Our data provide insights into the divergence of the insect sex determination pathways related to the most conserved downstream component *dsx*.

1. Introduction

Sex determination cascades are some of the most rapidly evolving developmental regulatory pathways and drive the differentiation of sexually dimorphic traits. The *doublesex* and *mab-3-related* (DM) domain was first identified in comparisons between the *Drosophila melanogaster dsx* and *Caenorhabditis elegans mab-3* genes (Raymond et al., 1998). *Mab-3* is a direct target of the *transformer 1* (*tra-1*) gene, which regulates male sexual development and behavior in *C. elegans* (Yi et al., 2000). The discovery of functional and molecular similarities between *dsx* and *mab-3* led to the identification of the *Dsx*- and *mab-3*-related transcription factor 1 (*Dmrt1*) in mammalian species (Raymond et al., 2000). These three genes (*dsx*, *mab-3* and *DMRT1*) control the development of a number of significant sexually dimorphic traits through male-specific expression or sex-specific transcript splicing. *Mab-3* controls the male tail tip morphology of *C. elegans* (Mason et al., 2008). Sexual dimorphism of the external and internal genitalia in *Mus musculus* are regulated by *DMRT1* (Raymond et al., 2000; Kuroki et al., 2013). Gene knockout experiments have shown that *mab-3* or *DMRT1* affects male sexual development through sex-specific expression.

Insects have sex-specific splicing that generates a male- (dsx^M) and female-specific (dsx^F) *dsx* isoform. Studies of the fruit fly, *Drosophila melanogaster*, have shown that the development of numerous sexually-dimorphic traits is controlled by *Dmdsx* (Burtis et al., 1991; Williams et al., 2008; Tanaka et al., 2011; Luo and Baker, 2015). These traits include female-specific yolk protein transcription, male-specific abdominal pigmentation, male-specific sex combs and female-specific spermathecae. $Dmdsx^F$ and $Dmdsx^M$ exhibit antagonistic effects on gene expression and compete with each other to regulate specific genes (Coschigano and Wensink,

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1993). In the *Bombyx mori*, the *Bmdsx* has sex-specific splicing products, $Bmdsx^F$ and $Bmdsx^M$ (Ohbayashi et al., 2001), ectopic expression $Bmdsx^F$ or $Bmdsx^M$ proofed its functional in the sexual development (Suzuki et al., 2003, 2005). In other insects, the *dsx* gene also been identified, such as in *Anopheles gambiae* (Scali et al., 2005), *Aedes aegypti*. (Salvemini et al., 2011), *Lucilia cuprina* (Concha et al., 2010), *Culex pipiens* (Price et al., 2015), *Tribolium castaneum* (Shukla and Palli, 2012), *Nasonia vitripennis* (Oliveira et al., 2009) *Apis mellifera* (Cho et al., 2007), *Musca domestica* (Hediger et al., 2004), *Gnatocerus cornutus* (Gotoh et al., 2016), *Antheraea assama* and *Antheraea mylitta* (Shukla and Nagaraju, 2010). These insects *dsx* displayed the same sex-specific splicing between two sexes. Recent studies place the DM domain genes at the bottom of the sex determination cascade, and this is conserved in many metazoans (Matson and Zarkower, 2012). Comparisons of the genes among diverse phyla, including nematodes, echinodermata, vertebrata and arachnida, indicate that these genes are not spliced and do not have splice-variant forms (Fig. 1). In contrast, splicing at the C-terminal region (CTR) is characteristic of the products of the insect DM domain genes and they have relatively high rates of evolution (Eirín-López and Sánchez, 2015). Thus, a considerable amount of the phylogenetic variation and divergent evolution among insects is found in the CTR domain. This variation through splicing may provide the basis for the evolution of sex-specific effects on the expression of the gene isoforms. We introduced mutations into the *Bmdsx* male-specific isoform or common regions to explore their physiological functions.

In the previous study, we developed a binary transgenic TALENs system to target the female-specific isoform of *Bmdsx* gene ($Bmdsx^F$) in *B. mori* (Xu et al., 2014). Depletion the $Bmdsx^F$ induced female-specific sterility whereas no effect on males. In current study, we further targeted the male-specific isoform of *Bmdsx* gene ($Bmdsx^M$) by using transgenic TALENs technologies, resulting in male-specific sterility. Furthermore, we also targeted the DM domain region which is needed for both sexes ($Bmdsx^C$) by using the transgenic CRISPR/Cas9 system, which induced intersexual phenotypes in both sexes. Phenotypic consequences including morphologic characters of sex organs, courtship behavior and fecundity in mutant animals were also investigated. We present the evidence that the conserved downstream gene *dsx* in insect sex determination pathway regulates species-specific sexual dimorphic traits.

2. Materials and methods

2.1. Silkworm strains

The multivoltine, nondiapausing silkworm strain, Nistari, was used for all experiments. The larvae were reared on fresh mulberry leaves under standard conditions (Tan et al., 2005).

2.2. Phylogenetic analysis

Phylogenies were determined based on sequence alignment (CLUSTAL W2) and phylogenetic analysis using Mega 5.1 (Larkin et al., 2007; Tamura et al., 2011). A maximum likelihood tree of 27 sequences was developed, and the reliability of the tree was tested by bootstrap analysis with 1000 replications.

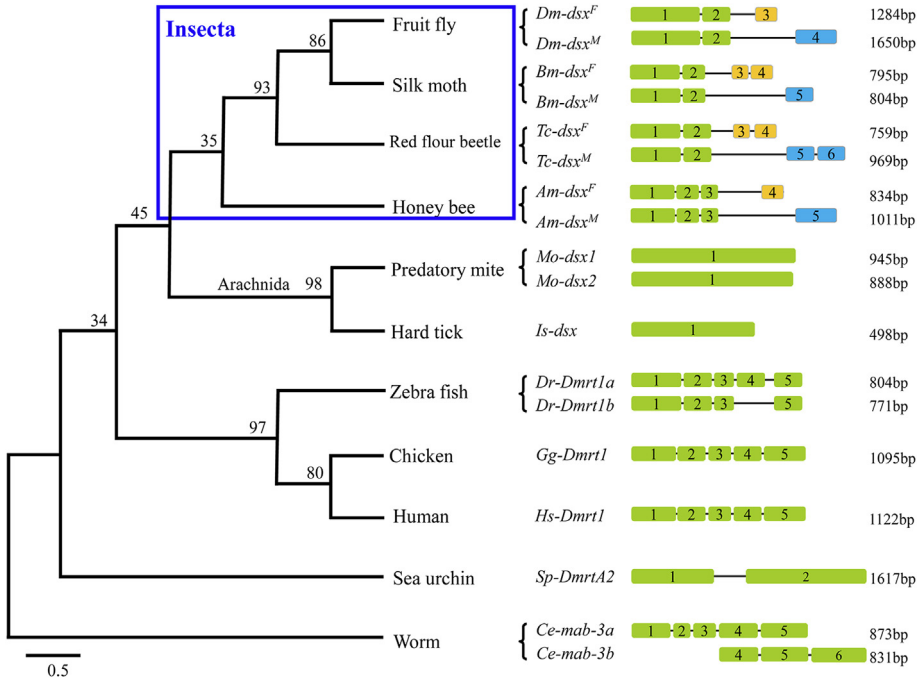

Fig. 1. Divergent evolution of the C-terminal regions of DM domain genes in insects and comparisons with other phyla. The display is a consensus of the most parsimonious trees derived from 1000 bootstrap resamplings of the original data using the complete sequences of DM domain proteins (Mab-3, Dsx or Dmrt1). The diagrams on the right are schematic representations of the corresponding gene structures. The green blocks represent the nonsex-specific spliced exons (1–6). The female- and male-specific exons (3–6) are indicated in yellow and blue blocks, respectively. The numbers at the nodes denote bootstrap values (%). Bootstrap values < 50% are not indicated. The abbreviations and GenBank accession numbers of the protein sequences are as follows: *Caenorhabditis elegans Ce-Mab-3a* (NP_001022464.1), *Ce-Mab-3b* (NP_871909.1); *Gallus gallus Gg-DMRT1* (NP_001095301.1); *Homo sapiens Hs-DMRT1* (AF130728); *Danio rerio Dr-DMRT1* (AF130728); *Drosophila melanogaster* $Dm\text{-}dsx^M$ (NP_731197.1), $Dm\text{-}dsx^F$ (NP_001287220.1); *Tribolium castaneum* $Tc\text{-}dsx^M$ (XP_001807448.1), $Tc\text{-}dsx^F$ (AFQ62106.1); *Bombyx mori* $Bm\text{-}dsx^M$ (NP_001104815.1), $Bm\text{-}dsx^F$ (NP_001036871.1); *Apis mellifera* $Am\text{-}dsx^M$ (NP_001104725.1), $Am\text{-}dsx^F$ (NP_001128407.1); *Ixodes scapularis Is-dsx* (ISCW007591-PA); *Metaseiulus occidentalis Mo-dsx1* (XP_003740430.1), *Mo-dsx2* (XP_003740429.1); *Strongylocentrotus purpuratus Sp-DMRTA2* (SPU_001255). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

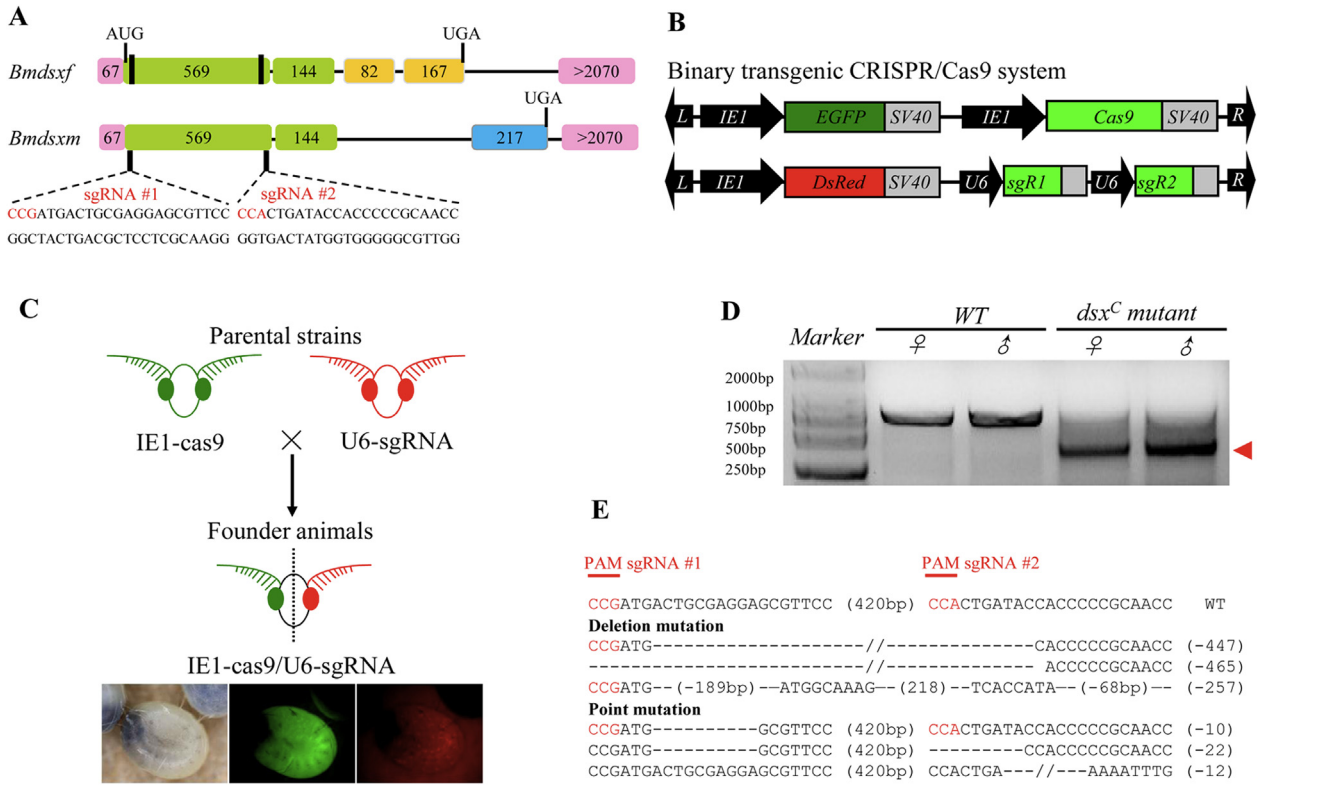

Fig. 2. Targeting the common region of *Bmdsx* gene using a binary transgenic CRISPR/cas9 system. (**A**) Alternative splicing of the *Bombyx mori doublesex* (*Bmdsx*) gene generates both female- (*Bmdsx*^{F}) and male-specific (*Bmdsx*^{M}) isoforms. Pink boxes: 5′- and 3′-UTRs; green boxes: *dsx* common region; red boxes: female-specific exons; gray boxes: male-specific exons. Numerals: number of nucleotides; AUG: start codon; UGA: stop codon; sgRNA #1/sgRNA #2: two CRISPR/Cas target sites and corresponding gRNA sequences. (**B**) The binary transgenic CRISPR/Cas9 system comprises one line with the full Cas9 open reading frame (ORF) driven by the *IE1* promoter, and the other has two U6 promoter-driven sgRNAs. These two lines also contain the reporter genes *EGFP* or *DsRed2* under the control of the *IE1* promoter. (**C**) Transgenic strains expressing Cas9 or sgRNA were established as parental strains. Somatic mutations were induced in the F_1 founder animals following crosses of these two strains. Red and green fluorescence in the entire body confirmed the presence of the appropriate transgene constructs. (**D**) Somatic mutations were induced in the F_1 founder animals following crosses of IE1-Cas9 and U6-sgRNA strains. PCR analyses with primers to amplify a region of 800 bp revealed deletion mutation events in the G_0 mutants. The red arrowhead indicates the deleted region. (**E**) Deletion mutations in the heterozygous offspring after crossing IE1-Cas9 and U6-Bmdsx^{C}sgRNA transgenic silkworm lines. The targeting sequence is noted in black, and the PAM sequence is presented in red. The deletion size is indicated by the negative number of nucleotides. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Construction of the transgenic TALEN and CRISPR/Cas9 plasmids

The transgenic TALEN plasmids PXLBacII-3 × P3-DsRed-IE1-TALEN-R (dsx^{M} -TALEN-R) and PXLBacII-3 × P3-EGFP-IE1-TALEN-L (dsx^{M}-TALEN-L) were constructed as described previously (Xu et al., 2014). The establishment of a binary transgenic CRISPR/Cas9 system was performed as described previously (Li et al., 2015) with modifications. Briefly, an activator plasmid, *pBac[IE1-EGFP -IE1-Cas9]* (*IE1-Cas9*), which expresses Cas9 constitutively under the control of a baculovirus immediate-early gene promoter IE1, and an effector plasmid, *pBac[*IE1-DsRed2-*U6-2xDsx^{c}sgRNA]* (*U6-Dsx^{c}sgRNA*), that expresses two sgRNAs targeting *Dsx^{c}* under the control of the silkworm small nuclear RNA promoter U6, were constructed through a series of cloning steps.

2.4. Silkworm germline transformation

Germline transformation was performed by microinjection of plasmid solutions into preblastoderm embryos followed by subsequent incubation and rearing. Putative transgenic G_0 adults were mated to WT moths, and the G_1 progeny were scored for the presence of the eye-specific or ubiquitous fluorescence marker using fluorescence microscopy (Nikon AZ100).

2.5. Mutagenesis analysis

The genomic DNA of the founder animals was extracted from the larvae in standard sodium dodecyl sulfate (SDS) lysis-phenol buffer after incubation with proteinase K followed by RNase treatment and purification. Gene amplification was performed using 50 ng of genomic DNA as the template. The following primer sets for amplification of the target region of male-specific region by the TALENs were used: forward primer, 5′-CTGTTAA-GATGACGCATGGCCTAAACCAGCG-3′; reverse primer, 5′-TAGGTAC-CATCGTACCACCTGCGTATTTCTG-3′. The primers amplified a 914 base pair (bp) fragment that encompassed the target site. The following primers were used for amplification of the target region of the CRISPR/Cas9 system: forward primer, 5′-GCATCGTTG-CACTGGAATAAATGCAGT-3′; reverse primer, 5′- GCTTCGT GTTCATTTAGGAAACTGT-3′. The primers amplified a region of 800 bp in length that encompassed the target site. The PCR products were sub-cloned into the pJET-1.2 vector (Fermentas) and sequenced.

2.6. Photography, scanning electron microscopy and paraffin sectioning

Testes of mutant or wild-type animals were dissected from the larval, pupal and adult stages and subjected to morphological

investigation under a microscope (Nikon, Tokyo, Japan). External genitalia of the adults were photographed using a digital camera (Nikon DS-Ri1, Japan).

The dissected body segments and external genitalia were fixed overnight in FAA (70% ethanol 90 ml, acetic acid 5 ml, 37% methyl aldehyde 5 ml), dehydrated in a series of 70%, 80%, 90%, and 100% ethanol for 5 min, and dried (CO_2 for 6 h in the Critical Point Dryer). The dissected materials were coated with gold and observed under a JSM-6360LV SEM (JEOL, Rigaku, Japan). The photographs are representative of samples obtained from biological triplicate experiments.

Tissue sectioning was performed as described previously (Zhang et al., 2014). Gonads were dissected at the fourth day of the fifth instar stage and fixed in Qurnah's fixative (anhydrous ethanol: acetic acid: chloroform = 6:1:3v/v/v) overnight. Sections were dehydrated and cleared three times using anhydrous ethanol and xylene. Tissues were embedded in paraffin overnight and sectioned using a Leica microtome (RM2235). Sections were stained using a mixture of hematoxylin and eosin to visualize morphology. All pictures were captured with a microscope (Olympus BX51) using differential interference contrast and the appropriate filter.

2.7. Courtship behavior analysis

WT virgin females and males, *Bmdsx*^{M} mutant females and males, and *Bmdsx*^{C} mutant females and males were crossed with each other. Courtship behavior was videotaped for 30 min after a target and a test were introduced into the courtship chamber. The courtship index was evaluated by calculating the time until the male first copulated with the female while observing mating behavior over a 30-min period. The behavioral assays were performed at 25 °C and 60% relative humidity under normal ambient light.

2.8. Semi quantitative RT-PCR

Total RNA was extracted from silkworm larvae using the Trizol reagent (Invitrogen) and treated with RNase-free DNAse I (Ambion). The cDNAs were synthesized using the Omniscript Reverse transcriptase kit (Qiagen) in a 20-μl reaction mixture containing 1 μg of total RNA. The PCR reactions were performed with gene-specific primers (forward, 5′- AACCATGCCACCACTGATACCAAC-3′ reverse, 5′- GCACAACGAATACTGCTGCAATCG-3′) to amplify the *Bmdsx* gene. The PCR conditions included 2 min of denaturation at 95 °C; 35 cycles of 1 min at 95 °C, 30 s at 55 °C and 30 s at 72 °C; followed by a final extension at 72 °C for 10 min.

2.9. Quantitative real-time PCR (qRT-PCR)

The qRT-PCR reactions were performed using gene-specific primers (PBP, forward, 5′-CATGGAGCCGATGAGACGAT-3′; reverse, 5′-TCATCGTTAGCTGGAGTGGACTT-3′). The PCR conditions included incubation at 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 30 s. A 10-fold serial dilution of pooled cDNAs was used as the template to generate a standard curve. The mRNA measurements were quantitated in three independent biological replicates and normalized to the *Bmrp49* mRNA (Tan et al., 2013).

2.10. Statistics

Experimental data were analyzed with Student's *t*-test. *t*-test: *, $P < 0.05$. At least three independent replicates were used for each treatment, and error bars indicate the mean ± standard error of the mean (SEM).

3. Results

3.1. Targeted mutagenesis by using transgene-based TALENs or CRISPR/cas9 system

A binary transgenic CRISPR/Cas9 system was designed to target exon 1 of *Bmdsx*, which is the common region (designated *Bmdsx*^{C}) of all splicing isoforms (Fig. 2A). One *piggyBac*-based transformation vector contains the Cas9-nuclease open reading frame (ORF) driven by the immediate-early 1 (IE1) baculovirus promoter (IE1-Cas9), which provides ubiquitous and constitutive expression of the enzyme throughout all tissues, and the enhanced green fluorescent protein (EGFP) driven by the same IE1 promoter as a marker gene for screening. A second transformation vector contains a single guide RNA (sgRNA) driven by the silkworm U6 promoter (U6-sgRNA) and the DsRed2 fluorescent marker gene under control of the IE1 promoter (Fig. 2B). IE1-Cas9 and U6-sgRNA parental transgenic lines were established separately (Supplementary Table 1 and Table 2) and crossed with each other to obtain F_1 founder moths (Fig. 2C). Characterization of the somatic mutations by polymerase chain reaction (PCR) using gene-specific primer pairs indicated that both male and female animals had mutations at the target site caused by non-homologous end joining (NHEJ)-induced indels, and most of these were deletions (Fig. 2D and E). Reverse transcription-PCR (RT-PCR) detected female-and male-specific isoforms in both sexes (Fig. S2).

A *piggyBac*-based transgenic TALEN system (Xu et al., 2014) was used to target *Bmdsx* exon 5, which is present in only the male-specific splicing isoform (designated *Bmdsx*^{M}; Fig. S1A). TALEN activity was validated *in vitro* using the luciferase single-strand annealing (SSA) assay in 293T cells (Fig. S1B). The TALEN left-arm (TALEN-L) and TALEN right-arm (TALEN-R) constructs contain sequences encoding the *Fok*I DNA endonuclease and a sequence-specific DNA recognition repeat domain, both of which are driven by the IE1 promoter. Genes encoding fluorescent marker proteins also were integrated into the vectors (EGFP for TALEN-L and DsRed2 for TALEN-R). Both EGFP and DsRed2 expression are under the control of the eye-specific promoter, 3 × P3 (Fig. S1C) (Thomas et al., 2002). TALEN-L or TALEN-R transgenic silkworm lines were established separately (Supplementary Material Table S1 and Table S2). Somatic mutagenesis analysis performed on the progeny of a cross between the two transgenic lines identified male and female individuals with mutations at the target site (Figs. S1D and E). RT-PCR analysis revealed the absence of the male-specific isoform in the mutant male animals (Fig. S2).

3.2. Bmdsx mutation induced gonad abnormality

Most phenotypic analyses of knockouts in the *dsx* gene family focus on gonad development (Hilderth, 1965; Smith et al., 2009). We confirmed that testis and ovary development was severely affected in sub-adult insects with the *Bmdsx*^{C} mutation (Fig. 3A). The testicular lobe in larvae appeared degenerated and separated from the testis envelope. The ovary exhibited a testicular lobe-like structure that was structurally equivalent to sexual homoeosis (Fig. 3). The larval stage mutant testes and ovaries also exhibited similarities in their gross morphology (Fig. 4A). Adult female *B. mori* have paired ovaries attached by lateral oviducts (each has four) to a common oviduct that joins the spermathecae. The *Bmdsx*^{C} mutant females exhibited degenerated ovaries with no eggs in the oviducts, and the spermathecae failed to differentiate. Adult male *B. mori* have a pair of reniform testes, each of which is joined to a vas deferens, the latter of which enters the anterior ejaculatory duct. The *Bmdsx*^{C} mutant male gonads exhibited serious defects, with degenerated testes and abnormalities in the vas deferens. The

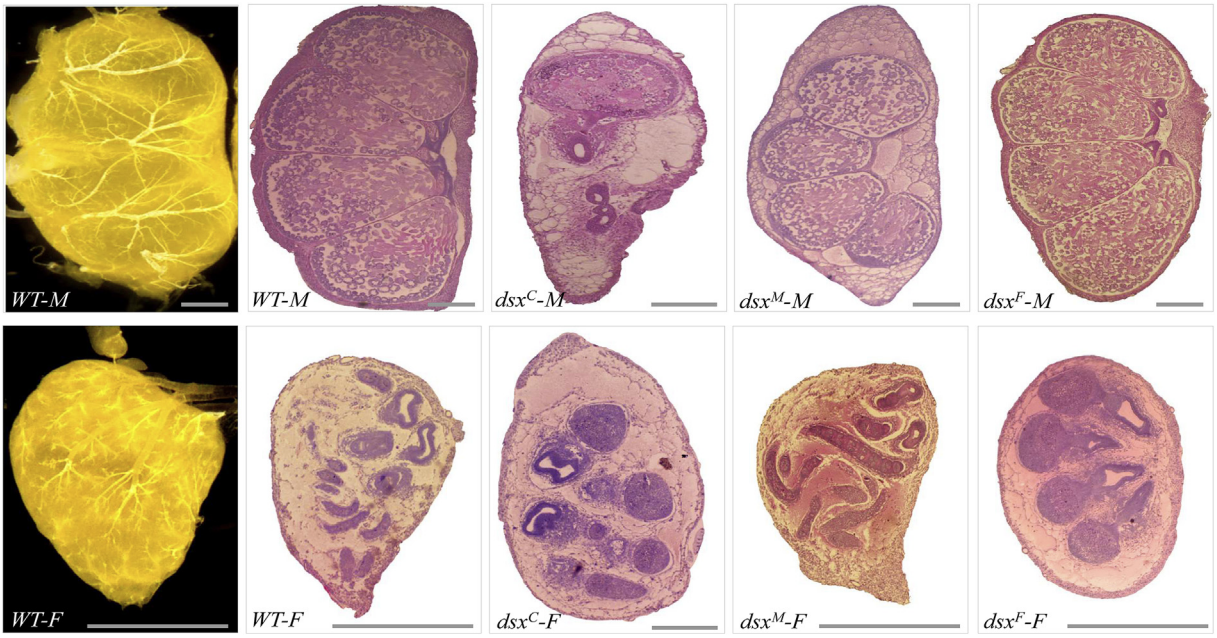

Fig. 3. Gonad development in *dsx* knockout larvae mutants. The upper panel shows the gross morphology of the intact wild-type (WT-M) testes and the internal structures of the WT and three mutant (dsx^C-*M*, dsx^M-*M*, dsx^F-*M*) testes in paraffin sections stained with hematoxylin and eosin. The lower panel shows the structures of the WT (WT-F) and three mutant (dsx^C-*M*, dsx^M-*M*, dsx^F-*F*) ovaries. Scale bar: 250 μm.

mutant testes and ovaries appear similar and were irregularly-shaped spheres (Fig. 4B).

In the $Bmdsx^M$ mutants, we observed that the testes were smaller than wild-type (WT) organs at the last larval stage (Fig. 5A). Furthermore, five testis chambers were evident in the mutant animals compared with the four normally observed in WT individuals (Fig. 5B and C). The testis chambers lost their structural integrity and appeared thinner at the third day after pupation (Fig. 5D and E). The testes of mutant $Bmdsx^M$ adults developed malformed chambers and slender sperm ducts (Fig. 5D and E). Moreover, four testicular lobes adjoined the sperm chamber and duct instead of the single lobe that is characteristic of WT animals (Fig. 5E). The testes in the $Bmdsx^F$ mutant pupae were phenotypically normal; however, the ovaries exhibited a testicular lobe-like structure (Fig. 3). These results support the conclusion that $Bmdsx^F$ and $Bmdsx^M$ are required for proper gonad development.

3.3. *Bmdsx* mutation induced external genital structures and fecundity abnormality

Sex-specific adult external characteristics include a ventral chitin plate and genital papilla in females and claspers in males, all of which are essential for copulation (Fig. 6). Genital papillae and clasper structures are evident in both sexes of the $Bmdsx^C$ mutants. The external genitalia of the $Bmdsx^C$ mutant male resemble female-specific genital papillae. The ventral chitin plate of the external genitalia of the $Bmdsx^C$ mutant female is similar to a male-specific clasper. Moreover, the mutated genital papillae in both sexes exhibited structural features characteristic of intersexual fusions. The mating apparatus was deformed and this prevented successful copulation and confers sterility to both sexes. The male-specific external genitalia (claspers and penis) in $Bmdsx^M$ mutant males exhibited severe structural defects, with shorter lengths compared with WT males (Fig. 7). These defective structures contribute to the failed courtship behavior of orientation and led to subsequent rejection of males by females (Fig. S3A and B). $Bmdsx^M$ males could not perform mating behavior even in the presence of wild-type levels of pheromone binding protein (PBP) expression (Fig. S3C). $Bmdsx^M$ males produce no progeny, whereas the mutant females exhibited normal fecundity (Fig. 8). This suggested that the defective structures and the sex behavior contribute to male sterility in $Bmdsx^M$ mutant.

We investigated $Bmdsx^F$-ablated adult females in a previous study and found that the dorsal chitin plate was absent and that the genital papilla exhibited severe morphological abnormalities (Fig. 6). However, the male structures appeared normal (Xu et al., 2014). These results supported our conclusion that the female-specific isoforms were required for sex-specific structure development in the corresponding sex, but had no effect on the other sex.

3.4. Sexually dimorphism of abdominal segments disappeared in the $Bmdsx^C$ mutant females

Abdominal segment numbers are another sexually dimorphic trait in *B. mori*. Both females and males have eight abdominal segments during the larval and early pupal stages (Fig. S4). The female eighth abdominal segment is reshaped at the adult stage into a naked chitin plate without scaly hairs. However, the $Bmdsx^C$ mutant females still had eight abdominal segments, which is the same morphology as the WT males (Fig. 9). In contrast, the number of abdominal segments was not altered in $Bmdsx^M$ mutant females (Fig. 9). These results support the conclusion that $Bmdsx^F$, which includes $Bmdsx^C$, is responsible for controlling the reshaping of the eighth abdominal segment in females.

4. Discussion

Loss-of-function analyses showed that *dsx* orthologs in *C. elegans* affected only male sexual dimorphic development due to its male-specific expression and *D. melanogaster* affected both sexual dimorphic development due to its sex-specific splicing (Yi et al., 2000; Raymond et al., 2000). Insect *dsx* genes exhibited sex-specific splicing to generate male- (dsx^M) and female-specific

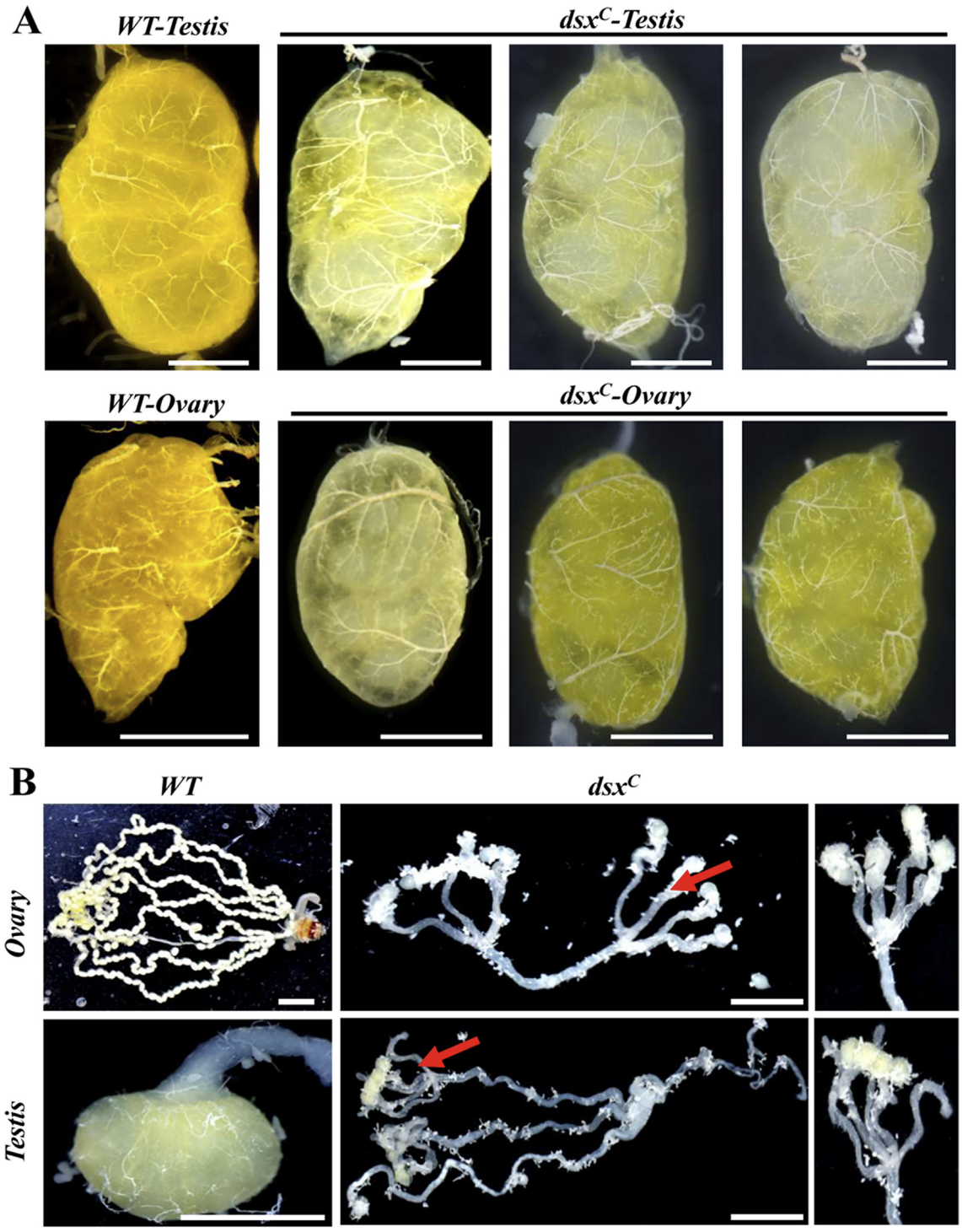

Fig. 4. Morphology of the gonads dissected from wild-type and *Bmdsx*^{C} mutants. (**A**) The gonads of one wild-type and three *Bmdsx*^{C} mutants were dissected on the third day of the fifth instar stage. Scale bars: 300 μm. (**B**) Both sexes of *Bmdsx*^{C} mutant moths exhibit intersexual phenotypes in their internal genitalia. The WT adult female internal genitalia exhibited normal oogenesis in the oviduct, and the WT adult testes are kidney-shaped. Scale bar: 1 mm.

(*dsx*^{F}) isoforms that control separately corresponding sex-specific dimorphic traits (Williams and Carroll, 2009). Functional analyses of sex specific isoforms has been performed by using RNAi, but it has been difficult previously to generate sex-specific *dsx* mutations in insects, even in the model organism *Drosophila* (Ito et al., 2013; Mysore et al., 2015; Shukla and Nagaraju, 2010). However, the precisely-targeted disruption of *Bmdsx* with the transgenic CRISPR/Cas9 and TALENs systems allows us to dissect the sex-specific biological functions of the *dsx* alternatively-spliced isoforms. Firstly, the regulatory mechanisms of sex-specific alternative splicing of *Bmdsx* that of exon skipping is distinct from that of *Dmdsx* that of 3' alternative splice. Secondly, the *Bmdsx* was female default pattern and the *Dmdsx* was male default pattern. Thirdly, we produced male-specific sexually-dimorphic defects via

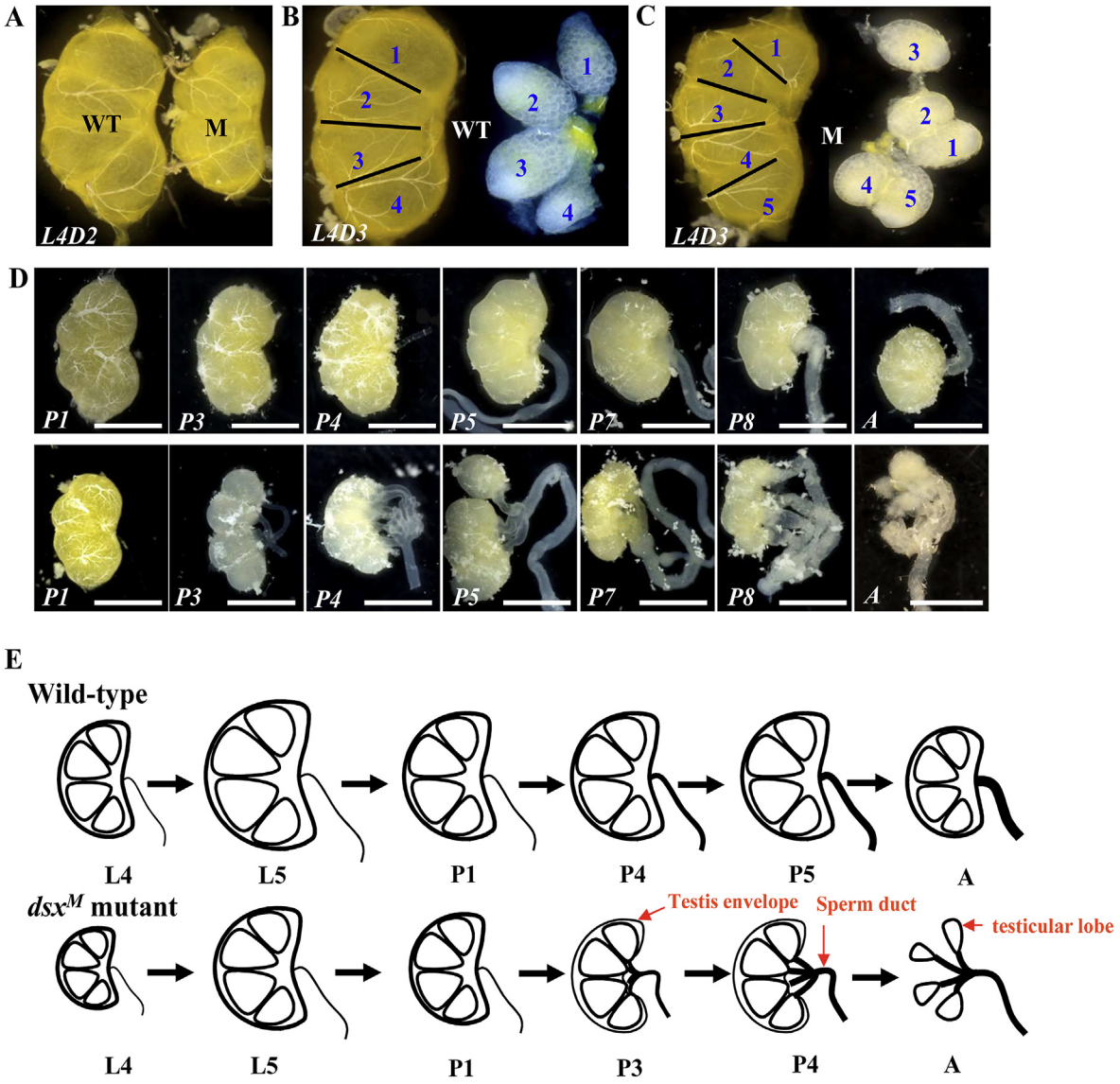

Fig. 5. Loss-of-function of *Bmdsx*^{M} caused developmental defects in the testes. (**A**) The mutant testes are smaller than those of WT at the fourth instar stage. (**B** and **C**) The WT testis has four sperm chambers. One of the mutant testes contained five sperm chambers. (**D**) Developmental morphology of the testes from pupal to adult stage. Scale bar: 500 μm. (**E**) Comparison of the morphological changes in the testes between WT and *Bmdsx*^{M} mutants from the larval to adult stages. The red arrows indicate the testis envelope. The sperm ducts and sperm chambers are abnormal in the mutant testis. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

knockout the male-specific isoform which was not reported in the previous studies.

4.1. DM domain genes control sexual dimorphism traits and undergo divergent evolution

Sex-specific traits are considered by genetic regulated via sex-specific expression proteins. *Mab-3* and *DMRT1* had been proofed that controlled almost all of these traits based on male-specific expression or sex-specific splicing expression. The knockout experiment showed *mab-3* or *DMRT1* only affected the male sexual dimorphism development due to the male-specific expression. The situation in insects displayed that the *dsx* gene has sex-specific splicing expression to generate the male-specific isoform (*dsx*^{M}) and female-specific isoform (*dsx*^{F}). The reports in *Drosophila* showed many sex dimorphism traits were controlled by the *dsx* gene such as abdominal pigmentation, sex segments and sex comb. Also in the model insect of Lepidoptera, *B. mori*, the loss function of *dsx*^{F} revealed that sex segments and genital are controlled by the *dsx* (Xu et al., 2014). DM (Doublesex/MAB-3) domain genes have the DNA binding domain (DM) and the C-termini region (CTR), sex-specific of CTR in insects allow them to have different effects on gene expression. Phylogenetic analysis of the protein sequences of CTR revealed that *dsx*^{M} and *dsx*^{F} might undergo divergent evolution at CTR but not DM domain (Fig. 1). Considered that *Dmdsx*^{F} regulated female sex development but *Dmdsx*^{M} is not so clear on regulating male sex development (Williams and Carroll, 2009), the situation was reported in the *Tribolium* that *Tcdsx*^{F} and *Tcdsx*^{M} affecting development of ovary and testis, respectively (Shukla and Palli, 2012). These means that DM domain gene *dsx* got function at C-termini regulation regions during evolution at least in the insect.

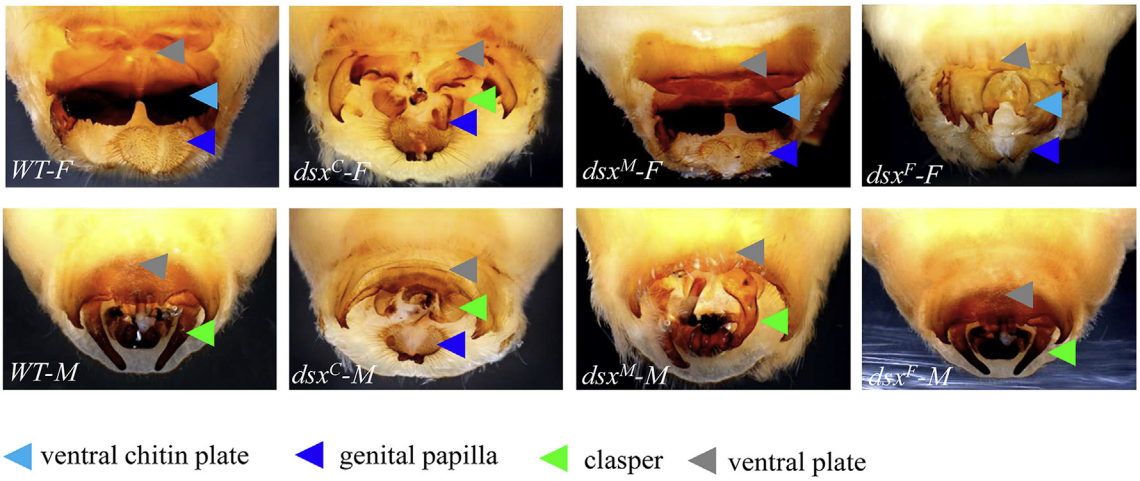

Fig. 6. Gross morphology of the external genitalia of the WT and three *Bmdsx* mutant insects. The WT female external genitalia consist of a ventral chitin plate, genital papilla and ventral plate. The WT adult male external genitalia consist of claspers and a ventral plate. The ventral chitin plate of the external genitalia of the $Bmdsx^C$ mutant female is similar to a male-specific clasper. The external genitalia of the $Bmdsx^C$ mutant male resemble female-specific genital papillae. The $Bmdsx^M$ mutant males and $Bmdsx^F$ mutant females have abnormal external genitalia. The blue arrows indicate the ventral chitin plate, the purple arrows indicate the genital papillae, the green arrows denote the claspers, and the gray arrows indicate the ventral plates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

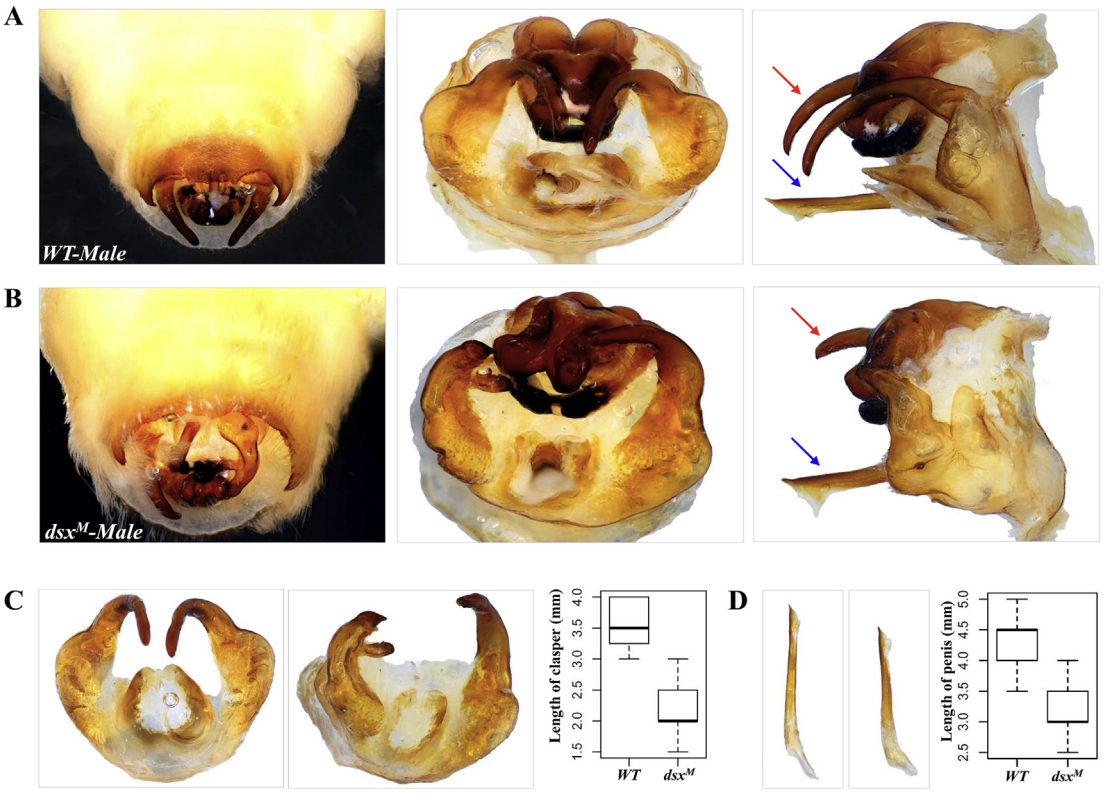

Fig. 7. Loss-of-function of $Bmdsx^M$ causes developmental defects in the male external genitalia. (**A** and **B**) Gross morphology of the male external genitalia of a wild-type male and a mutant male. The red arrows indicate the claspers, and the blue arrows denote the penis. (**C** and **D**) Dissection of the clasper and penis reveal that the lengths of those organs in the mutant males are shorter than in the controls (data from fifteen WT and fifteen dsx^M moths). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.2. Sterile insect technologies based on regulating sexually dimorphic traits

Traditional sterile insect technologies (SIT) involve the mass release of sterile males obtained by radiological or chemical treatments (Knipling, 1955). The paradigm of this approach has achieved success in eradication of the New World screwworm *Cochliomyia hominivorax* in USA and Mexico (Wyss, 2000). However, radiological or chemical treatments caused the genetically unstability, thus limited its further applications. New technologies, especially recombinant DNA techniques, were considered as essentially safe and efficacious approaches for improving the success of sterile insect

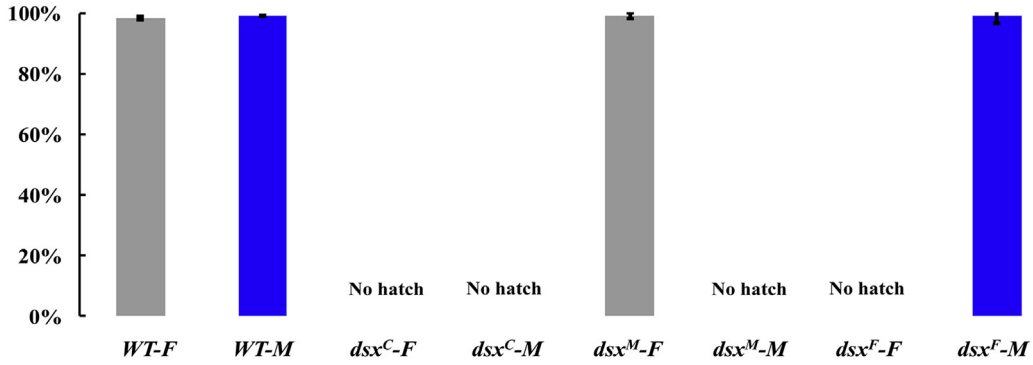

Fig. 8. Numbers of abdominal segments in the WT and three different *Bmdsx* mutant insects. WT females and males contain seven and eight abdominal segments, respectively. Both the $Bmdsx^C$ and $Bmdsx^F$ mutant females have eight abdominal segments, whereas the $Bmdsx^M$ mutant females possess seven segments similar to the WT.

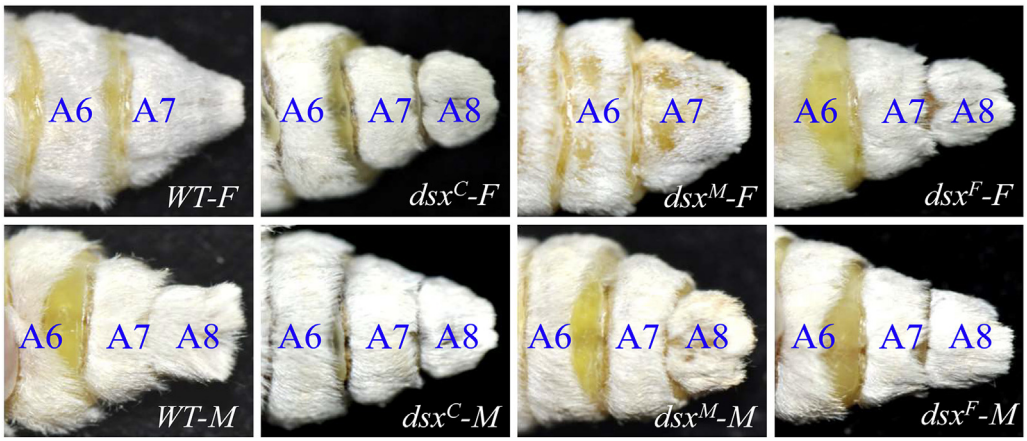

Fig. 9. Fertility of three *dsx* mutants. Numbers of hatching embryos from the oviposited eggs. No hatch shows the mating-failed caused sterility. All data represent the average of three independent experiments ± SEM.

releases (Benedict and Robinson, 2003). Successful paradigms had been reported in the Diptera including *Ceratitis capitata* and *Aedes aegypti* (Gong et al., 2005). These sex-specific sterile or lethality system were based on introducing the cell apoptosis genes (such as *hid*, *reaper* and *grim*), or toxicity genes (such as *Ras64B* or *tTA*), controlled by sex-specific promoters or sex-specific alternative splicing factors (Schetelig and Handler, 2012; White et al., 1996; Chen et al., 1996; Thomas et al., 2000; Fu et al., 2007). Recently developed genome editing techniques, TALENs and the CRISPR/Cas9 might improve and bring revolution SIT (Alphey, 2016; Gantz et al., 2015). These would realize the precisely modification on sterile or lethality genes. *Tra* and *dsx* as two key sex-determination genes of insects, were both regulated by sex-specific alternative splicing (Black, 2003). Using alternative splicing of *tra* had engineered female-specific insect lethality in *A. aegypti* (Fu et al., 2007) and *C. capitata*. However, *tra* only exists in some finite species of insects (Geuverink and Beukeboom, 2014). This would be restricted its applications in SIT especially for the Lepidoptera pests in which no *tra* homologs were found. The *dsx* displayed more ambitious in SIT than other possible approaches due to its conserved exist in all insects and its applications in SIT for lepidoptera has been reported (Tan et al., 2013; Jin et al., 2013). Targeted mutagenesis in the current study provides direct evidence that the sex-specific isoform of *Bmdsx* regulates sexual dimorphism in a sex-specific manner. As the representative model insect in lepidoptera, the sex-specific sterility induced by *Bmdsx* depletion might be applicable in lepidoptera pest control.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2016.11.005.

References

Alphey, L., 2016. Can CRISPR-Cas9 gene drives curb malaria? Nat. Biotechnol. 34, 149–150.
Benedict, M.Q., Robinson, A.S., 2003. The first releases of transgenic mosquitoes: an argument for the sterile insect technique. Trends. Parasitol. 9, 349–355.
Black, D.L., 2003. Mechanisms of alternative pre-messenger RNA splicing. Annu. Rev. Biochem. 72, 291–336.
Burtis, K.C., Coschigano, K.T., Baker, B.S., Wensink, P.C., 1991. The doublesex proteins of *Drosophila melanogaster* bind directly to a sex-specific yolk protein gene enhancer. EMBO J. 10, 2577–2582.
Chen, P., Nordstrom, W., Gish, B., Abrams, J.M., 1996. *Grim*, a novel cell death gene in *Drosophila*. Genes Dev. 10, 1773–1782.
Cho, S., Huang, Z.Y., Zhang, J., 2007. Sex-specific splicing of the honeybee *doublesex* gene reveals 300 million years of evolution at the bottom of the insect sex-determination pathway. Genetics 177, 1733–1741.
Concha, C., Li, F., Scott, M.J., 2010. Conservation and sex-specific splicing of the *doublesex* gene in the economically important pest species *Lucilia cuprina*.

J. Genet. 89, 279–285.
Coschigano, K.T., Wensink, P.C., 1993. Sex-specific transcriptional regulation by the male and female doublesex proteins of *Drosophila*. Genes Dev. 7, 42–54.
Eirín-López, J.M., Sánchez, L., 2015. The comparative study of five sex-determining proteins across insects unveils high rates of evolution at basal components of the sex determination cascade. Dev. Genes Evol. 225, 23–30.
Fu, G., Condon, K.C., Epton, M.J., Gong, P., Jin, L., Condon, G.C., Morrison, N.I., Dafa'alla, T.H., Alphey, L., 2007. Female-specific insect lethality engineered using alternative splicing. Nat. Biotechnol. 25, 353–357.
Gantz, V.M., Jasinskiene, N., Tatarenkova, O., Fazekas, A., Macias, V.M., Bier, E., James, A.A., 2015. Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito Anopheles stephensi. Proc. Natl. Acad. Sci. U. S. A. 112, E6736–E6743.
Geuverink, E., Beukeboom, L.W., 2014. Phylogenetic distribution and evolutionary dynamics of the sex determination genes *doublesex* and *transformer* in insects. Sex. Dev. 8, 38–49.
Gong, P., Epton, M.J., Fu, G., Scaife, S., Hiscox, A., Condon, K.C., Condon, G.C., Morrison, N.I., Kelly, D.W., Dafa'alla, T., Coleman, P.G., Alphey, L., 2005. A dominant lethal genetic system for autocidal control of the Mediterranean fruitfly. Nat. Biotechnol. 23, 453–456.
Gotoh, H., Ishiguro, M., Nishikawa, H., Morita, S., Okada, K., Miyatake, T., Yaginuma, T., Niimi, T., 2016. Molecular cloning and functional characterization of the sex-determination gene *doublesex* in the sexually dimorphic broad-horned beetle *Gnatocerus cornutus* (Coleoptera, Tenebrionidae). Sci. Rep. 6, 29337.
Hediger, M., Burghardt, G., Siegenthaler, C., Buser, N., Hilfiker-Kleiner, D., Dübendorfer, A., Bopp, D., 2004. Sex determination in *Drosophila melanogaster* and *Musca domestica* converges at the level of the terminal regulator doublesex. Dev. Genes Evol. 214, 29–42.
Hilderth, P.E., 1965. Doublesex, a recessive gene that transforms both males and females of drosophila into intersexes. Genetics 51, 659–678.
Ito, Y., Harigai, A., Nakata, M., Hosoya, T., Araya, K., Oba, Y., Ito, A., Ohde, T., Yaginuma, T., Niimi, T., 2013. The role of *doublesex* in the evolution of exaggerated horns in the Japanese rhinoceros beetle. EMBO Rep. 14, 561–567.
Jin, L., Walker, A.S., Fu, G., Harvey-Samuel, T., Dafa'alla, T., Miles, A., Marubbi, T., Granville, D., Humphrey-Jones, N., O'Connell, S., Morrison, N.I., Alphey, L., 2013. Engineered female-specific lethality for control of pest Lepidoptera. ACS Synth. Biol. 2, 160–166.
Knipling, E.F., 1955. Possibilities of insect control or eradication through the use of sexually sterile males. J. Econ. Entomol. 48, 459–462.
Kuroki, S., Matoba, S., Akiyoshi, M., Matsumura, Y., Miyachi, H., Mise, N., Abe, K., Ogura, A., Wilhelm, D., Koopman, P., Nozaki, M., Kanai, Y., Shinkai, Y., Tachibana, M., 2013. Epigenetic regulation of mouse sex determination by the histone demethylase Jmjd1a. Science 341, 1106–1109.
Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and clustal X version 2.0. Bioinformatics 23, 2947–2948.
Li, Z., You, L., Zeng, B., Ling, L., Xu, J., Chen, X., Zhang, Z., Palli, S.R., Huang, Y., Tan, A., 2015. Ectopic expression of ecdysone oxidase impairs tissue degeneration in *Bombyx mori*. Proc. Biol. Sci. 282, 20150513.
Luo, S.D., Baker, B.S., 2015. Constraints on the evolution of a *doublesex* target gene arising from doublesex's pleiotropic deployment. Proc. Natl. Acad. Sci. U. S. A. 112, E852–E861.
Mason, D.A., Rabinowitz, J.S., Portman, D.S., 2008. *dmd-3*, a *doublesex*-related gene regulated by *tra-1*, governs sex-specific morphogenesis in *C. elegans*. Development 135, 2373–2382.
Matson, C.K., Zarkower, D., 2012. Sex and the singular DM domain: insights into sexual regulation, evolution and plasticity. Nat. Rev. Genet. 13, 163–174.
Mysore, K., Sun, L., Tomchaney, M., Sullivan, G., Adams, H., Piscoya, A.S., Severson, D.W., Syed, Z., Duman-Scheel, M., 2015. siRNA-Mediated silencing of *doublesex* during female development of the dengue vector mosquito Aedes aegypti. PLoS Negl. Trop. Dis. 9, e0004213.
Ohbayashi, F., Suzuki, M.G., Mita, K., Okano, K., Shimada, T., 2001. A homologue of the *Drosophila doublesex* gene is transcribed into sex-specific mRNA isoforms in the silkworm, *Bombyx mori*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 128, 145–158.
Oliveira, D.C., Werren, J.H., Verhulst, E.C., Giebel, J.D., Kamping, A., Beukeboom, L.W., van de Zande, L., 2009. Identification and characterization of the *doublesex* gene of *Nasonia*. Insect Mol. Biol. 18, 315–324.
Price, D.C., Egizi, A., Fonseca, D.M., 2015. Characterization of the *doublesex* gene within the *Culex pipiens* complex suggests regulatory plasticity at the base of the mosquito sex determination cascade. BMC Evol. Biol. 15, 108.
Raymond, C.S., Murphy, M.W., O'Sullivan, M.G., Bardwell, V.J., Zarkower, D., 2000. *Dmrt1*, a gene related to worm and fly sexual regulators, is required for mammalian testis differentiation. Genes Dev. 14, 2587–2595.
Raymond, C.S., Shamu, C.E., Shen, M.M., Seifert, K.J., Hirsch, B., Hodgkin, J., Zarkower, D., 1998. Evidence for evolutionary conservation of sex-determining genes. Nature 391, 691–695.
Salvemini, M., Mauro, U., Lombardo, F., Milano, A., Zazzaro, V., Arcà, B., Polito, L.C., Saccone, G., 2011. Genomic organization and splicing evolution of the *doublesex* gene, a *Drosophila* regulator of sexual differentiation, in the dengue and yellow fever mosquito *Aedes aegypti*. BMC Evol. Biol. 11, 41.
Scali, C., Catteruccia, F., Li, Q., Crisanti, A., 2005. Identification of sex-specific transcripts of the *Anopheles gambiae doublesex* gene. J. Exp. Biol. 208, 3701–3709.
Schetelig, M.F., Handler, A.M., 2012. A transgenic embryonic sexing system for *Anastrepha suspensa* (Diptera: Tephritidae). Insect Biochem. Mol. Biol. 42, 790–795.
Shukla, J.N., Nagaraju, J., 2010. Two female-specific DSX proteins are encoded by the sex-specific transcripts of *dsx*, and are required for female sexual differentiation in two wild silkmoth species, *Antheraea assama* and *Antheraea mylitta* (Lepidoptera, Saturniidae). Insect Biochem. Mol. Biol. 40, 672–682.
Shukla, J.N., Palli, S.R., 2012. Doublesex target genes in the red flour beetle, *Tribolium castaneum*. Sci. Rep. 2, 948.
Smith, C.A., Roeszler, K.N., Ohnesorg, T., Cummins, D.M., Farlie, P.G., Doran, T.J., Sinclair, A.H., 2009. The avian Z-linked gene *DMRT1* is required for male sex determination in the chicken. Nature 461, 267–271.
Suzuki, M.G., Funaguma, S., Kanda, T., Tamura, T., Shimada, T., 2003. Analysis of the biological functions of a *doublesex* homologue in *Bombyx mori*. Dev. Genes Evol. 213, 345–354.
Suzuki, M.G., Funaguma, S., Kanda, T., Tamura, T., Shimada, T., 2005. Role of the male BmDSX protein in the sexual differentiation of *Bombyx mori*. Evol. Dev. 7, 58–68.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.
Tanaka, K., Barmina, O., Sanders, L.E., Arbeitman, M.N., Kopp, A., 2011. Evolution of sex-specific traits through changes in HOX-dependent *doublesex* expression. PLoS Biol. 9, e1001131.
Tan, A., Fu, G., Jin, L., Guo, Q., Li, Z., Niu, B., Meng, Z., Morrison, N.I., Alphey, L., Huang, Y., 2013. Transgene-based, female-specific lethality system for genetic sexing of the silkworm, *Bombyx mori*. Proc. Natl. Acad. Sci. U. S. A. 110, 6766–6770.
Tan, A.J., Tanaka, H., Tamura, T., Shiotsuki, T., 2005. Precocious metamorphosis in transgenic silkworms overexpressing juvenile hormone esterase. Proc. Natl. Acad. Sci. U. S. A. 102, 11751–11756.
Thomas, J.L., Da Rocha, M., Besse, A., Mauchamp, B., Chavancy, G., 2002. 3×P3-EGFP marker facilitates screening for transgenic silkworm *Bombyx mori* L. from the embryonic stage onwards. Insect Biochem. Mol. Biol. 32, 247–253.
Thomas, D.D., Donnelly, C.A., Wood, R.J., Alphey, L.S., 2000. Insect population control using a dominant, repressible, lethal genetic system. Science 287, 2474–2476.
White, K., Tahaoglu, E., Steller, H., 1996. Cell killing by the *Drosophila* gene *reaper*. Science 271, 5250–5253.
Williams, T.M., Carroll, S.B., 2009. Genetic and molecular insights into the development and evolution of sexual dimorphism. Nat. Rev. Genet. 10, 797–804.
Williams, T.M., Selegue, J.E., Werner, T., Gompel, N., Kopp, A., Carroll, S.B., 2008. The regulation and evolution of a genetic switch controlling sexually dimorphic traits in *Drosophila*. Cell 134, 610–623.
Wyss, J.H., 2000. Screwworm eradication in the Americas. Ann. N. Y. Acad. Sci. 916, 186–193.
Xu, J., Wang, Y., Li, Z., Ling, L., Zeng, B., James, A.A., Tan, A., Huang, Y., 2014. Transcription activator-like effector nuclease (TALEN)-mediated female-specific sterility in the silkworm, *Bombyx mori*. Insect Mol. Biol. 23, 800–807.
Yi, W., Ross, J.M., Zarkower, D., 2000. *Mab-3* is a direct *tra-1* target gene regulating diverse aspects of *C. elegans* male sexual development and behavior. Development 127, 4469–4480.
Zhang, J., Lu, A., Kong, L., Zhang, Q., Ling, E., 2014. Functional analysis of insect molting fluid proteins on the protection and regulation of ecdysis. J. Biol. Chem. 289, 35891–35906.