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ORIGINAL ARTICLE

Identification and characterization of *doublesex* from the pumpkin fruit fly, *Bactrocera tau* (Diptera: Tephritidae)

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Abstract. The sex determination cascades in insects are diversified at the top of the cascade, where different primary molecular signals are employed, while at the bottom of the cascades, particularly the *doublesex* genes, are highly conserved. Here, we identified the *doublesex* ortholog (*Btau-dsx*) of *Bactrocera tau*, a pumpkin fruit fly, and found that *Btau-dsx* is composed of six exons and five introns with an additional short "m" exon located in the second intron. *Btau-dsx* is different from its orthologs in most dipteran insects: Its pre-mRNA is sex-specifically spliced to yield three (two male and one female) instead of two transcript variants. The two deduced proteins produced by the male-specific transcripts are a functional (Btau-DSX^{M1}) and a truncated (Btau-DSX^{M2}) protein, while the female-specific transcript produces the functional Btau-DSX^{F} protein. These three proteins contain all conserved domains except Btau-DSX^{M2} which has no OD2 domain. The female-specific transcript is detected in both fertilized and unfertilized eggs and in both somatic and germ cells of the adult females, while the male-specific transcript is detected only in fertilized eggs and in the abdominal tissues and testes of adult males. The presence of the *Btau-dsx^{M1}* transcript in fertilized eggs at the early syncytium stage suggests that in XY embryos, the *Y-linked M factor* gene may function quite soon after fertilization to alter the splicing pattern of *Btau-dsx* pre-mRNA from the female-specific to the male-specific mode. Injection of *Btau-dsx^{F}* dsRNA into recently emerging females can reduce the expression of *vitellogenin* (*Btau-Vg*) and causes some defects in the ovaries, indicating that *Btau-dsx^{F}* works upstream of *Btau-Vg*.

INTRODUCTION

The most clearly known mechanism of sex determination among insects is obtained from studies of *Drosophila melanogaster*, whose sex determination cascade contains four key genes, *sex lethal* (*Sxl*), *transformer* (*tra*), *transformer2* (*tra2*) and *doublesex* (*dsx*). These genes function in a hierarchical order, from the top one to the bottom gene (Cline, 1993; Bopp et al., 2014) . The uppermost gene, *Sxl*, is ON or OFF depending on the number of X chromosome. In XX flies, X-linked signaling elements (XSEs) act as a primary signal to activate *Sxl* and induce this gene to produce SXL protein. In XY flies, the concentration of XSEs is insufficient to activate *Sxl* expression and therefore the SXL protein is not produced. When Sxl protein is present, the downstream gene *tra* produces the female-specific protein, TRA^{F}, which combines with TRA2, a non sex-specific protein, generating the TRA/TRA2 complex protein. This protein complex regulates *dsx* splicing in the female mode to produce DSX^{F}, which directs female embryonic development. In XY embryos that do not have the SXL protein, both *tra* and *dsx* genes produce male-specific proteins, TRA^{M} and DSX^{M}, which induce embryos to develop as male flies (Baker & Wolfner, 1988; Burtis & Baker, 1989; Hoshijima et al., 1991; Salz & Erickson, 2010).

Sex determination cascades of other insects do, however, seem to be different from that of *Drosophila*. In tephritid insects for example, the *Sxl* genes identified from *Ceratitis capitata* and *Bactrocera oleae* do not function in sex determination, although their *Sxl* sequences are highly similar to that of *Drosophila* (Saccone et al., 1998; Lagos et al.,

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2005). Instead, the maternal *tra* genes identified in several tephritid insects are suggested to be the primary signals promoting embryonic sex fate. In XX embryos, functional TRA protein (TRA^F) and TRA2 protein are both maternally provided and form a maternal TRA/TRA2 complex, ensuring via an autoregulation feedback loop that all zygotic *tra* pre-mRNA is spliced into the female-specific mode (Pane et al., 2002; Verhulst et al., 2010). tra^F mRNA produces the TRA^F protein that, in turn, regulates female-specific splicing of *dsx* in order to produce the female specific protein, DSX^F. In the presence of DSX^F, XX embryos develop as females. In XY embryos, the presence of M factors on Y chromosomes was suggested to turn off the *tra* autoregulatory feedback loop and, therefore, in the absence of TRA, *dsx* produces the male-specific protein, DSX^M, promoting male development of XY embryos (Hoshijima et al., 1991; Lagos et al., 2007; Concha & Scott, 2009; Salvemini et al., 2009; Sarno et al., 2010).

Besides dipteran insects, sex determination has been studied in seven insect orders (Price et al., 2015) including Lepidoptera (Ohbayashi et al., 2001; Shukla & Nagaraju, 2010), Hymenoptera (Cho et al., 2007; Oliveira et al., 2009) and Coleoptera (Shukla & Palli, 2012; Ito et al., 2013). The studies of sex determination in all insect species have thus far revealed that the primary signals initiating the sex determination cascade seem to vary, while genes, such as *doublesex*, functioning at the bottom of the cascades, are quite conserved. *Doublesex* (*dsx*), has been identified and characterized in a number of different insects. In most dipteran insects, *dsx* genes are transcribed in both sexes but their pre-mRNAs are spliced in a sex-specific manner, producing one male- and one female-specific transcript that is translated into a DSX^M protein in males and a DSX^F protein in females (Baker & Wolfner, 1988; Burtis & Baker, 1989; Hediger et al., 2004; Lagos et al., 2005; Chen et al., 2008). DSX proteins are members of the doublesex/mab-3 related (Dmrt) family. Like other proteins belonging to the Dmrt family, DSX proteins have two conserved functional domains, an N-terminal DNA binding domain (DM or OD1 domain) and a C-terminal dimerization domain (DSX dimer or OD2 domain). OD1 is a common domain found in both DSX^M and DSX^F, while the C-terminal regions of the OD2 domain are different in the two proteins and direct an embryo containing DSX^M towards the male sex fate or an embryo containing DSX^F towards the female sex fate.

dsx genes have been identified and characterized in many tephritid insects such as *Bactrocera tryoni* (Shearman & Frommer, 1998), *Anastrepha oblique* (Ruiz et al., 2005), *B. oleae* (Lagos et al., 2005), *Ceratitis capitata* (Saccone et al., 2008), *B. dorsalis* (Chen et al., 2008; Permpoon et al., 2011), *B. jarvisi* (Morrow et al., 2014), and *B. correcta* (Permpoon et al., 2011). The structures of *Bactrocera* and *Ceratitis dsx* genes are similar since they are composed of six exons and five introns, while *Anastrepha dsx* genes are instead composed of four exons and three introns. However, in all cases *dsx* genes are alternatively spliced in a sex-specific manner, producing one male (DSX^M) and one female-specific (DSX^F) protein.

The pumpkin fruit fly, *Bactrocera tau* (Walker), is an important pest widely distributed throughout South East Asian countries including Thailand (Chen, 2001). The sex determination genes have been suggested as potential targets for creating genetic sexing strains or for RNAi technology used in the pest management programmes (Dafa'alla et al., 2010; Whyard et al., 2015; Leftwich el al., 2016). In this study, we identify and characterize the *dsx* homologue of *B. tau* and find that the structure of *Btau-dsx* is the same as that of other *Bactrocera dsx* genes except for the existence of a short "m" exon located in the second intron. Interestingly, we detect the male-specific $Btau\text{-}dsx^{M1}$ mRNA in fertilized embryos just after egg-laying. We also examine the function of *Btau-dsx* in regulating the downstream target gene, *vitellogenin*, by using adult abdominal double-strand RNA injection. We hope, therefore, that in the future the *Btau-dsx* gene may be utilized as a means to control the dispersion of this insect species.

MATERIAL AND METHODS

Insect culturing

Pumpkin fruit flies, *Bactrocera tau*, were kindly provided by Prof. Dr. Sangvorn Kitthawee, Department of Biology, Faculty of Science, Mahidol University. Flies were cultured on artificial food, and kept at 25°C, 70% relative humidity, and with a photoperiod of 12L : 12D. Cucumbers were used for egg collection.

Cloning and sequencing of *Btau-dsx*

Total RNA was individually extracted from the 15-day-old adult females and males separately using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). One unit of DNase I per mg of RNA was added to the total RNA obtained. One microgram of total RNA was used for synthesis of the first cDNA strand of *Btau-dsx* using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) and following the manufacture's protocol. Primers were designed based on *Bactrocera dsx* sequences available in the NCBI database. The first cDNA strand was used as a template to amplify the *Btau-dsx* gene with primers BtdsxF and BtdsxR (Table 1) under the following conditions: preheating at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min: with a final extension at 72°C for 10 min. The PCR product was cloned in $pGEM^T$ Easy plasmid vector (Promega, Madison, WI, USA) and sent to Macrogen, Seoul, Korea, for sequencing. The BLAST program (NCBI) was used to check that the sequence obtained from Marcrogen was that of the *dsx* gene.

To obtain the full-length *Btau-dsx* cDNA sequence, both 5' and 3' Rapid Amplifications of cDNA Ends (RACE) were conducted using the smart cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to manufacturer's instructions and the gene-specific primers, BtdsxR5'RACE for *dsx* 5' RACE and BtdsxF3'RACE for *dsx* 3' RACE (Table 1). The nested PCR for the *dsx* 5' RACE was performed using the same kit and a specific primer, BtdsxnestR5'RACE (Table 1). The 5' RACE PCR and 3' RACE PCR fragments were cloned and sequenced at Macrogen. All obtained PCR sequences were assembled to get the full length *Btau-dsx* cDNA sequence using the CAP3 Sequence Assembly Program (http://pbil.univ-lyon1.fr/cap3.php).

Identification of the *Btau-dsx* genomic structure

The genomic DNA sequence of *B. cucurbitae* (NW_011863834.1) available in the NCBI database was used

Table 1. Primers used in this study.

Primer name	Sequence primer 3'–5'
BtdsxF	GGC-CAC-AAA-CGC-TAT-TGT-AAA-TAC-CCG
BtdsxR	AAT-GGC-ATC-ATC-TCC-CAT-GGA-TAT-CG
BcdsxR5′RACE	GGG-CAA-TAC-CGC-TCC-CGA-AGT-GGA
BcdsxF3′RACE	GCA-GAC-AGC-ATT-GAG-GCG-GGC-ACA-G
UMP	CTA-ATA-CGA-CTC-ACT-ATA-GGG-CAA-GCA-GTG-GTA-TCA-ACG-CAG-AGT
BtdsxnestR5′RACE	GCG-GGG-GAG-GAG-ATA-GAG-CTG-CCA
UMP short	CTA-ATA-CGA-CTC-ACT-ATA-GGG-C
dsx-dsRNAi2-F	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-TAA-GTG-TGA-ATA-CCA-GCA-TTT-CTG
dsx-dsRNAi2-R	TAA-TAC-GAC-TCA-CTA-TAG-GGA-GAC-CAC-CCT-AAA-ATA-TTT-TTT-ATT-ATT-GTA
BttradsRNA-F	GTA-ATA-CGA-CTC-ACT-ATA-GGG-GGA-GAG-TGA-AGT-TGG-ACC-CA
dsDsRed_F_T7	CCG-GAT-CCT-AAT-ACG-ACT-CAC-TAT-AGG-GCG-GTG-CTT-GTC-AAT-GCG-GTA-AGT-G
dsDsRed_R_T7	CCG-GAT-CCT-AAT-ACG-ACT-CAC-TAT-AGG-GCG-CTC-GAG-ATC-TGA-CAA-TGT-TCA-G
BtdsxFF	TTG-CCC-ATA-TCG-GTA-GTT-GGC
R-dsxF	TCA-TCC-GCA-TTG-CCT-TGT-CGT-AC
R-dsxM	CGG-CTG-CTG-GCG-GTG-ACA-TC
BTdsxF-qRT	TGG-TTT-CGG-AGG-ATA-GTT-G
BTdsxR-qRT	GTA-TCT-GCA-GAA-CCC-TTT-GT
BTvgF-qRT	AGC-GTT-TAG-CTC-TCA-TCG
BTvgR-qRT	GAT-GGC-GTG-TAC-CCA-TTC
BTactF-qRT	GAG-GAG-CAC-CCA-GTT-CTG-C
BTactR-qRT	GTA-GCC-GCG-CTC-AGT-CAG
BTactF	GAG-GAG-CAC-CCA-GTT-CTG-C
BTactR	GTA-GCC-GCG-CTC-AGT-CA

as a template for alignment with all *Btau-dsx* full-length cDNA sequences using ClustalW2 version 2.0.10 to get the number of exons and introns.

Multiple alignment and phylogenetic analysis

Full length *Btau-dsx* cDNA sequences were translated into the deduced protein sequences using the Expasy-Translation tool (web.expasy.org/translate/) and were then aligned with a selection of insect Dsx protein sequences (Table S1) available in the NCBI database using clustalW2 version 2.0.10 to highlight sequence identities. Phylogenetic trees were constructed using the neighbour-joining method in MEGA version 7.0.21 (Kumar et al., 2016). The reliability of clusters within the tree was evaluated based upon 1000 bootstrap replications.

Expression analysis using RT-PCR

Total RNA was extracted from both fertilized and unfertilized eggs of *B. tau* at several development stages (0.5–24 h), from whole bodies of adult males and females 2–10 days old, and from some tissues (heads, thoraxes, abdomens, ovaries and testes) of adult males and females at 15, 20 and 25 days post-eclosion, using GenUP™ Total RNA Kit (Biotechrabbit, Hennigsdorf, Germany) following the manufacture's instruction. Three micrograms of total RNA was reverse-transcribed (Reverse transcription System Kit; Promega). Multiplex RT-PCRs were performed using these cDNAs as templates and three primers, BtdsxFF, R-dsxF and R-dsxM (Table 1), for amplifying dsx^F and dsx^M transcripts. The *β-actin* gene was used as a reference gene and amplified with primers β-actinF and β-actinR (Table 1). Amplification was carried out in a total volume of 50 µl, including 1 µg of cDNA, 2 µl of each primer (10 mM), 1 µl of dNTP (10 mM), 0.5 µl of *Taq* DNA polymerase, and 33.5 µl of distilled water. The PCR reaction was performed at 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final step of 5 min at 72°C. The PCR products were analyzed on 1% agarose gels.

Preparation of double-stranded RNAs and microinjection procedures

The region common to all *dsx* genes containing the OD1 domain sequence (752 bp long) was amplified with primers dsx-dsRNAi2-F and dsx-dsRNAi2-R (Table 1) and cloned into a pGEM^{T}Easy plasmid vector. This clone was used as a template for preparing *Btau-dsx* dsRNAs using a MEGA script T7 kit (Ambion, Foster City, CA, USA), according to the manufacturer's instructions. As a control, 562 bp of the *DsRed* gene was amplified using primers dsDsRedF and dsDsRedR with the amplification product being used as a template for synthesis the *DsRed* dsRNA using the same protocol (Table 1). One microgram per microliter of either *Btau-dsx* dsRNA or *DsRed* dsRNA was injected into the third abdominal segment of each adult female within 1 h of eclosion using a 10 µl Hamilton microsyringe. Two females were collected on each of the 2nd, 4th, 6th, 8th and 10th day post-injection and kept at –80°C for analyzing the expression of *Btau-dsx* and *Btau-Vitellogenin* genes. Some remaining females were dissected on the 10th day post-injection, their ovaries were observed under an Olympus stereomicroscope and photographs were taken with a DP72 Olympus digital camera.

Real-time PCR experiment

Total RNA was separately extracted from whole bodies of all collected *Btau-dsx* or *DsRed* dsRNAs injected *B. tau* females using TRIzol reagent and following the manufacturer's protocol. Three micrograms of total RNA were reverse-transcribed to cDNAs using a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The expression of *Btau-dsx* and *Btau-Vg* genes were examined by qPCR experiments using the Mastercycler® ep realplex (Eppendorf Co., Ltd, Hamburg, Germany). qPCR reactions were performed using RBC ThermOne™ Real-Time Premix (with SYBR Green). The qPCR program for amplifying the cDNAs was one cycle of 94°C for 3 min and 40 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s, followed by melting curve analysis to detect a specific amplification product. The primers used for Real-time PCR were BTdsxF-qRT, BTdsxR-qRT (Table 1) for assaying the transcription level of *Btau-dsx*^{F}, BTvgF-qRT, BTvgF-qRT and BTvgR-qRT (Table 1) for detecting the *Btau-vitellogenin* (*Btau-Vg*) gene and BTactF-qRT and BTactR-qRT (Table 1) for detecting the expression of the *β-actin* gene. Each sample was analyzed in triplicate and normalized to the *β-actin* expression of *B. tau* as an internal control. Real-time data were calculated using the relative expression level by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Primers for amplifying the *Btau-Vg* gene were designed using the sequences from a selection of insect *vitellogenin* genes avail-

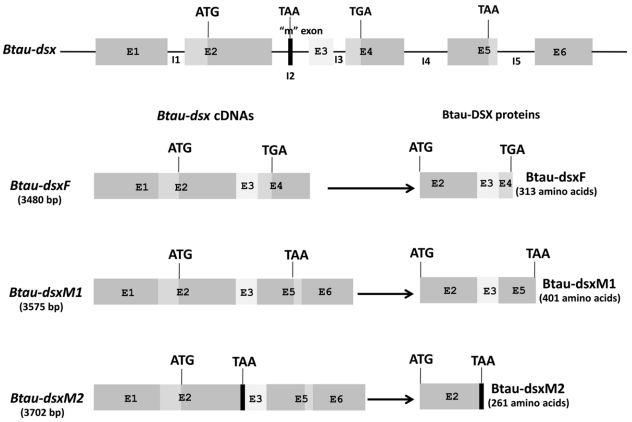

Fig. 1. Schematic representation of the genomic organization of the *Btau-dsx* gene, and *Btau-dsx* cDNAs and Btau-DSX proteins. Gray boxes (E1–E6) and straight lines (I1–I5) indicate exons and introns. The narrow black box indicates the 127 bp sequence inserted in the second intron (I2).

able on the NCBI database. RT-PCR was performed using the *B.tau* female cDNA as a template and amplified with the designed primers. The obtained PCR fragment was cloned into a pGEM^{T} easy vector and sent to Macrogen for sequencing. The obtained nucleotide sequence was blasted using the BLAST program (NCBI) to confirm that it was the sequence of the *vitellogenin* gene.

RESULTS

Identification of the *Btau-dsx* gene structure and its transcript variants

The structure of the *Btau-dsx* gene was identified by aligning the cDNA sequences of all *Btau-dsx* transcripts with the genomic sequence of the *Bactrocera cucurbitae dsx* gene (NW_011863834.1). The result showed that *Btau-dsx* consisted of six exons and five introns, the same genomic structure as that of *D. melanogaster* and of other *Bactrocera* insects reported earlier (Fig. 1). The *Btau-dsx* gene was transcribed in both sexes but its pre-mRNA was spliced in sex-specific manners, resulting in three transcripts: one female (*Btau-dsx^{F}*) and two male (*Btau-dsx^{M1}* and *Btau-dsx^{M2}*). The female transcript consisted of four exons 1, 2, 3 and 4, while the male transcripts harbored five exons, 1, 2, 3, 5 and 6 (Fig. 1). However, only the *Btau-dsxM2* transcript contained an additional small exon (127 bp long, called the "m" exon) located within the second intron. Alignment of these two male-specific transcripts with the genomic sequence of the *B. cucurbitae dsx* gene revealed that this gene contained the "m" exon in the second intron. The full length cDNA sequence of *Btau-dsx^{F}* (accession number: KP844899) was 3477 bp long and contained a 969 bp ORF encoding a deduced protein of 322 amino acid residues. While, the complete cDNA sequences of *Btau-dsx^{M1}* (accession number: KP844901) and *Btau-dsx^{M2}* (accession number: KP844900) were 3575 and 3436 bp long, respectively. In the *Btau-dsx^{M2}* transcript, the 127 bp "m" exon was located between exons 2 and 3, and contained a stop codon (TAA). Thus, the 786 bp long ORF of *Btau-dsx^{M2}* was quite short in comparison to *Btau-dsx^{M1}* (1206 bp) and encoded a short protein of 261 amino acid

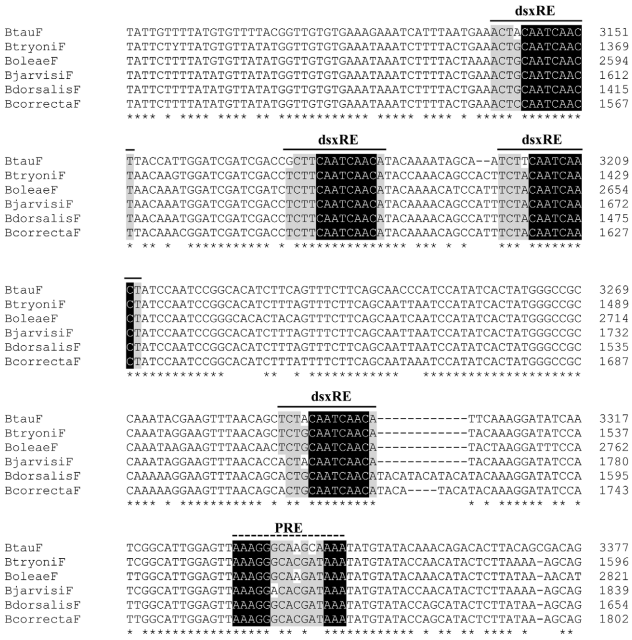

Fig. 2. Comparison of the 3' UTR of *Bactrocera dsx* female-specific transcripts show five conserved domains, four dsxRE elements (13 nucleotides) indicated by black lines above the domains and the purine-rich element (PRE) indicated by a dashed line above the corresponding domain, while white letters in black boxes show the identical eight nucleotide (CAATCAAC) sequences present in all *Bactrocera* dsxRE elements.

residues, while *Btau-dsx^{M1}* produced a deduced 401 amino acid functional protein.

The 3' UTR of the *Btau-dsx^{F}* transcript contained five conserved domains, four putative 13 nt *dsx* repeat elements (dsxRE) and a purine rich element (PRE), followed by a poly (A) signal. Comparison of the *Btau-dsx^{F}* mRNA sequence and other *Bactrocera dsx^{F}* mRNA sequences available in the NCBI database indicated that the *dsx^{F}* mRNA of *Bactrocera* species commonly contained four dsxRE elements, with an identical eight nucleotide sequence (CAATCAAC) present in all *Bacterocera* dsxRE elements (Fig. 2). Moreover, the PRE element of *Btau-dsx^{F}* was composed of 15 nucleotides and 13 of the 15 nucleotides were purines, while other *Bactrocera* PRE elements contained 12 purine nucleotides (Fig. 2).

Amino acid sequence alignment and phylogenetics

The amino acid sequences of these three Btau-DSX proteins and those of some other insect DSX proteins available in the NCBI database were aligned using the clustalw2 program. The identity of Btau-DSX^{F} to the two male-specific DSX proteins, Btau-DSX^{M1} and Btau-DSX^{M2}, was 93.48 and 95.40%, respectively, while the identity between Btau-DSX^{M1} and Btau-DSX^{M2} was 95.40%. The amino acid sequences of the three Btau-DSX proteins (at amino acid position 1–246) were identical at the N terminal (containing the DNA binding domains, OD1, at position 38–100) until the beginning of the oligomerization (OD2) domains. The three Btau-DSX proteins were different at their C terminals, where the OD2 domains and sex-specific regions were located. However, the common region of the OD2 domains

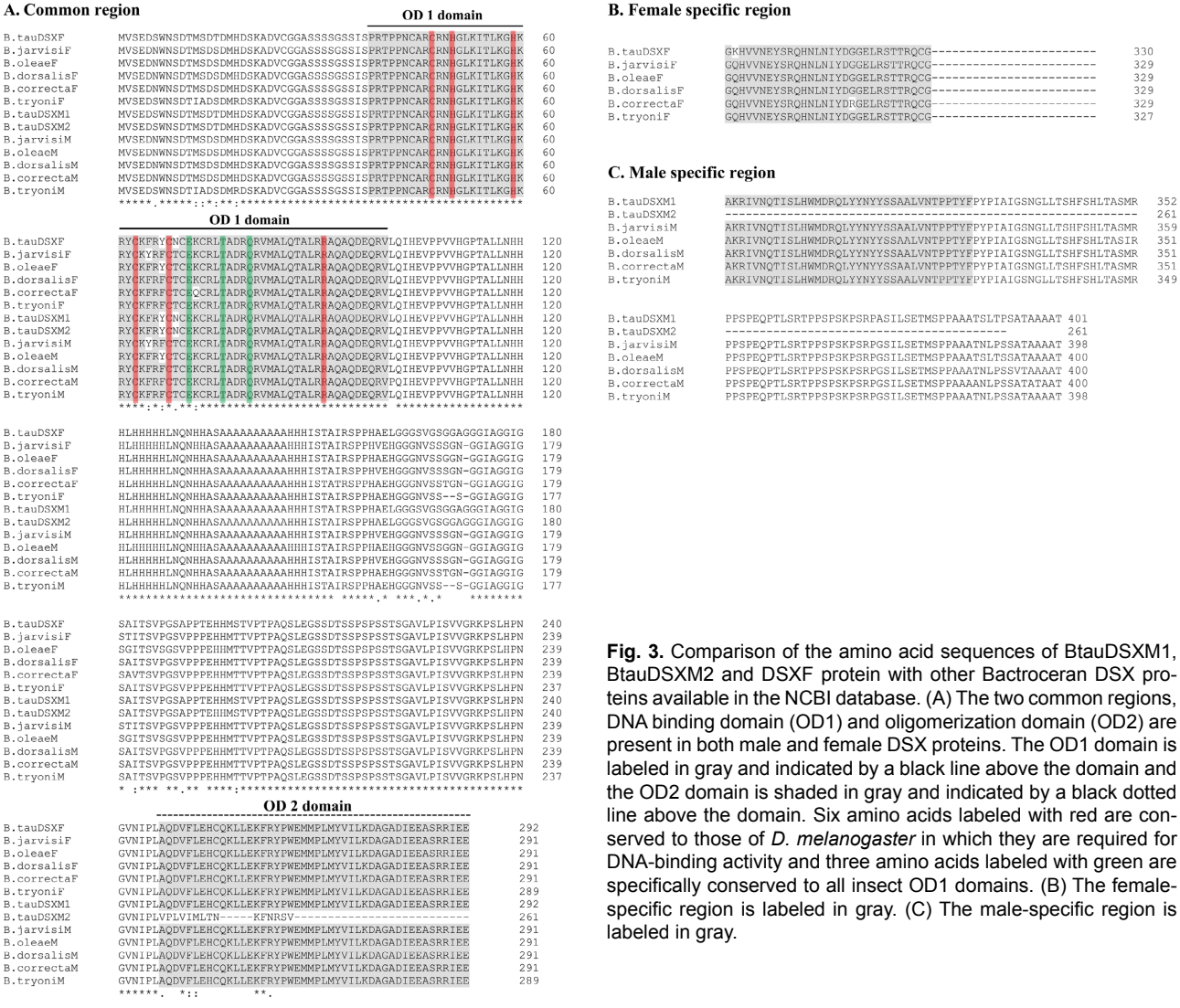

Fig. 3. Comparison of the amino acid sequences of BtauDSXM1, BtauDSXM2 and DSXF protein with other Bactroceran DSX proteins available in the NCBI database. (A) The two common regions, DNA binding domain (OD1) and oligomerization domain (OD2) are present in both male and female DSX proteins. The OD1 domain is labeled in gray and indicated by a black line above the domain and the OD2 domain is shaded in gray and indicated by a black dotted line above the domain. Six amino acids labeled with red are conserved to those of *D. melanogaster* in which they are required for DNA-binding activity and three amino acids labeled with green are specifically conserved to all insect OD1 domains. (B) The female-specific region is labeled in gray. (C) The male-specific region is labeled in gray.

(46 amino acids at position 247–292) of Btau-DSX^{F} and Btau-DSX^{M1}were also identical, while Btau-DSX^{M2} lacked the OD2 domain including the sex-specific region, indicating that it was a truncated protein. Alignment of these three Btau-DSXs with all other tephritid DSX proteins available in the NCBI database showed a percentage identity ranging from 85.17 to 95.95%, while the identities to some DSX proteins of other insect orders were between 34.21 and 57.99%. Comparison of all *Bactrocera* DSX proteins available in the NCBI database, including the three Btau-DSX proteins, showed that the amino acid sequences at the N-terminal regions were highly conserved. Furthermore, at the C-terminal regions, if Btau-DSX^{M2} was excluded, the amino acid sequences of the OD2 domains including the male-specific regions were completely identical, while the sequences of female-specific regions were also highly conserved (Fig. 3).

Moreover, the OD1 and the OD2 domains of *B. tau* DSX proteins (Btau-DSX^{F} and Btau-DSX^{M1}) were aligned with those of another 16 insect species available in the NCBI database. The alignment showed that at OD1 domains, 20 of 63 amino acid residues were conserved. The 20 conserved amino acids included six essential amino acid residues (C, H, H, C, C and R) necessary for DNA-binding activity in *D. melanogaster* (Erdman & Burtis, 1993) and three amino acid residues (E, T, Q) specific to the insect OD1 domains (Oliveira et al., 2009) (Fig. 4). In contrast, OD2 domains showed high variability, with only two positions out of 47 amino acid residues conserved (Fig. 4).

A phylogenetic tree was constructed using the deduced amino acid sequences of Btau-DSXs (Btau-DSX^{F}, Btau-DSX^{M1} and Btau-DSX^{M2}) and the DSX proteins of other insects available in the NCBI database, by the neighbor-joining method replicated 1000 times with bootstrap resampling. The tree topology showed that sequences of the DSX proteins could be used to separate insects at the order and genus levels. Tephritid insects were grouped in the same clade that was, using a classical taxonomy, separated into three subclades, namely *Bactrocera*, *Anastrepha* and *Ceratitis* (Fig. 5).

Analysis of *Btau-dsx* gene expression patterns using RT-PCR

Expression levels of the three sex-specific *Btau-dsx* transcripts at embryonic stages were assessed, this included both male and female embryos, since they could not be

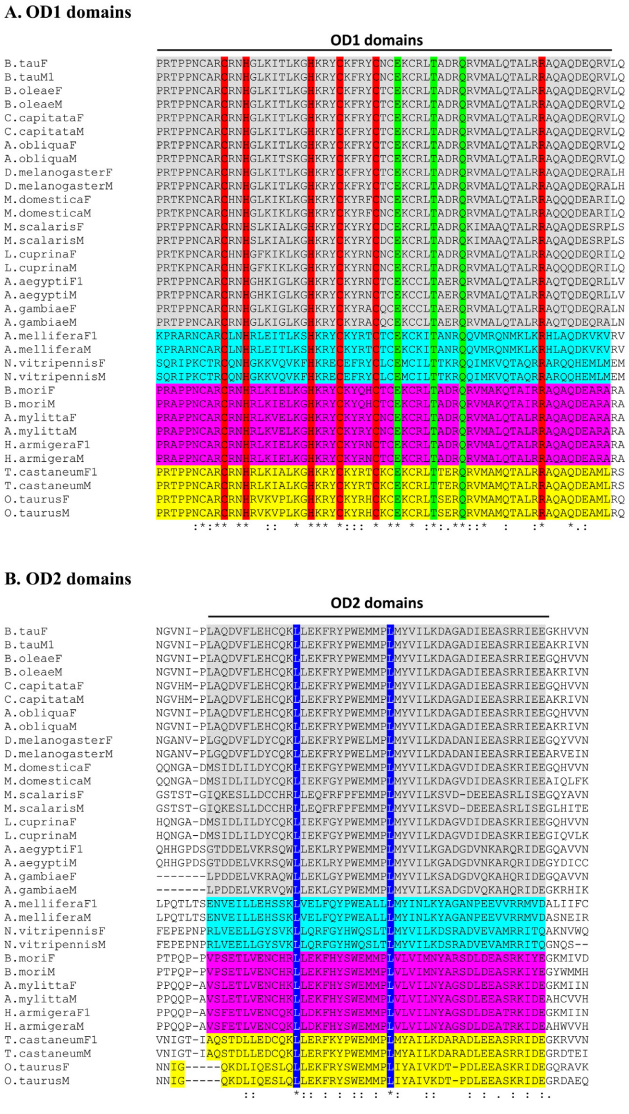

Fig. 4. Alignment of the OD1 and the OD2 domains of Btau-DSXs and some insect DSX proteins available in the NCBI database. (A) The OD1 domains. Amino acid residues labelled with red are essential for DNA-binding activity in *D. melanogaster* and the three amino acids labelled with green are specific to the insect OD1 domains. (B) The OD2 domains. Amino acid residues labelled with blue are conserved residues found in all insect DSX proteins present here.

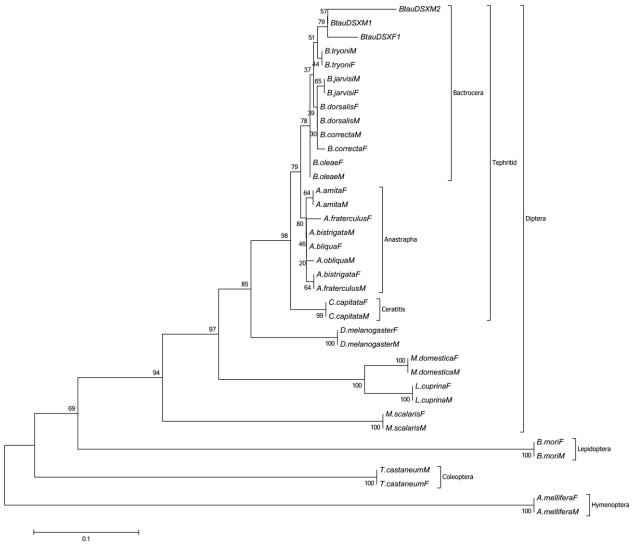

Fig. 5. Phylogenetic tree constructed using amino acid sequences of DSX proteins of a selection of insects available in the NCBI database using the neighbor-joining method in MEGA 7.0.21.

separated from each other. The results showed that fertilized eggs after 0.5–24 h contained both *Btau-dsx*^{F} (295 bp band) and *Btau-dsx*^{M1} (487 bp band) transcripts but the band of *Btau-dsx*^{F} was stronger than that of *Btau-dsx*^{M1} at all stages detected (Fig. 6A). In unfertilized eggs at 0.5, 3, 6 and 12 h after oviposition, only the *Btau-dsx*^{F} transcript was detected, but this transcript disappeared at 24 h (Fig. 6B). In adult stages, the *Btau-dsx*^{F} transcript was detected in adult females on each examination day (2, 4, 6, 8 and 10 days post-eclosion). In adult males, the *Btau-dsx*^{M1} transcript was detected in all examination days (2, 4, 6, 8 and 10 days post-eclosion), while the *Btau-dsx*^{M2} transcript was detected only in adult males at 8 and 10 days post-eclosion (Fig. 6C). We found that *Btau-dsx* expression in some adult tissues of both sexes 15, 20 and 25 days after eclosion gradually increased and that the *Btau-dsx*^{F} transcript was detected in all tissues examined (heads, thoraxes, abdomens and ovaries). Expression levels were similar in tissues from flies examined on the same day. In male tissues (heads, thoraxes, abdomens and testes), the *Btau-dsx*^{M1} transcript was strongly detected only in abdomens and testes, while the *Btau-dsx*^{M2} transcript was detected at comparatively very low levels (Fig. 6D).

Functional analysis of *Btau-dsx*^{F} using RNA interference

The *Btau-dsx*^{F} transcript was only present in female flies. In this study, we reduced the quantity of *Btau-dsx*^{F} transcripts in female flies by injecting early emerged females with *Btau-dsx* dsRNA. Ten days after injection, females were dissected and their ovaries were examined. The results showed that the ovaries of some injected females were mildly deformed: for example, eggs were shorter and their number was reduced, while the ovaries of females injected with *DsRed* dsRNA were relatively normal and were similar to those of uninjected females (Fig. 7). Moreover, the efficacy of *Btau-dsx* dsRNA in the reducing the levels of endogenous *Btau-dsx*^{F} mRNA was examined by quantitative real-time PCR (qPCR). Samples of females injected with *Btau-dsx* dsRNA were examined at 2, 4, 6, 8 and 10 days post-eclosion; the result showed that the levels of endogenous *Btau-dsx*^{F} mRNA were significantly lower than those of females injected with *DsRed* dsRNA ($P < 0.01$, Student *t*-test) (Fig. 8) except at 2 and 10 day post-injection. Furthermore, we also examined the effect of *Btau-dsx* dsRNA on the expression of the *Btau-vitellogenin* (*Btau-Vg*) gene. In oviparous animals including insects, the *Vg* gene is expressed in females to produce a vitellogenin protein that is accumulated in the yolk of the eggs and is an essential nutrient for embryos during their development. We found that the level of *Btau-Vg* mRNA in females injected with *Btau-dsx* dsRNA was dramatically reduced compared to that of females injected with *DsRed* dsRNA ($P < 0.01$,

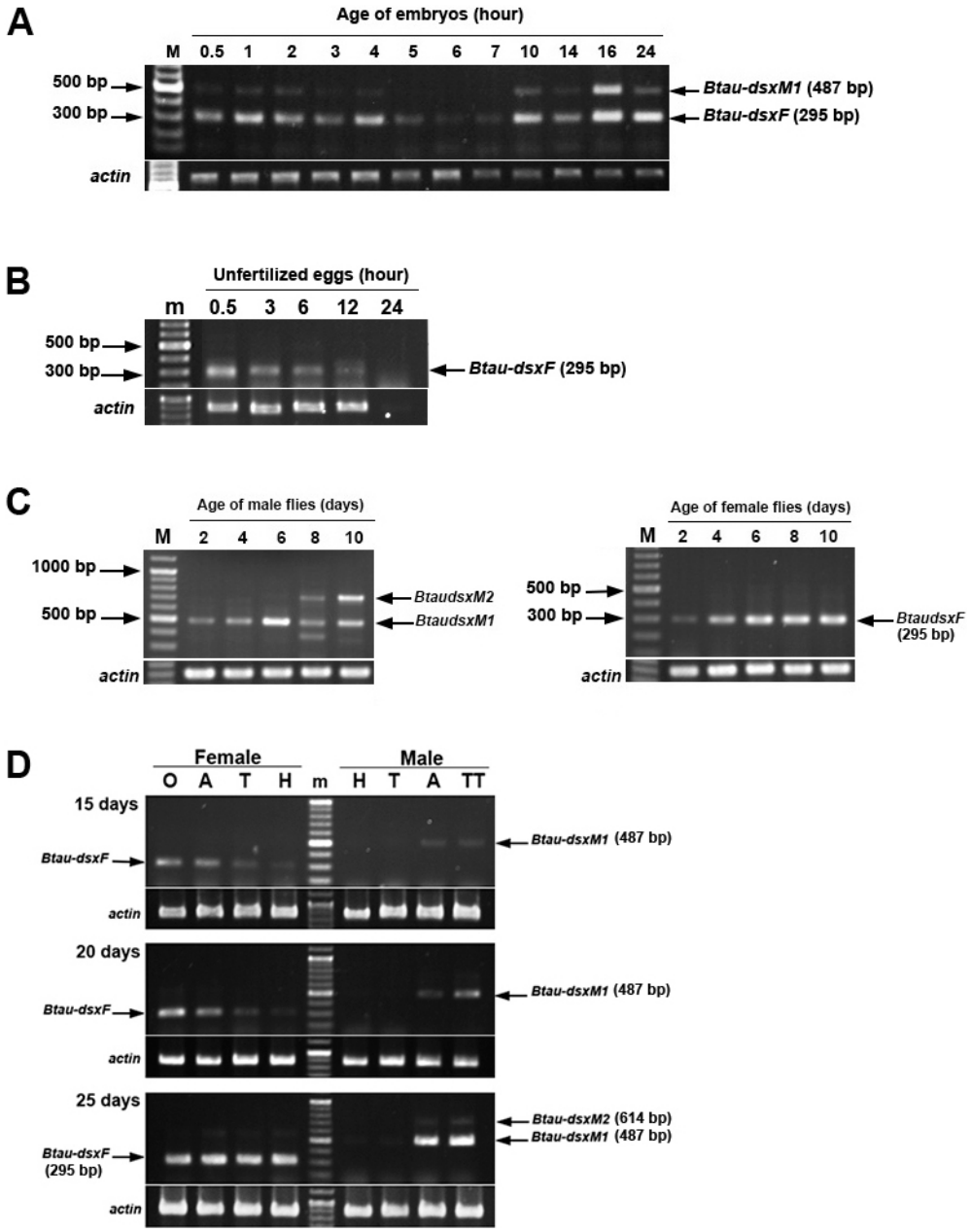

Fig. 6. The expression profiles of the *Btau-dsx* gene at several stages using RT-PCR. (A) 0.5–24 h fertilized eggs. (B) 0.5–24 h unfertilized eggs. (C) Adult males and females 2–10 days old. (D) Adult female and male tissues. H – head, T – thorax, A – abdomen, O – ovary, TT – testis.

Student *t*-test), indicating that *Btau-dsx* functions as an upstream gene regulating the expression of *Btau-Vg* (Fig. 9).

DISCUSSION

In this study, we identified the *doublesex* (*Btau-dsx*) gene from the pumpkin fruit fly, *Bactrocera tau* (Walker), an invasive insect pest distributed worldwide. The structure and function of *Btau-dsx* was similar to most dipteran *dsx* genes, however *Btau-dsx* showed some different aspects from most dipteran and other *Bactrocera dsx* genes such as the number of transcript variants, the presence of the "m" exon, and the presence of the male-specific transcript at the early syncytial stages of fertilized eggs.

The structure and transcript variants of the *B. tau doublesex* gene

The structure of the *Btau-dsx* gene was found to be similar to that of *D. melanogaster*, *M. domestica* and *Bactrocera* insects. *Btau-dsx* consisted of six exons and five introns and was alternatively spliced in a sex-specific manner. The first three exons were common to both male and female transcript variants, while exon 4 was a female-specific exon, and exon 5 and 6 were male-specific (Burtis & Baker, 1989; Lagos et al., 2005; Saccone et al., 2008). Similar to the *dsx* genes of *M. domestica* and *L. cuprina*, *Btau-dsx* also contained the 127 bp "m" exon located in the second intron between exon 2 and 3, and after splicing, the "m" exon remained only in one of the two male-specific transcripts, *Btau-dsx*^{M2}. The "m" exons of *M. domestica*

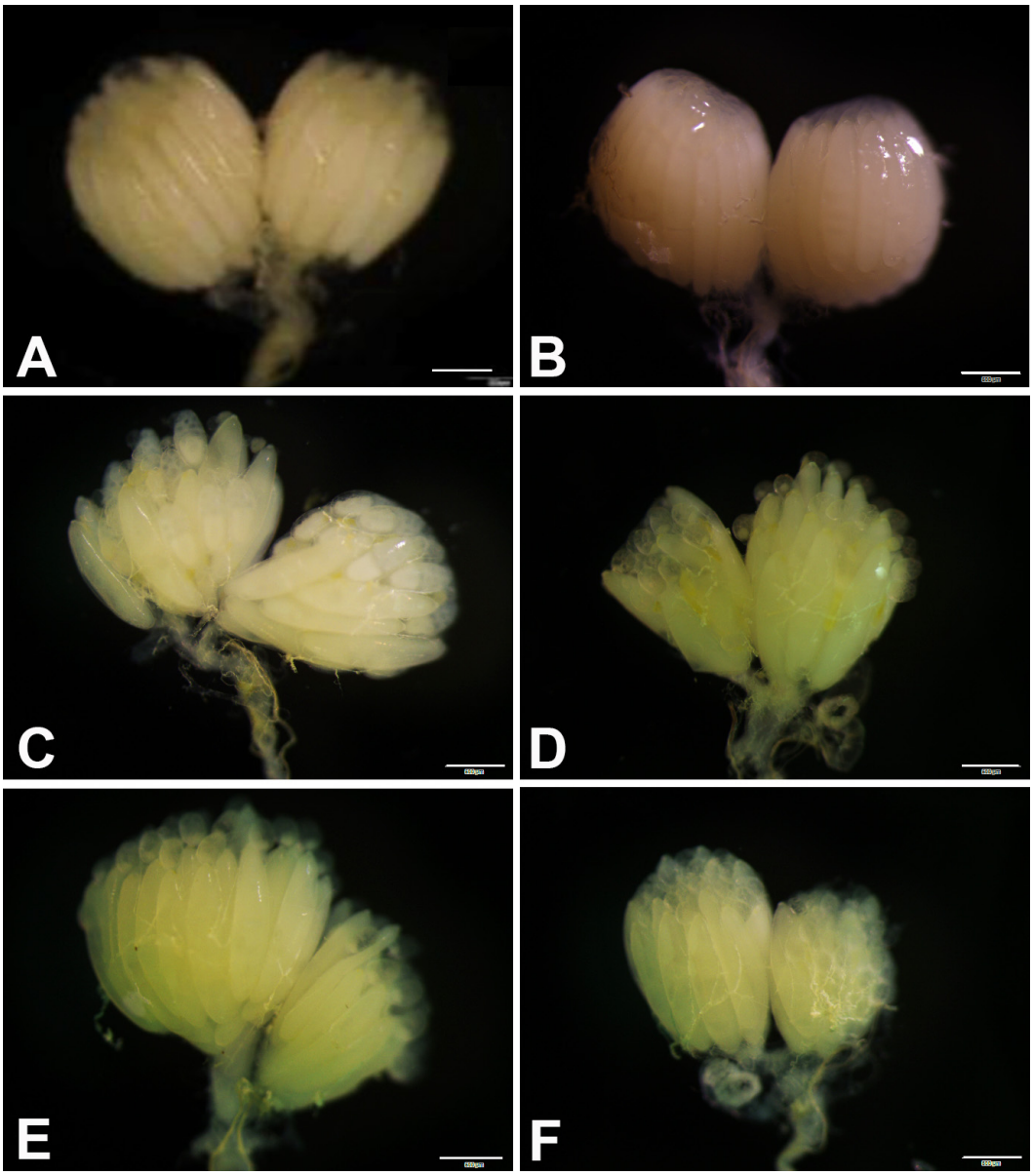

Fig. 7. Effect of *Btau-dsx* dsRNA on ovaries. (A) Ovary of an uninjected female. (B) Ovary of a female injected with *DsRed* dsRNA. (C–F) Ovaries of females injected with *Btau-dsx* dsRNA. Bars = 500 µm.

and *L. cuprina dsx* genes were found to be 142 and 141 bp long, respectively, and located in the intron between exon 3 and 4 (Hediger et al., 2004; Concha & Scott, 2010). In *M. domestica*, after processing the *dsx* pre-mRNA, the "m" exon was present only in the male-specific and minor common transcript (Hediger et al., 2004). In fact, in *Bactrocera* insects, the "m" exon was also present in the second intron of the *dsx* genomic region of *Bactrocera cucurbitae*, but was not found in that of *Bactrocera oleae*.

In contrast to most dipteran *dsx* genes, *Btau-dsx* was transcribed into three (one female and two male) instead of two (one male and one female) transcript variants (An et al., 1996; Shearman & Frommer, 1998; Hediger et al., 2004; Lagos et al., 2005; Chen et al., 2008; Saccone et al., 2008; Permpoon et al., 2011; Morrow et al., 2014). The splicing of the *dsx* pre-mRNAs to produce more than two transcript variants had been reported in many insect species, however, almost all additional *dsx* transcripts are female-specific transcripts, such as two in *Aedes aegypti* (Salvemini et al., 2011), *Apis mellifera* (Cho et al., 2007), *Bombyx mori* (Ohbayashi et al., 2001), *Antheraea mylitta* (Shukla & Nagaraju, 2010), three in *Tribolium castaneum* (Shukla & Palli, 2012), and six in *A. assama* (Shukla & Nagaraju, 2010). Two examples of a *dsx* gene producing more than one male-specific transcript have been reported in two insect species, a Japanese rhinoceros beetle, *Trypoxylus dichotomus*, 6 (F) female-specific transcripts, and 2 (M) male-specific transcripts (Ito et al., 2013), and a fungus fly, *Sciara ocellaris* (2F and 2M) (Ruiz et al., 2015). Therefore, splicing of *Btau-dsx* pre-mRNA to produce two male-specific transcripts may be the first multiple male-specific *dsx* pre-mRNA identified in tephritid insects.

In *D. melanogaster*, the 3' UTR of the *dsx* female-specific exon (exon 4) harbored six 13 nt repeat elements (dsxRE)

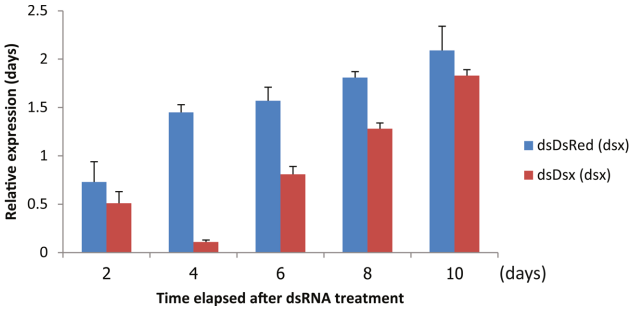

Fig. 8. Relative *doublesex* expression in females injected with either *Btau-dsx* dsRNA or *DsRed* dsRNA on 2, 4, 6, 8 and 10 days post-injection. Bars represent the mean ± SD obtained from qPCR experiment.

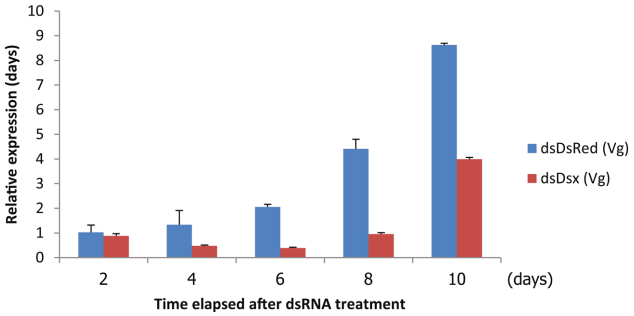

Fig. 9. Relative *vitellogenin* (*Btau-Vg*) expression in females injected with either *Btau-dsx* dsRNA or *DsRed* dsRNA on 2, 4, 6, 8 and 10 days post-injection. Bars represent the mean ± SD obtained from qPCR experiment.

and a purine-rich element (PRE) that were the binding sites for the Tra/Tra2 protein complex. This protein complex is necessary for activation of the female-specific mode processing of *dsx* pre-mRNA (Hoshijima et al., 1991; Inoue et al., 1992). Currently, these conserved domains have been identified in many dipteran *dsx* female-specific transcripts including *Btau-dsx*^{F}. The *Btau-dsx*^{F} transcript harbors four 13 nt repeat elements (dsxRE) and a purine-rich element (PRE) at its 3' UTR same as did the 3' UTR of *M. domestica* and other *Bactrocera* insects. This suggests that the *Btau-dsx* female-specific splicing may also be regulated by the combined protein products of the two upstream genes, *Btau-tra* and *Btau-tra2*, which bind to the dsxREs and the PRE. Moreover, the copy number of the dsxRE domain seems to vary to as much as eight in *Lucilia capurina* (Concha et al., 2010); six in *D. melanogaster* (Inoue et al., 1992), *Megaselia scalaris* (Kuhn et al., 2000) and *Anopheles gambiae* (Scali et al., 2005); five in *Aedes aegypti* (Salvemini et al., 2011); four in *M. domestica* (Hediger et al., 2004), *C. ceratitis* (Saccone et al., 2008) and *Bactrocera* species (Shearman & Frommer, 1998; Chen et al., 2008; Permpoon et al., 2011), and three in *Anastrepha obligua* (Ruiz et al., 2005). However, all *Bactrocera dsx* female-specific transcripts including *Btau-dsx*^{F} contain four dsxRE copies and one PRE domain. Therefore, the presence of dsxRE and PRE domains found in the *Btau-dsx*^{F} transcript also strongly suggests that the TRA/TRA2 protein complex is required for *dsx* female-specific splicing in *B. tau* as with most dipteran insects.

Expression of *Btau-dsx*

In *D. melanogaster*, the *dsx* gene is transcribed in both sexes but its pre-mRNA was spliced in sex specific manners, producing one *dsx* male-specific mode in males and one female-specific mode in females. The *dsx* male-specific transcript is the default state while the female-specific mode is a regulated state since the *dsx* pre-mRNA spliced into the female-specific mode requires the activity of the TRA/TRA2 protein complex (Burtis & Baker, 1989; Hedley & Maniatis, 1991; Hoshijima et al., 1991; Inoue et al., 1992). In this study, both *Btau-dsx*^{M1} and *Btau-dsx*^{F} transcripts were detected in early fertilized eggs, but only *Btau-dsx*^{F} was detected in unfertilized eggs. The presence in unfertilized eggs of only the *Btau-dsx*^{F} transcript suggests that *Btau-dsx* pre-mRNA may be a maternal substrate transferred to the eggs during oogenesis. Since we also detected the maternal *transformer* female-specific (*Btau-tra*^{F}) transcript (our unpublished data) and the maternal *transformer2* (*Btau-tra2*) transcript (Thongsaiklaing et al., 2018) in early laid unfertilized and fertilized eggs, it is likely that in *B. tau* female embryos and in unfertilized eggs, the maternal *Btau-dsx* pre-mRNAs may be spliced into the female mode upon activation of the maternal Btau-TRA/Btau-TRA2 protein complex. In early fertilized eggs, the *Btau-dsx*^{M1} transcript was also detected, albeit at low levels, indicating that the *Btau-dsx*^{M1} transcript may exist only in male embryos. In XY fertilized eggs of *C. ceratitis*, presence of the *Ccdsx* male-specific transcript suggested that the *male determining M factor* on the Y chromosome could prevent maternal *Cctra* activity, thus leading to an absence of the CcTRA/CcTRA2 protein complex required for zygotic *Cctra* pre-mRNA splicing into the female mode via the positive feedback loop. As in *D. melanogaster*, in the absence of the female-specific *Cctra*^{F} transcript, the CcTRA/CcTRA2 protein complex could not form and thus *Ccdsx* was spliced by default to produce the CcDSX male-specific protein, which in turn induced male differentiation of XY embryos (Hoshijima et al., 1991; Pane et al., 2002; Salvemini et al., 2009). Moreover, the *Btau-dsx*^{M1} transcript appeared in early laid eggs (0–0.5 h), much earlier than in embryos of other insects such as *D. melanogaster*, *C. ceratitis* and *B. jarvisi*. In the former two insect species, the *dsx* male-specific transcripts were first detected in male embryos 10 h after oviposition (Hempel & Oliver, 2007; Gabrieli et al., 2010), while in the latter, the *dsx*^{M} transcript was first detected in male embryos at 6 h after oviposition (Morrow et al., 2014). However, the expression of the *dsx* male-specific transcript (*Aadsx*^{M}) in *A. aegypti* was similar to that of *Btau-dsx*^{M1} since it was detected in early laid eggs (0.5 h after oviposition) and in the ovaries of adult females. The explanation for the presence of the *Aadsx*^{M} transcript in the adult ovaries was that the *Aadsx*^{M} was not present in female tissues but presented in the fertilized eggs located within the ovaries of fertilized females (Salvemini et al., 2011). The presence of the *Btau-dsx*^{M1} transcript in early fertilized eggs suggests that the *male determining M factor* on the Y chromosome may function at the early syncytial stage

of XY fertilized eggs and that the M factor produced may function as a repressor to prevent the activity of maternal *Btau-tra*, resulting in the maternal Btau-TRA/Btau-TRA2 protein complex not being formed. Without this protein complex, the splicing pattern of the *Btau-dsx* pre-mRNA may be switched from the female (alternative) mode to the male (default) mode. In contrast, the *Btau-dsx^{M2}* transcript was first detected in adult males eighth days post-eclosion. Since the *Btau-dsx^{M2}* transcript encoded a truncated protein without the OD2 domain and sex-specific region, the Btau-DSX^{M2} protein likely has no function, at least not in sex determination. Male *dsx* transcripts without the OD2 domain were reported in *S. coprophila* and *S. ocellaris* but in these two insects, male and female *dsx* transcripts were present in both sexes (Ruiz et al., 2015). However, Btau-DSX^{M2} contained the OD1 domain that functions as a DNA binding domain so the presence of the *Btau-dsx^{M2}* transcript in the adult stage could mean that this protein might be required for some other unknown function.

Function of the *Btau-dsx* gene

We examined the function of the *Btau-dsx* gene by injecting *Btau-dsx* dsRNA into the ventral side of the abdomens of just-emerged females, since this technique was quite suitable for examining the function and expression of genes, particularly genes expressed in the fat body such as *vitellogenin* (*Vg*) genes (Amdam et al., 2003). The efficiency of this technique was confirmed in our experiment by comparing the levels of endogenous *Btau-dsx^{F}* mRNA in females injected with *Btau-dsx* dsRNA and females injected with *DsRed* dsRNA. We found that on all days post-eclosion, *Btau-dsx* dsRNA injected females had lower levels of endogenous *Btau-dsx* mRNA than that of the *DsRed* dsRNA injected control females, particularly on the fourth day post-injection.

We also examined the level of *Vitellogenin* (*Btau-Vg*) mRNA in females injected with either *Btau-dsx* or *DsRed* dsRNAs since the expression of *Vitellogenin* was under the control of the female-specific DSX protein (Coschigano & Wensink, 1993; Chen et al., 2008; Shukla & Nagaraju, 2010). Vitellogenin (Vg) is a precursor of the major yolk protein, an essential nutrient for the embryonic development of oviparous animals including insects (Valle, 1993). Vg is synthesized in the fat body and then accumulates in the eggs during oogenesis (Raikhel & Dhadialla, 1992). In other insects, decreased expression of *Vg* genes affects the development of the oocytes, which in turn affects the morphology of ovaries (Chen et al., 2008; Shukla & Nagaraju, 2010; Veerana et al., 2014). In this study, the ovaries dissected from females injected with *Btau-dsx* dsRNA showed only mild defects. In *B. dorsalis*, the ovaries of females injected with 2 µg/µl dsRNA per female were abnormal (Chen et al., 2008) and in *Antheraea assama*, ovaries dissected from females injected with 70 µg of *Aadsx* dsRNA per larva were deformed (Shukla & Nagaraju, 2010). Therefore, it is likely that the concentration of *Btau-dsx* dsRNA (1 µg/µl per female) used in this study may not be enough to efficiently suppress expression of the *Btau-dsx* gene, such insufficient suppression would not lower *Btau-Vg* expression levels under the threshold necessary to cause strong ovary defects.

In conclusion, the present study shows that the structure of the *Btau-dsx* gene is similar to that of most dipteran insects. In contrast to other tephritid orthologs, *Btau-dsx* produces three (one female and two male) instead of two (one female and one male) transcript variants. The novel male-specific protein (Btau-DSX^{M2}) has no OD2 domain. The *Btau-dsx^{M2}* is first detected in eight day-old adult males, indicating that *Btau-dsx^{M2}* might have a role and is required only during the development of mature adult males, after sex has already been determined by DSX^{M1}. Indeed, similar to *A. aegypti dsx*, *Btau-dsx^{M1}* is detected at the early syncytium stage, but differs from the *dsx* genes of most insects previously studied. The knowledge obtained from this study might be useful for developing advanced biotechnological methods to control the spread of *B. tau* and other insect pests, since sex determination genes can be used to develop transgenic sexing strains for male sterile insect techniques or pest management programmes using RNAi technology (Raphael et al., 2004; Dafa'alla et al., 2010; Fu et al., 2010; Whyard et al., 2015; Leftwich et al., 2016).

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Table S1. Doublesex proteins of a selection of insect species available on the NCBI database used for alignment. Supplementary data Spreadsheet containing the raw data obtained from qPCR analysis for *Btau-dsx* and *Btau-Vg* expression levels, comparing adult females injected with the *Btau-dsx* dsRNA to adult females injected with *DsRed* dsRNA, examined at 2, 4, 6, 8 and 10 days post-injection. The results for detecting the expression of *dsx* and *Vg* are displayed in Figs 8 and 9, respectively.

Insect species	Accession numbers	
Aedes aegypti	ABD96571.1	ABD96573.2
Anastrepha obliqua	AAY25166	AAY25168
Anastrepha amita	ABF50961	ABF50951
Anastrepha bistrigata	ABF50960	ABF50950
Anastrepha fraterculus	ABF50953	ABF50943
Anopheles gambiae	AIY68269.2	AIY68268.3
Antheraea mylitta	ADL40853.1	ADL40855.2
Apis mellifera	ABV55180.1	ABW99102.2
Bactrocera correcta	ACN73403	ACN73403
Bactrocera dorsalis	AAV85891	AAV85891
Bactrocera jarvisi	AIK66583	KJ816790
Bactrocera oleae	AJ547621	CAD67988
Bactrocera tryoni	AAB99948	AAB99948
Ceratitis capitata	AAN63598	AAN63598
Drosophila melanogaster	NP_731198	NP_731199
Helicoverpa armigera	AHF81652.1	AHF81649.2
Lucilia cuprina	ADG37649.1	ADG37648.2
Megaselia scalaris	AAK38831.1	AAK38832.2
Musca domestica	AAR23812.1	AAR23813.2
Nasonia vitripennis	ACJ65507.1	ACJ65510.2
Onthophagus taurus	AEX92939.1	AEX92938.2
Tribolium castaneum	AFQ62106.1	AFQ62105.2