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## ***Tribolium castaneum* Transformer-2 regulates sex determination and development in both males and females**

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### **Abstract**

*Tribolium castaneum* Transformer (TcTra) is essential for female sex determination and maintenance through the regulation of sex-specific splicing of *doublesex* (*dsx*) pre-mRNA. In females, TcTra also regulates the sex-specific splicing of its own pre-mRNA to ensure continuous production of functional Tra protein. Transformer protein is absent in males and hence *dsx* pre-mRNA is spliced in a default mode. The mechanisms by which males inhibit the production of functional Tra protein are not known. Here, we report on functional characterization of *transformer-2* (*tra-2*) gene (an ortholog of *Drosophila transformer-2*) in *T. castaneum*. RNA interference-mediated knockdown in the expression of gene coding for *tra-2* in female pupae or adults resulted in the production of male-specific isoform of *dsx* and both female and male isoforms of *tra* suggesting that Tra-2 is essential for the female-specific splicing of *tra* and *dsx* pre-mRNAs. Interestingly, knockdown of *tra-2* in males did not affect the splicing of *dsx* but resulted in the production of both female and male isoforms of *tra* suggesting that Tra-2 suppresses female-specific splicing of *tra* pre-mRNA in males. This dual regulation of sex-specific splicing of *tra* pre-mRNA ensures a tight regulation of sex determination and maintenance. These data suggest a critical role for Tra-2 in suppression of female sex determination cascade in males. In addition, RNAi studies showed that Tra-2 is also required for successful embryonic and larval development in both sexes.

### **Keywords**

Sex determination; RNAi; Transformer; Doublesex; Alternative splicing

## **1. Introduction**

Sex in insects is determined by a wide range of mechanisms (Gempe and Beye, 2011; Saccone et al., 2002; Sanchez, 2008; Shearman, 2002). This diversity comes from the presence of different upstream signals (Verhulst et al., 2010). In *Drosophila melanogaster* the X:A ratio (X:A = 1) or the X chromosome dose acts as a signal to initiate the female determination cascade in XX embryos (Erickson and Quintero, 2007; Penalva and Sanchez, 2003). As a result, *sex-lethal* (*sxl*) is turned on, which later acts as a memory device for the female determination cascade (Keyes et al., 1992; Penalva and Sanchez, 2003). Sxl, besides regulating the female-specific splicing of its own pre-mRNA, also regulates the female-specific splicing of the pre-mRNA of *transformer* (*tra*), resulting in the production of

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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.2013.08.010>.

functional Tra protein only in females (Boggs et al., 1987; Inoue et al., 1990). Transformer-2 (Tra-2), a protein containing a RNA Recognition Motif (RRM) domain and SR (serine/arginine) rich regions heterodimerizes with Tra and binds to exonic splicing enhancer (ESE) sequences present in the 4th exon (female-specific exon) of *doublesex* (*dsx*) pre-mRNA (Hoshijima et al., 1991; Lynch and Maniatis, 1996; Tian and Maniatis, 1992, 1993). This results in the assembly of spliceosomal complex at the adjacent 3' splice site and, in turn, inclusion of exon 4 in the *dsxf* transcript. Due to the absence of Tra protein in males, the pre-mRNA of *dsx* splices in a default male mode resulting in the production of male-specific Dsx protein. Sex specific Dsx proteins (a transcription factor) differ at their C-terminal ends, hence sex-specifically regulate target genes to control different aspect of somatic sexual differentiation (Burtis and Baker, 1989; Burtis et al., 1991; Coschigano and Wensink, 1993; Erdman and Burtis, 1993; Erdman et al., 1996; Luo et al., 2011).

Dsx has been found to be involved in the sex determination cascade in all the insect species studied to date (Graham et al., 2003; Salz, 2011; Shukla and Nagaraju, 2010). In all insects except silk moths, Tra forms a conserved central axis of sex determination (Verhulst et al., 2010); downstream to *tra* is *dsx* and upstream to *tra* are diverse sex-determining signals. Tra-2 has been characterized in several dipterans including *D. melanogaster*, *Musca domestica* (Burghardt et al., 2005), *Bactrocera oleae* (Lagos et al., 2005), *Anastrepha obliqua* (Sarno et al., 2010), *Ceratitidis capitata* (Salvemini et al., 2009), *Lucilia cuprina* (Concha and Scott, 2009), and *Sciara ocellaris* (Martin et al., 2011). Tra-2 has been identified in a hymenopteran [*Apis mellifera* (Nissen et al., 2012)] and in a lepidopteran insect [*Bombyx mori* (Niu et al., 2005; Suzuki et al., 2012)]. In all these insects *tra-2* is expressed in a non-sex specific manner, producing the same protein in both males and females. Involvement of Tra-2 in the female-specific splicing of *dsx* pre-mRNA has been found to be conserved in all the dipteran and hymenopteran insect species studied. In some of the dipteran insects, *M. domestica*, *C. capitata*, *Anastrepha suspensa*, *A. obliqua*, and *A. mellifera* Tra-2 has been found to be involved in the female-specific splicing of *tra* pre-mRNA (Burghardt et al., 2005; Concha and Scott, 2009; Martin et al., 2011; Nissen et al., 2012; Salvemini et al., 2009; Sarno et al., 2010). In *B. mori* Tra-2 is not involved in the sex-specific splicing of *dsx* pre-mRNA, but it plays an essential role in the testis development (Suzuki et al., 2012). Recent report showed that Tra-2 in *A. mellifera* is required for the sex-specific splicing of *tra* pre-mRNA in both male and female. The authors of this publication also proposed a role for Tra-2 in embryonic development (Nissen et al., 2012).

Red flour beetle, *Tribolium castaneum* is a coleopteran model insect which follows XX/XY sex determination system (XX female and XY male). Recently, we characterized *dsx* and *tra* homologs (*Tcdsx* and *Tctra*) from this insect (Shukla and Palli, 2012a,b). Functional TcTra protein is produced only in females, which besides regulating the female-specific splicing of *Tcdsx*, also regulates the female-specific splicing of its own pre-mRNA. TcTra lacks any RNA-binding domain indicating the involvement of a co-factor (with RNA binding properties) that might be working with TcTra in the splicing of *Tcdsx* and *Tctra* pre-mRNA (Shukla and Palli, 2012b). The studies presented here identified and characterized such a protein, *Transformer-2* homolog (*Tctra-2*), which is essential for sex-specific splicing of both *Tctra* and *Tcdsx* pre-mRNAs. This is the first study on identification and characterization of *tra-2* homolog in a coleopteran insect. Unlike in dipteran insects but similar to that in *A. mellifera* (Nissen et al., 2012), Tra-2 is required for female-specific splicing of *Tcdsx* and *Tctra* pre-mRNAs, male-specific splicing of *Tctra* pre-mRNA and embryonic development. TcTra-2 also plays an important role in regulation of larval development in *T. castaneum*.

## 2. Material and methods

### 2.1. Insect rearing, RNA isolation and RT-PCR

Strain GA-1 of *T. castaneum* was used in all the studies presented here. Beetles were reared on organic whole wheat flour supplemented with 10% yeast. Sexing of pupa and adults was done on the basis of the presence of sex-specific structures described previously (Shukla and Palli, 2012b). Trizol (Invitrogen Corporation, USA) was used to isolate total RNA. Total RNA was DNase treated, denatured at 75 °C for 5 min and then immediately chilled on ice. First strand cDNA was synthesized using MMLV reverse transcriptase (Invitrogen, USA), using the mixture of 17-mers polyT and random primers. PCR conditions with initial denaturation at 94 °C for 2 min, 32 cycles of 94 °C for 30s, 58°C for 30s, 72 °C for 2 min, and final extension at 72 °C for 10 min were used for amplification.

### 2.2. Sequence analysis

Exons and introns, of *Tetra-2* were identified by aligning sequences of RT-PCR products with their corresponding genomic DNA sequences obtained from the Beetlebase (<http://beetlebase.org/>) and the NCBI (<http://www.ncbi.nlm.nih.gov/>). Exon–intron boundaries were confirmed by aligning the sequences through the Spidey program (<http://www.ncbi.nlm.nih.gov/spidey/>).

### 2.3. Developmental expression of *Tetra-2*

Under rearing conditions used, embryonic development is completed in about 70 h, final instar larval stage and pupal stages are completed in approximately 7 and 6 days respectively. Eggs laid by mated females were collected and staged. Total RNA was isolated from eggs sampled at 0–1, 5–6, 10–11, 15–16, 20–25, 30–35, 40–45, and 60–65 h after laying and from the newly hatched larvae. RNA was also isolated from several individual day 0 larvae, sex-separated day 0 pupae, and day 0 adults, and the RNAs were converted to cDNAs and used as templates to perform reverse transcriptase PCR (RT-PCR) using primers specific to the common region of *Tetra-2* (Fig.1). *T. castaneumrp49* was used as an endogenous control. Sex of larvae was determined by performing RT-PCR using cDNA made from RNA isolated from 10 individual larvae, as the template and *Tcdsx* primers [*Tcdsx* is sex-specifically spliced to produce three female and one male-specific isoforms (Shukla and Palli, 2012b)].

### 2.4. Double stranded RNA (dsRNA) synthesis and injections

Primers flanked with T7 promoter sequence at their 5' ends were used to amplify two different regions (dsRNA1 and dsRNA2), common to all the *Tetra-2* isoforms (Fig. 1). Purified PCR products were used as templates to synthesize dsRNA using the MEGAscript T7 kit (Ambion, Austin, Texas). Control dsRNA was prepared for a region corresponding to the *Escherichia coli malE* gene. dsRNAs were injected into newly ecdysed larvae, pupae, or adults as described previously (Shukla and Palli, 2012b). Knockdown efficiency of *Tetra-2* expression in the RNAi insects was calculated as the ratio of *Tetra-2* expression between insects injected with dsRNA specific to *Tetra-2* or *malE* genes.

### 2.5. Quantitative real-time PCR

The SYBR Green kit (Roche, USA) was used to perform quantitative PCR according to the manufacturer's instructions. Three independent biological replicates were analyzed for each treatment. Expression of *rp49* gene of *T. castaneum* was used as an endogenous control for the normalization of the expression data. Gene expression levels were analyzed by  $2^{-\Delta\Delta Ct}$  method (Livak and Schmittgen, 2001).

## 2.6. Imaging and documentation

The gonads were dissected from the injected beetles on 6th day post-adult eclosion (PAE). Images of gonads were taken using an Olympus 1 × 71 Inverted Research Microscope. Megna Fire software (version 1.5) was used to control the microscope, image acquisition, and exportation of TIFF files. Adobe Photoshop Element 9 was used to assemble all micrographs.

## 2.7. Analysis of parental RNAi

To analyze the effect of depletion of *Tctra-2* in early embryos, parental RNAi of *Tctra-2* was performed as described previously (Shukla and Palli, 2012b).

## 3. Results

### 3.1. Genomic organization of *T. castaneum* transformer-2 gene

To identify *tra-2* homolog (*Tctra-2*) in *T. castaneum*, Blast (tblastn) searches were performed in the Beetlebase (<http://beetlebase.org/>) and in the NCBI (<http://www.ncbi.nlm.nih.gov/>) using *D. melanogaster* Tra-2 protein sequence (NP\_476764.1) as a query. Two ESTs (98999753 and 99004500) and two predicted *Tctra-2* sequences (XM\_963457.2 and GLEAN\_12340) with overlapping regions were identified. Primers were designed at different locations of these sequences, and RT-PCR was performed using cDNAs prepared from RNA isolated from female and male beetles. Cloning, sequencing, and analysis of sequences identified the presence of three alternatively spliced isoforms of *Tctra-2* (Fig. 1). RT-PCR amplified three fragments when cDNA made using RNA isolated either from male or female and primers F1 and R1 were used. All the three amplicons were gel purified, cloned into pGEMT easy vector, and sequenced. Alignment of these sequences and blastx analysis confirmed them to be alternatively spliced isoforms of *Tctra-2*. Sequences of all three isoforms of *Tctra-2* were aligned with the *Tctra-2* genomic sequence (AAJJ01000348.1) and exon intron boundaries were identified. *Tctra-2* harbors at least 8 exons and 7 introns; *Tctra-2-1* contains all the exons whereas *TcTra-2-2* lacks a region of 48 bp from the middle of exon 5 and *Tctra-2-3* lacks 111 bp sequence from the 5' end of exon 4 (Fig. 1).

### 3.2. TcTra-2 protein is highly conserved within its RRM domain

The open reading frame (ORF) of all three *Tctra-2* isoforms (*Tctra-2-1*, 813 bp; *Tctra-2-2*, 765 bp; and *Tctra-2-3*, 702 bp) shares common starts (AUG) and stop codons (UAA). The conceptual translation of ORFs of all three isoforms showed the presence of a single RRM domain (RBD-RNA binding domain) and two RS-rich regions on either sides of the RRM domain, a characteristic feature of Tra-2 proteins. The lengths of deduced amino acid sequences of *Tctra-2-1*, *Tctra-2-2* and *Tctra-2-3* are 270, 254, and 230 amino acids, respectively. TcTra-2-2 lacks most of the N-terminal SR-rich region, whereas TcTra-2-3 has a smaller RRM domain in its C-terminus (Fig. S1). The cDNA sequences of *Tctra-2* isoforms have been submitted to GenBank (accession numbers for *Tctra-2-1*, *Tctra-2-2* and *Tctra-2-3* are KF235892, KF235893, and KF235894, respectively).

Multiple sequence alignment of TcTra-2 proteins with the Tra-2 proteins from other insects [*M. domestica* (AAW34233.1), *L. cuprina* (ACS34688.1), *C. capitata* (ACC68674.1), *B. mori* (NP\_001119708.1), *A. mellifera* (AFJ15561.1), and *D. melanogaster* (NP\_476764.1)] showed a high degree of sequence conservation within the RRM domain (Fig. 2). The amino acid sequence flanking the RRM domain is highly variable among different Tra-2 proteins.

### 3.3. *Tctra-2* gene is expressed constitutively throughout the development in a non sex-specific manner

Relative *Tctra-2* mRNA levels did not show significant differences during different developmental stages tested. All the three *Tctra-2* mRNAs were detected during embryonic, larval, pupal, and adult stages (Fig. 3A and B). Expression of *Tctra-2* during very early embryonic stage, i.e., during 0–1 h of embryonic growth suggests maternal transfer of *Tctra-2* mRNAs. We also detected all three isoforms of *Tctra-2* in testis and ovaries dissected from adults (Fig. 3C).

### 3.4. Knockdown in the expression of gene coding for TcTra-2 affects the female-specific splicing of *Tcdsx* and both female- and male-specific splicing of *Tctra*

To confirm the predicted function of Tra-2 as a splice regulator of *dsx* pre-mRNA, dsRNA (dsRNA1) targeting the common region of *Tctra-2* was injected into sex-separated newly ecdysed pupae and adults. Total RNA was isolated on the fifth day after injection of dsRNA and was used to analyze the splicing status of *Tcdsx* and *Tctra* pre-mRNAs. The pre-mRNA of *Tcdsx* is spliced to produce three female- and one male-specific isoforms, whereas the pre-mRNA of *Tctra* is spliced to produce one female and two male isoforms (Shukla and Palli, 2012a, b). Injection of *Tctra-2* dsRNA1 caused an efficient knockdown in the expression of gene coding for TcTra-2 (Fig. S2). Injection of *Tctra-2* dsRNA into newly ecdysed male pupae or male adults caused an appearance of both male and female isoforms of *Tctra* in male RNAi adults (Fig. 4A and B). Similarly, injection of *Tctra-2* dsRNA into newly ecdysed female pupae or female adults caused an appearance of both female and male *Tctra* isoforms in female RNAi adults (Fig. 4A and B). Injection of *Tctra-2* dsRNA into newly ecdysed male pupae or male adults did not affect *Tcdsx* isoform status as only male isoform was detected (Fig. 4C and D). In contrast, injection of *Tctra-2* dsRNA into newly ecdysed female pupae or female adults caused an appearance of male but not female isoforms of *Tcdsx* in these female pupae and adults (Fig. 4C and D).

The ovary and testes dissected from adults developed from the pupae injected with *Tctra-2* dsRNA were highly regressed compared to those in control adults developed from *maleE* dsRNA injected pupae (Fig. 5). The ovaries (Fig. 5B) were affected more than the testes in RNAi insects (Fig. 5D). Consistent with this, *Tctra-2* RNAi females did not produce even a single egg when they were mated with virgin control males developed from *maleE* dsRNA injected pupae (Table 1A). On the other hand, virgin control females developed from *maleE* dsRNA injected pupae laid reduced number of eggs when mated with *Tctra-2* RNAi males. All the eggs laid successfully hatched, and the hatched larvae completed their life cycle (Table 1A). Similar results were obtained when dsRNA was injected into newly eclosed adults (Fig. S3 and Table 1B). These data suggest that TcTra-2 is required for sex-specific splicing of *Tctra* in both sexes and sex-specific splicing of *Tcdsx* in females but not in males. Also, TcTra-2 has an important role in the development of reproductive organs (either directly or through *Tcdsx*) in both male and female and, in turn, regulates fertility.

### 3.5. *Tctra-2* has a vital role during development

Knockdown in the expression of *Tctra-2* in females, during pupal or adult stages exhibited the same splicing pattern of *Tctra* and *Tcdsx* as observed previously in *Tctra* RNAi beetles (Shukla and Palli, 2012b). Further, knockdown in the expression of *Tctra* during the larval stages (but not during pupal and adult stages) resulted in the development of male sexually dimorphic structures in genetic females (Shukla and Palli, 2012b). We performed injections of *Tctra-2* dsRNA into final instar larvae expecting the same change in sexually dimorphic structures as in *Tctra* knockdown. Surprisingly, none of the *Tctra-2* RNAi larvae developed to pupal stage, and all of them died on the fifth or sixth day after injection (Table 2 and Fig.

6). All the *male* dsRNA injected larvae developed to become pupae within 5–6 days. These data suggest that TcTra-2 is required for development of both male and female larvae. To confirm that the larval deaths by *Tctra-2* RNAi is not due to off target effects of TcTra-2 dsRNA, we designed and prepared dsRNA (dsRNA2) targeting a different region of *Tctra-2* than the region targeted by dsRNA1 (Fig. 1). Injection of dsRNA2 into newly ecdysed final instar larvae showed the similar phenotype as seen in dsRNA1 injected larvae, suggesting that the larval developmental arrest phenotype observed in TcTra-2 RNAi larvae is specific to knockdown in the expression of TcTra-2.

Parental RNAi of *Tctra* causes production of all male progenies. To determine whether the parental RNAi of *Tctra-2* also induces the same phenotype, 5 day-old female adults were injected with either *Tctra-2* or *male* dsRNA. After 24 h, five *male* or five *Tctra-2* dsRNA injected females were mated with an equal number of un-injected virgin males in separate cups. Eggs laid by *male* or *Tctra-2* RNAi females were counted from each cup on 15<sup>th</sup> day after initiation of mating. Number of eggs laid by *Tctra-2* RNAi females was low compared to the number of eggs laid by *male* RNAi females (Table 3). Also, eggs laid by *Tctra-2* RNAi females did not develop into larvae (Table 3). These data suggest that TcTra-2 is required for embryonic development.

#### 4. Discussion

Comparative and evolutionary studies on sex-determination mechanisms in insects belonging to different orders suggest existence of a conserved central axis around which sex is determined. This central axis is formed by Tra protein (Verhulst et al., 2010). Tra, along with Tra-2 regulates splicing of *dsx* pre-mRNA in females. In males, *dsx* pre-mRNA splices in a default male mode in the absence of functional Tra. The domesticated silk moth, *B. mori*, presents an exception to this rule where *tra* homologs have not been identified in its genome (Mita et al., 2004), and BmPSI and BmIMP have been implicated in the splicing of *Bmdsx* pre-mRNA (Suzuki et al., 2008, 2010).

*T. castaneum* represents an excellent model insect for the order Coleoptera where RNAi has been demonstrated to work efficiently (Posnien et al., 2009). Recently, we reported on the characterization of the homologs of *tra* and *dsx* from *T. castaneum* (Shukla and Palli, 2012a,b). In this paper, we report on the identification and characterization of *tra-2* homolog (*Tctra-2*) from *T. castaneum* and show its role in the regulation of sex determination and development. Data included in this paper showed the requirement of TcTra-2 in the sex-specific splicing of *Tctra* and *Tcdsx* pre-mRNA in females and also in the sex-specific splicing of *Tctra* in males. In addition, we propose a vital role for TcTra-2 during embryonic and larval development in *T. castaneum*.

Blast searches in the *Tribolium* genome and transcriptome databases led to the identification of *Tctra-2*, which codes for three isoforms. Alignment of TcTra-2 (isoform1) with Tra-2 protein sequences from other insects showed the similarity only within the RRM domain (Fig. 2), confirming suggestions in previous reports that the adjoining regions to the RRM evolve faster than RRM (Nissen et al., 2012). All three isoforms of *Tctra-2* are expressed in both male and female beetles during all the stages tested (Fig. 3). Detection of *Tctra-2* mRNA during early embryonic stages (0–1 h) prior to initiation of zygotic expression suggests the maternal transfer of *Tctra-2*. Similar predictions of maternal transfer of *tra-2* mRNA have been suggested for *M. domestica* (Burghardt et al., 2005), *C. capitata* (Salvemini et al., 2009) and *A. obliqua* (Sarno et al., 2010). In many dipteran insects studied so far, *tra-2* pre-mRNA is not spliced alternatively and only a single transcript has been detected (Burghardt et al., 2005; Concha and Scott, 2009; Sarno et al., 2010; Schetelig et al., 2012). However, alternative splicing and production of multiple *tra-2* isoforms is not

uncommon as multiple isoforms of *tra-2* have been reported in *S. ocellaris*, *Bradysia coprophila*, *B. mori*, and *A. mellifera* (Martin et al., 2011; Nissen et al., 2012; Suzuki et al., 2012). All the three isoforms of TcTra-2 (TcTra-2-1, TcTra-2-2, and TcTra-2-3) contain an RRM domain flanked by RS rich regions (Fig. S1).

Knockdown in the expression of *Tctra-2* during pupal and adult stages altered the splicing status of *Tctra*, in both female and male insects (Fig. 4A and B). The regulation of *Tctra* pre-mRNA splicing by *Tctra-2* in beetles is different from that in dipteran insects where Tra-2 regulates only the female-specific splicing of *tra* pre-mRNA (Burghardt et al., 2005; Concha and Scott, 2009; Martin et al., 2011; Ruiz et al., 2007; Salvemini et al., 2009; Sarno et al., 2010). Regulation of sex-specific splicing of *fem/tra* pre-mRNA by *tra-2* in both male and female has recently been reported in *A. mellifera* (Nissen et al., 2012). How does the same protein Tra-2 promote splicing in females and suppress splicing in males? Serine and arginine rich proteins are known to act as both splicing activators (Graveley and Maniatis, 1998; Shen et al., 2004) as well as splicing suppressors (Chandler et al., 2003; Qi et al., 2007). In *D. melanogaster* Tra-2 (a SR protein), together with Tra act as a splicing activator for splicing of *dsx* pre-mRNA into female mode (Nagoshi et al., 1988). Tra-2, together with Puf68, act as splicing repressor of male-specific exon of its own pre-mRNA in testis (Wang et al., 2013).

TcTra-2 also promotes the female-specific splicing of *Tcdsx* pre-mRNA as knockdown in the expression of *Tctra-2* led to the detection of only male *Tcdsx* isoform in females (Fig. 4C and D). In spite of the presence of female isoform of *Tctra* in *Tctra-2* RNAi males (Fig. 4A and B), we did not detect any female-specific isoform of *Tcdsx* in the RNAi males (Fig. 4C and D). This may be due to lower levels of female TcTra protein that may not have reached the threshold levels essential for the female-specific splicing of *Tcdsx* pre-mRNA resulting in the production of only male isoform of *Tcdsx* mRNA in *Tctra-2* RNAi males. This hypothesis is also supported by the data that showed the presence of only male isoform of *Tcdsx* in *Tctra-2* RNAi females (Fig. 4C and D) in spite of the presence of higher levels of female-specific isoform of *Tctra* compared to the levels of male-specific isoforms (Fig. 4A and B). Additionally, these RNAi females showed lower levels of female-specific isoforms of *Tctra* compared to control females (Fig. 4A and B).

In females, knockdown in the expression of *Tctra-2* (present study) produced similar results at the molecular levels as that of knockdown of *Tctra* (Shukla and Palli, 2012b), i.e., presence of male-specific isoform of *Tcdsx* and both female and male isoforms of *Tctra* (Fig. 4). In contrast, we did not notice any testis-like lobes (Fig. 5B) seen in the ovaries of *Tctra* RNAi insects (Shukla and Palli, 2012b). This observation suggests the requirement of TcTra-2 for the development of gonads in both male and female insects. TcTra-2 is indeed required for testis development in *T. castaneum*, as evident by smaller testis and other male reproductive organs in adult males developed from *Tctra-2* RNAi pupae (Fig. 5D).

Injection of *Tctra-2* dsRNA into embryos led to the transformation of XX female into pseudo males in *C. capitata* and *Anastrepha species* (Salvemini et al., 2009; Sarno et al., 2010). These pseudo males displayed gonad morphology that included male and female internal genital structures (Salvemini et al., 2009; Sarno et al., 2010). In *M. domestica*, genetic female embryos were converted to fertile males as a result of *tra-2* dsRNA injection during embryonic stage (Burghardt et al., 2005). In contrast, knockdown of *tra-2* during the early embryonic stage of *A. mellifera* resulted in the death of embryos after approximately 70 h of development (Nissen et al., 2012), leading to a suggestion of a vital developmental role for Tra-2 during embryonic development in this insect (Nissen et al., 2012). Parental RNAi of *Tctra-2* led to the production of a few eggs which failed to hatch (Table 3), suggesting that TcTra-2 is required for embryonic development in *T. castaneum* as well. The

mechanism of action of TcTra-2 in embryonic development is not known at this time. However, it is likely that the role of TcTra-2 during embryonic development may be related to regulation of Tra/Tra-2 target genes coding for proteins such as those involved in the formation of dosage compensation complex. Dosage compensation is a process by which the overall amount of X-linked gene products are equalized in females (2X) and males (1X), in *D. melanogaster* (Cline and Meyer, 1996; Lucchesi, 1973). Fig. 7 illustrates the model of sex determination in *T. castaneum* based on current understanding of data obtained in this and previous studies (Shukla and Palli, 2012a,b).

RNAi experiments clearly demonstrated the function of Tra2 in sex determination and development in both sexes of *T. castaneum* (present study) and *A. mellifera* (Nissen et al., 2012). Hence, mis-regulation of *Tctra/tra-2* target genes likely occurs in both males and females as a result of Tctra-2 knockdown. Future studies on global identification of TcTra and Tctra-2 target genes in *T. castaneum* should clarify the sex determination and development roles of these proteins.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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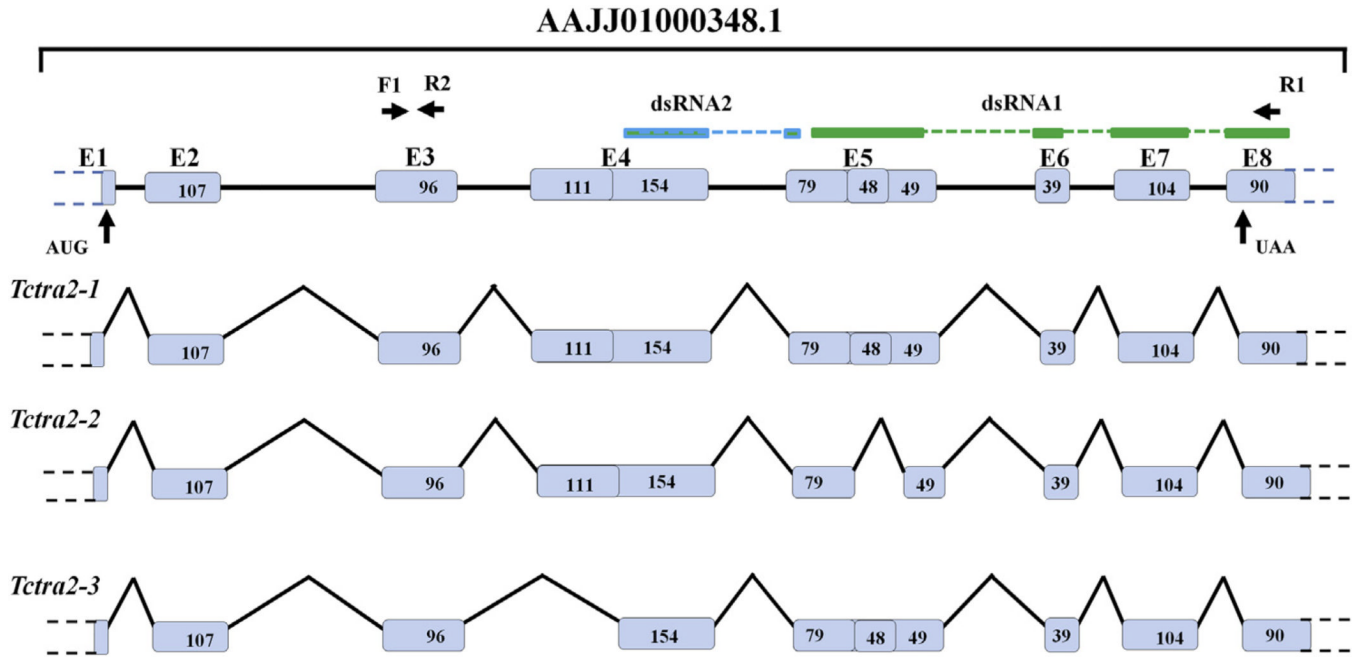
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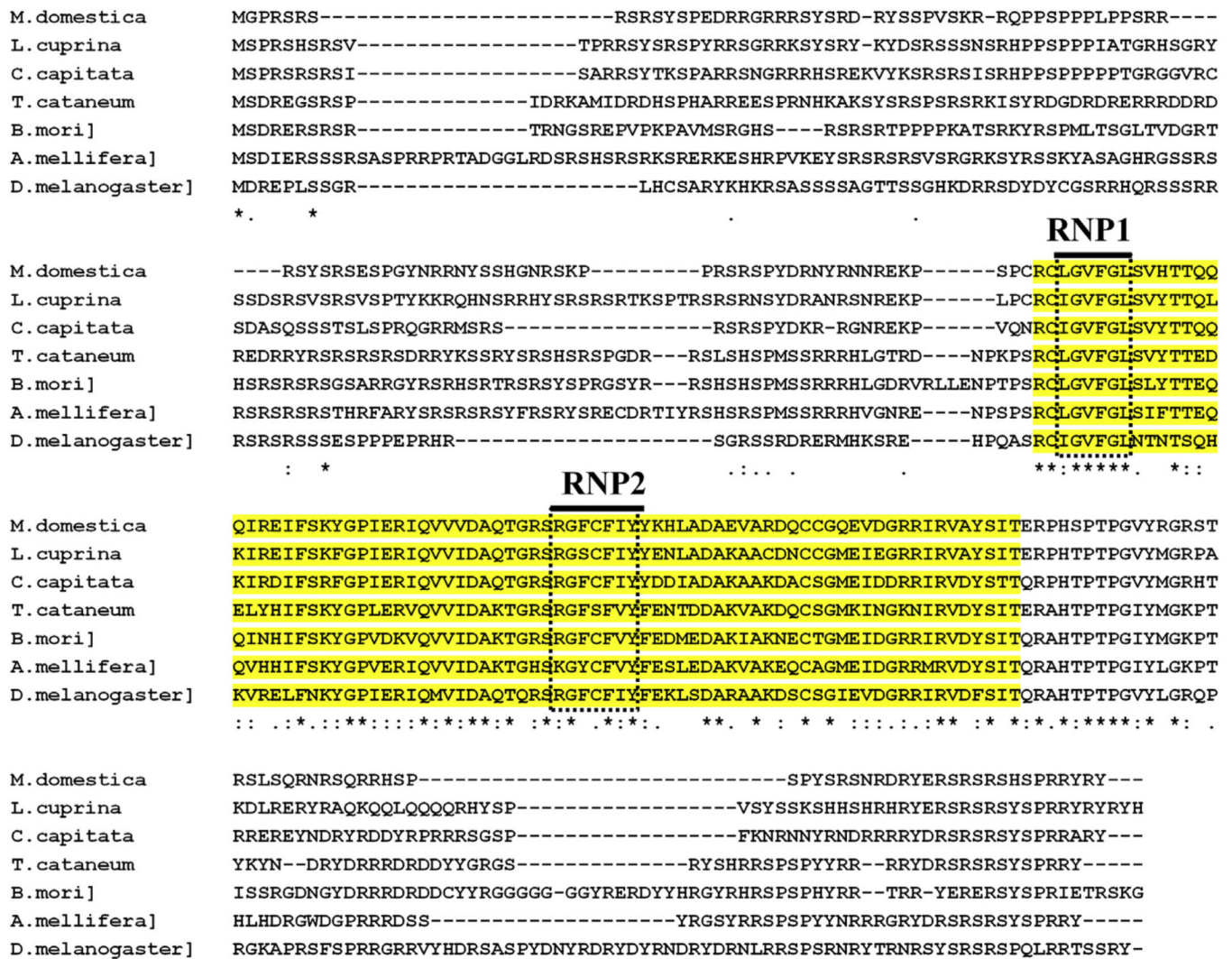


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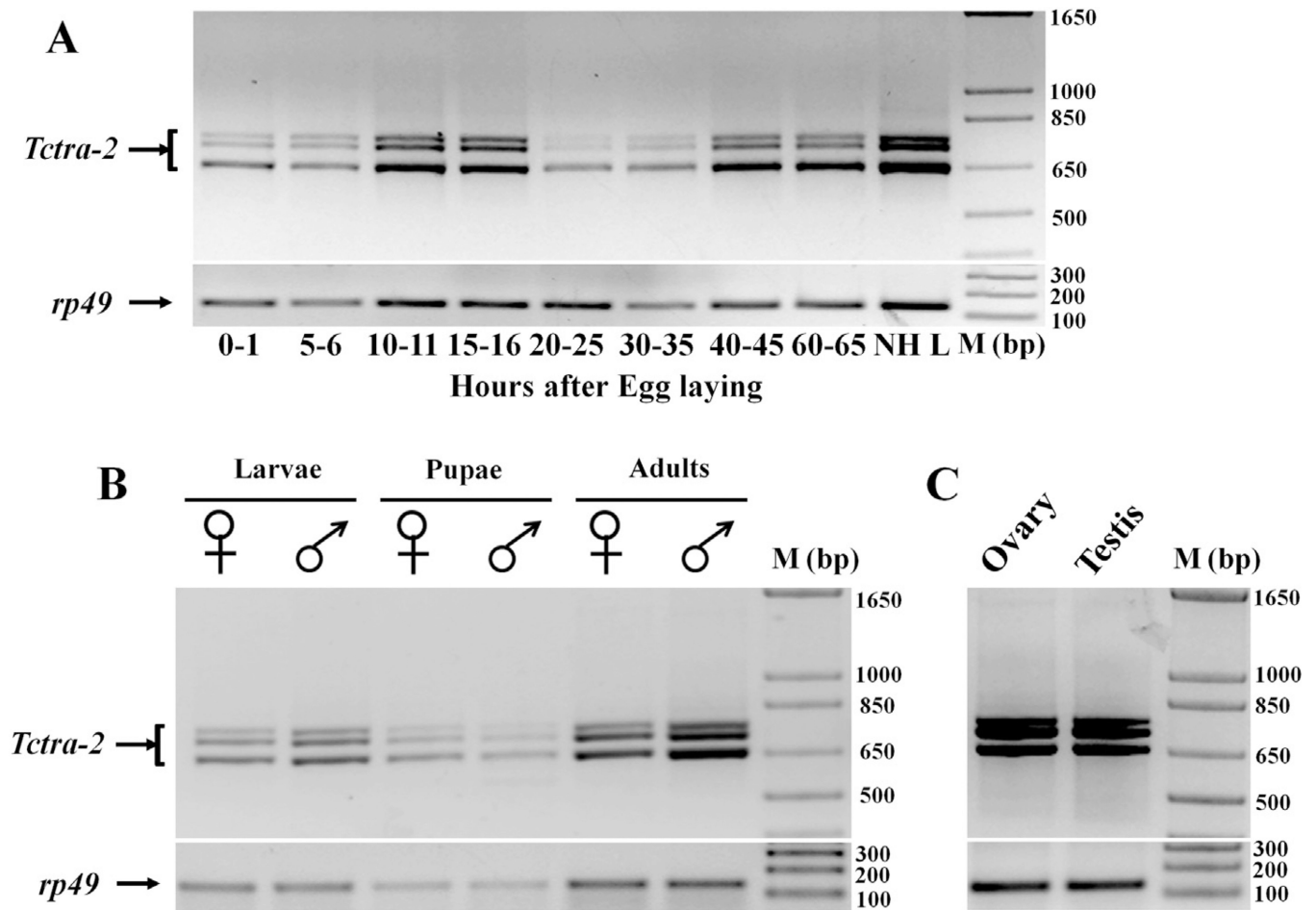


**Fig. 1.** Schematic representation of the genomic organization of *Tctra-2* gene showing exons (boxes-E1 to E8), introns (lines), and primer positions (horizontal arrows). Green and blue bars represent the regions used for the synthesis of dsRNA1 and dsRNA2, respectively. Vertical arrows represent start and stop codon positions. Numbers within the boxes represent the length of the exons, and the dashed line represents the gap. Primers F1 and R2 were used for qRT PCR, whereas F1 and R1 were used for semi-quantitative PCR. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

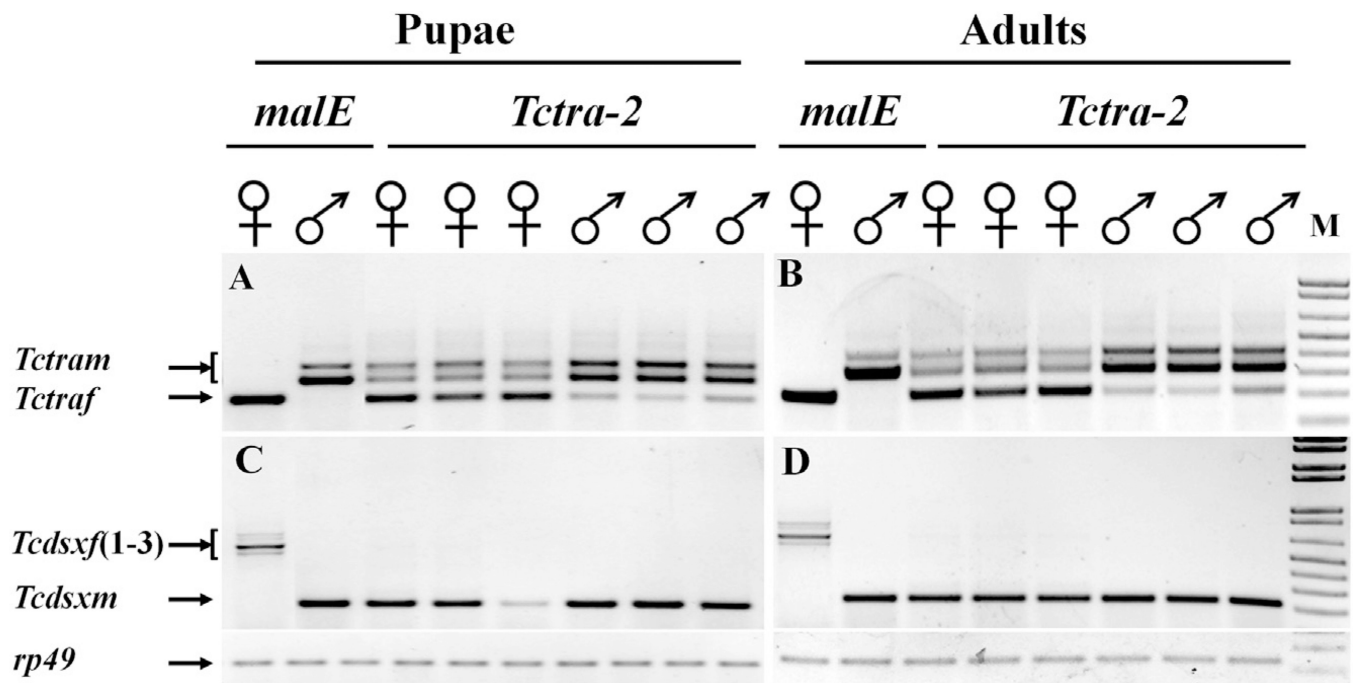


**Fig. 2.**

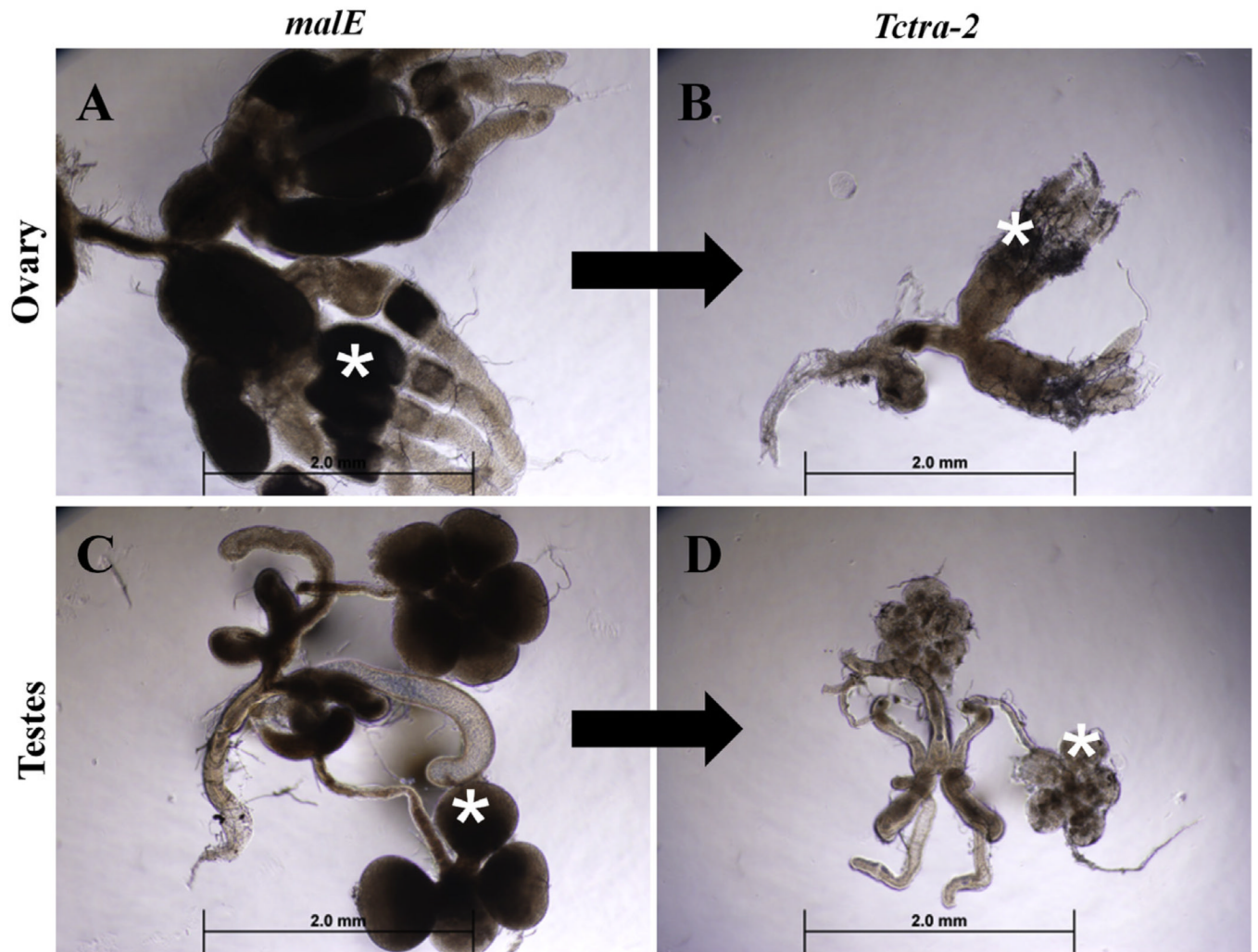
Amino acid alignment of Tra-2 proteins of insect species from Diptera (*D. melanogaster*-NP\_476764.1, *M. domestica*-AAW34233.1, *C. capitata*-ACC68674.1, and *L. cuprina*-ACS34688.1), Hymenoptera (*A. mellifera*-AFJ15561.1), Lepidoptera (*B. mori*-NP\_001119708.1), and Coleoptera (*T. castaneum*). RRM (RBD) domain (yellow shaded) but not the flanking regions showed amino acid similarity among proteins compared. Conserved amino acid sequences (ribonucleoprotein 1, RNP1 and ribonucleoprotein 1, RNP2) shown in the boxes with dotted lines are the characteristic features of RRM domain. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



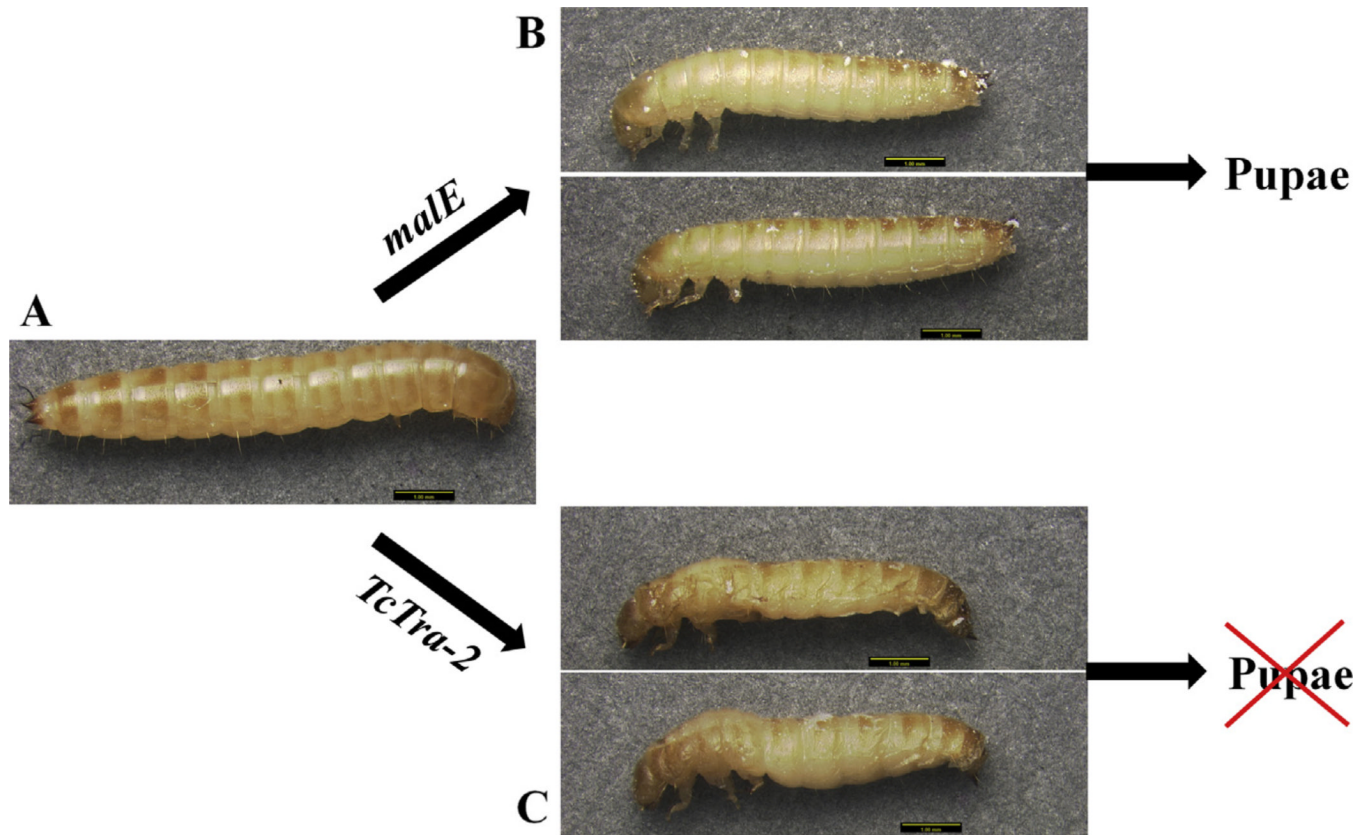
**Fig. 3.** Semi-quantitative RT-PCR analysis of *Tctra-2* mRNA levels. Primers F1 and R1 shown in Fig. 1 and cDNA copied from RNA isolated from staged embryos (A), larvae, pupae or adults (B) as well as ovary and testis (C) dissected from adults were used to amplify *tra-2*. Bottom panels in each Figure show *rp49* amplified using the same cDNA samples. NHL, newly hatched first instar larvae.



**Fig. 4.** Sex-specific splicing of *Tetra* (A & B) and *Tcdsx* (C & D) in insects injected with *maleE* or *Tctra-2* dsRNA. *maleE* or *Tctra-2* dsRNAs were injected into newly ecdysed pupa (A & C) or newly emerged adults (B & D). Total RNA was isolated on 6th day after injection of dsRNA and cDNAs were synthesized. RT-PCR was done to detect the splicing status of *Tetra* and *Tcdsx* using the primers reported previously (Shukla and Palli, 2012b). *Tetra* and *Tcdsx* isoforms were detected by resolving RT-PCR products on agarose gels. Bottom panels in each Figure show *rp49* amplified using the same cDNA samples.

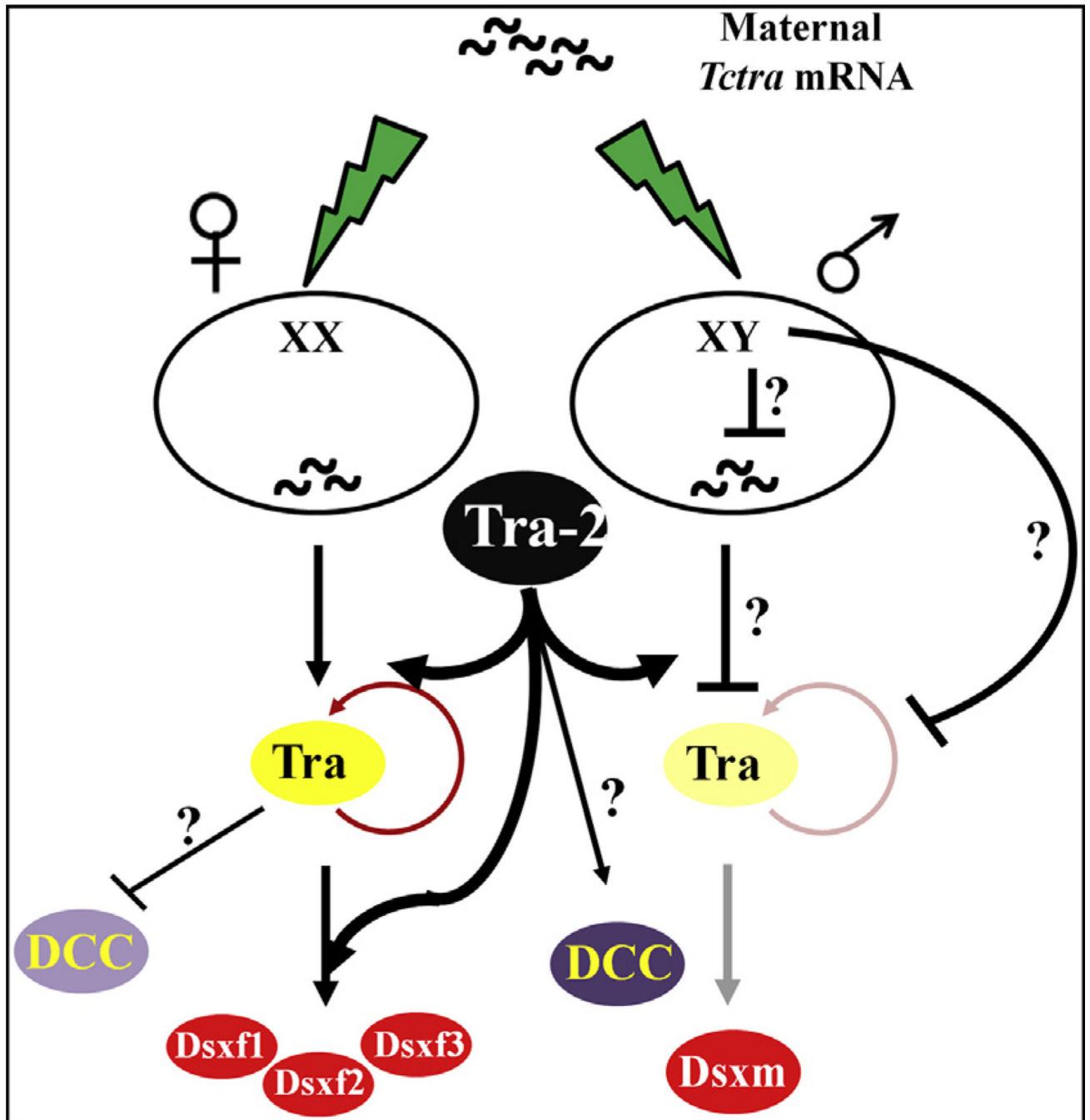


**Fig. 5.** Effect of *tra-2* knockdown on the development of gonads. Ovaries (A & B) or testes (C & D) dissected from insects injected with *maleE* (A & C) or *Tetra-2* (B & D) dsRNA. *Tetra-2* or *maleE* dsRNA were injected into sex-separated day 0 pupae, and gonads were dissected on 6th day PAE. Pictures were taken at 5× magnification. Asterisks are used to mark ovarioles and testis lobes.



**Fig. 6.** Effect of *tra-2* knockdown on the development of larvae. *Tetra-2* or *maleE* dsRNA were injected into day 0 final instar larvae (A). Control larvae injected with *maleE* dsRNA entered into quiescent stage and pupated in 5–6 days of injection of dsRNA (B). The development of *Tra-2* dsRNA injected larvae was arrested and the larvae died within 5–6 days after injection of dsRNA (B).





**Fig. 7.** Model of the sex-determination cascade in *T. castaneum* [the model published recently (Shukla and Palli, 2012b) was modified to show the function of TcTra-2]. TcTra-2 is required for the female-specific splicing of *Tetra* and *Tcdsx* pre-mRNAs in females. TcTra is also required for the male-specific splicing of *Tetra* pre-mRNA in males. Both Tra and Tra-2 may be required to inhibit the formation of dosage compensation complex in females whereas in males Tra-2 alone is required for the formation of dosage compensation complex. This is based on the fact that *Tetra* knockdown leads to death of only females

(Shukla and Palli, 2012b) whereas *Tetra-2* knockdown leads to death of both female and male (present study).

**Table 1**

**A**  
Effect of *Tetra-2* knockdown on the fecundity in the adults developed from *Tetra-2* dsRNA injected pupae.

Control female × control male	RNAi female × RNAi male	RNAi female × control male	Control female × RNAi male
59 ± 2.4	0	0	18 ± 2.16

**B**  
Effect of *Tetra-2* knockdown on the fecundity in the adults injected with *Tetra-2* dsRNA.

Control female × control male	RNAi female × RNAi male	RNAi female × control male	Control female × RNAi male
60 ± 2.9	0	0	25 ± 2.3

The values represent Mean ± S.D ( $n = 6$ ).

**Table 2**Effect of *Tetra-2* knockdown during the larval stage on larval development.

Injection	No of larvae injected	No of larvae metamorphosed to pupae and adult	No of larvae died
<i>malE</i>	20	20	0
TcTra-2	20	0	20

The values represent Mean  $\pm$  S.D ( $n = 3$ ).

**Table 3**Effect of parental RNAi of *Tetra-2* on fecundity and hatching.

dsRNA	Eggs laid	Eggs hatched
<i>malE</i>	295 ± 12.9	All
TcTra-2	12.5 ± 2.08	None

The values represent Mean ± S.D ( $n = 5$ ).