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Two of the three *Transformer-2* genes are required for ovarian development in *Aedes albopictus*

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Abstract

In *Drosophila melanogaster*, *transformer-2* (*tra2*) plays an essential role in the sex-specific splicing of *doublesex* (*dsx*) and *fruitless* (*fru*), two key transcription factor genes that program sexual differentiation and regulate sexual behavior. In the present study, the sequences and expression profiles of three *tra2* (*Aalbtra2*) genes in the Asian tiger mosquito, *Aedes albopictus* (*Ae. albopictus*) were characterized. Phylogenetic analysis revealed that these paralogs resulted from two duplication events. The first occurred in the common ancestor of Culicidae, giving rise to the *tra2-α* and *tra2-β* clades that are found across divergent mosquito genera, including *Aedes*, *Culex*, and *Anopheles*. The second occurred within the *tra2-α* clade, giving rise to *tra2-γ* in *Ae. albopictus*. In addition to the conserved RNA recognition motif (RRM), arginine-rich/serine-rich regions (RS domains) and a linker region, a glycine-rich region located between the RRM and RS2 was observed in Tra2–α and Tra2–γ of *Ae. albopictus* that has not yet been described in the Tra2 proteins of dipteran insects. Quantitative real-time PCR detected relatively high levels of transcripts from all three *tra2* paralogs in 0–2 hr embryos, suggesting maternal deposition of these transcripts. All three *Aalbtra2* genes were highly expressed in the ovary, while *Aalbtra2-β* was also highly expressed in the testis. RNAi–mediated knockdown of any or all *Aalbtra2* genes did not result in an obvious switch of the sex-specificity in *dsx* and *fru* splicing in the whole-body samples. However, knockdown of transcripts from all three *tra2* genes significantly reduced the female isoform of *dsx* mRNA and increased the male isoform of the *dsx* mRNA in both the ovary and the fat body in adult females. Furthermore, knockdown of either *Aalbtra2-α* or *Aalbtra2-γ* or all three *Aalbtra2* led to a decrease in ovariole number and ovary size after a blood meal. Taken together, these results indicate that two of the three *tra2* genes affect female ovarian development.

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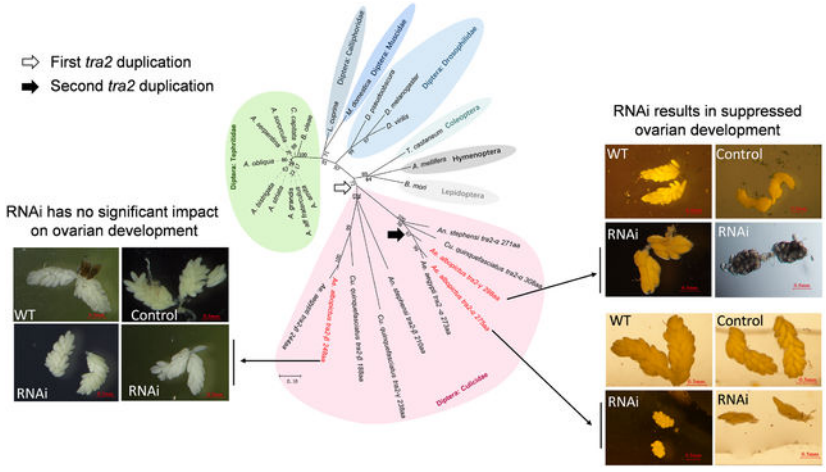
Graphical Abstract



Keywords

Aedes albopictus; gene duplication; sex determination; *transformer-2* (*tra2*); *doublesex* (*dsx*); ovarian development

1 Introduction

Sexual differentiation is controlled by an array of regulatory gene networks. These networks determine morphological, physiological, and behavioral dimorphism between the two sexes. In the fruit fly *Drosophila melanogaster*, initiation of the sex determination cascade is dependent upon X chromosome dosage, which determines the transcriptional activation of *Sex-lethal* (*Sxl*) (Erickson and Quintero, 2007). *Sxl* RNA splicing is then autoregulated to produce a transcript that codes for the full-length Sxl protein only in female embryos. The functional Sxl protein regulates the splicing of *transformer* (*tra*) pre-mRNA, creating the functional splicing factor Tra. Tra, together with the RNA-binding protein Transformer-2 (Tra2) and other splicing factors, controls the sex-specific splicing of the pre-mRNA of two transcription factor genes, *doublesex* (*dsx*) and *fruitless* (*fru*), which gives rise to female- and male-specific isoforms of *dsx* (dsx^F and dsx^M, respectively) and *fru* (fru^F and fru^M, respectively). The sex–specific isoforms of the Dsx and Fru proteins program sexual differentiation (Bell et al., 1991; Inoue et al., 1990).

Although sex–specific splicing of *dsx* and *fru* is highly conserved in insects, including mosquitoes (Biedler et al., 2016), the upstream regulators of the sex determination pathway appear to evolve rapidly. For example, *Sxl* has not been shown to be involved in sex determination outside Drosophilidae (Zhang et al., 2014), and the *tra* gene has not been found in a number of insects, including mosquitoes (Geuverink and Beukeboom, 2014; Salvemini et al., 2013). On the other hand, the *tra2* gene is well conserved among insects and has been reported in a number of insects, including *D. melanogaster* (Belote and Baker, 1982), *Drosophila virilis* (Chandler et al., 1997), the housefly *Musca domestica* (Burghardt et al., 2005), the tephritids *Ceratitis capitata* (Gomulski et al., 2008; Salvemini et al., 2009),

Anastrepha suspense (Schetelig et al., 2012) and *Anastrepha* spp. (Sarno et al., 2010), the calliphorid *Lucilia cuprina* (Concha and Scott, 2009), and the silkworm *Bombyx mori* (Niu et al., 2005). In all cases, *tra2* has been found to be transcribed in both sexes throughout development. The Tra2 protein structure consists of an RNA recognition motif (RRM) flanked by two arginine-rich/serine-rich regions (RS domains) that mediate protein-protein interactions and are required for the female-specific splicing of *dsx* pre–mRNA (Amrein et al., 1994; Mattox et al., 1996; Sarno et al., 2010; Wu and Maniatis, 1993).

Injection of *tra2* dsRNA into *C. capitata* (Salvemini et al., 2009) and *M. domestica* (Burghardt et al., 2005) embryos resulted in complete sex reversal of genotypically female individuals into fertile adult males, suggesting an essential role of *tra2* in female development. Transient knock-down of either *tra* or *tra-2* using corresponding dsRNA led to highly efficient sex reversion in the transgenic strain of *A. suspensa* chromosomal females with a Y–linked DsRed marker integration, indicating that *tra* and *tra-2* are both necessary for female development (Schetelig et al., 2012). The sex reversion tends to be associated with the switch of sex-specificity of *dsx* or *fru* splicing, as shown in *Tribolium castaneum* (Shukla and Palli, 2013). In *D. melanogaster*, *tra2* is necessary not only for female sexual differentiation but also for normal spermatogenesis in males (Amrein et al., 1988). Tra2 behaves as a splicing inhibitor for *tra*2 pre-mRNA by binding the specific intronic splicing silencer (*ISS*) sites of the M1 intron in the germline of *Drosophila* males (Mattox and Baker, 1991). In the mosquito *Ae. aegypti, nix*, a dominant male-determining factor on the M chromosome, acts as a primary signal in the sex determination hierarchy (Hall et al., 2015). Knockout of *nix*, a distant homolog of *tra2* results in a shift to female splice isoforms of both *dsx* and *fru*. It has not been determined how Nix alters the sex-specific splicing of *dsx* and *fru*. We have previously isolated and characterized two *tra2* homologous genes in *Ae. aegypti*, namely, *Aaegtra2-α* and *Aaegtra2-β* (Liu et al., 2013). Knockdown of *Aaegtra2-β* causes a bias in the sex ratio of the next generation due to segregation distortion acting at the level of gametic function and female-specific zygotic lethality (Hoang et al., 2016). However, it is not clear whether *dsx* and *fru* are also under the control of *tra2* gene in mosquitoes. Here, we report the cloning, evolutionary analysis, and functional characterization of three *tra2* genes in the Asian tiger mosquito *Aedes albopictus* (*Aalbtra2* genes).

2 Materials and methods

2.1 Mosquitoes

The Foshan strain of *Ae. albopictus* was collected in Foshan, Guangdong Province, P.R. China, and established in the laboratory in 1981. All mosquitoes were maintained in humidified incubators at 25 ± 1°C on a 12 hr:12 hr light:dark cycle. Larvae were reared in pans and fed on finely ground fish food mixed 1:1 with yeast powder. Adult mosquitoes were kept in 30 cm cube cages and allowed access to a cotton wick soaked in 20% sucrose as a carbohydrate source.

2.2 *Aalbtra2* cloning strategy

tBLASTn searches were performed using the *D. melanogaster* Tra2 *(Dmeltra2–264)* (*Swiss-Prot: P19018.1)* (Amrein et al., 1988) protein sequence as a query against the *Ae. albopictus* transcriptome and genome (http://www.vectorbase.org/). Furthermore, to explore the common characteristics of *tra2* in Culicidae, we also performed a series of tBLASTn searches against the *Anopheles stephensi* and *Culex quinquefasciatus* genomic databases. To determine the molecular organization of *Aalbtra2*, *Astetra2* and *Cquitra2*, reverse transcription PCR (RT–PCR), 3'-rapid amplification of cDNA ends (RACE) and 5'–RACE analyses were performed. The gene cloning strategies for *Ae. aegypti tra2* have been described in our previous work (Liu et al., 2013).

2.3 Expression analysis of *Aalbtra2*

Total RNA from different developmental stages (embryo, larva, pupa, adult male and adult female) and different tissues (head, midgut, Malpighian tubule, ovary and testis) of *Ae. albopictus* was prepared using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) followed by DNase treatment (Ambion, Inc., Austin, TX, USA). First-strand cDNA was synthesized using Superscript III (Invitrogen, USA) reverse transcriptase with oligo-dT primers. RT–PCR amplification of cDNA was performed using gene-specific primers. After PCR, all amplicons were analyzed by electrophoresis in agarose gels, cloned using a CloneJET PCR Cloning Kit (ThermoFisher Scientific, Waltham, MA USA) or a pGEM–T Easy Vector System (Promega, Madison, WI, USA) following the manufacturer's protocol, and sequenced. In all cases, the mRNA of the *Ae. albopictus* ribosomal protein 7 (*Aalb-RpS7*) gene (GenBank: JN132168) was used as an internal control.

2.4 5' and 3' RACE

5' RACE and 3' RACE were performed with gene-specific primers and a Smart RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA) or a FirstChoice RLM–RACE Kit (Ambion, Austin, TX, USA) according to the manufacturer's protocol. The sequences of the primers used in RT–PCR and RACE for amplification of nearly full-length cDNA sequences are shown in Supplementary Table 1. The amplified fragments were gel-eluted, subcloned into the pGEM-T Easy Vector and confirmed by sequencing (Beijing Genomics Institute, Beijing, China).

2.5 Molecular evolutionary analyses

The evolutionary analysis included 22 Tra2 sequences belonging to different insects and vertebrates with the following accession numbers:

Insecta: Diptera: *Anastrepha amita* (EMBL: FN658617), *Anastrepha bistrigata* (EMBL: FN658616), *Anastrepha fraterculus sp. 1* (EMBL: FN658608), *Anastrepha grandis* (EMBL: FN658612), *Anastrepha obliqua* (EMBL: FN658607), *Anastrepha serpentina* (EMBL: FN658613), *Anastrepha sororcula* (EMBL: FN658614), *Anastrepha striata* (EMBL: FN658615), *Ceratitis capitata* (EMBL: EU999754), *Bactrocera oleae* (EMBL: AJ547623), *Musca domestica* (EMBL: AY847518), *Lucilia cuprina* (EMBL: FJ461620), *Drosophila melanogaster* (EMBL: M23633), *Drosophila virilis* (EMBL: XM002049663), and

Drosophila pseudoobscura (EMBL: XM001360568); **Lepidoptera:** *Bombyx mori* (EMBL: NM001126233); **Hymenoptera:** *Apis mellifera* (EMBL: XM001121070); **Hemiptera:** *Acyrthosiphon pisum* (EMBL: XM_001944790.2); **Coleoptera:** *Tribolium castaneum* (EMBL: EFA10734.1).

Vertebrata: Mammalia: *Homo sapiens* (EMBL: U53209); **Aves:** *Gallus gallus* (EMBL: AADN02024287); and **Pisces:** *Oryzias latipes* (EMBL: AB079121).

The different domains of the Tra2 protein were defined as described by (Salvemini et al., 2009) as an RS-rich N-terminal region, an RRM, a linker region, and an RS–rich C–terminal region. The ExPASy ScanProsite tool (Gattiker et al., 2002) (http://prosite.expasy.org/scanprosite/) was used to carry out a search for RRMs. RRM and linker region amino acid sequences of Tra2 homologs were aligned using *ClustalX* version 1.81 (Thompson et al., 1997) with the default program parameters. A phylogenetic tree based on the amino acid sequences was constructed using the distance-based neighbor-joining (NJ) method in the *MEGA 4* program (Tamura et al., 2007) with 1000 bootstrap replicates. The tree was rooted with the Tra2 proteins of the non-dipteran insects *B. mori*, *A. mellifera*, and *T. castaneum*.

2.6 DsRNA synthesis and adult injection

Aalbtra2-α- and *Aalbtra2-β-*specific primers containing the T7 promoter sequence at their 5’ ends and cDNA were used to amplify the different *Aalbtra2* genes. The purified PCR products were used as templates to synthesize dsRNA using a MEGAscript T7 Kit (Ambion, USA). Green fluorescent protein (GFP) dsRNA was used as a control. The primers used to amplify the *Aalbtra2-α*, *Aalbtra2-β* and GFP templates are listed in Supplementary Table 2. A specific dsRNA was also designed to knock down *Aalbtra2-γ*; however, the preliminary experimental results showed that the designed dsRNA did not significantly reduce *Aalbtra2-γ* relative expression levels. As a result, a set of double-stranded *siRNAs* (designated *Aalbtra2-γ siRNA*) was designed to target the coding sequence of *Aalbtra2-γ*. A scrambled siRNA (targeting GFP) was used as a negative control. All double-stranded siRNAs were purchased from Convenience Biology (Changzhou, China), and the sequences are shown in Supplementary Table 2.

Approximately 500 nl of dsRNA and siRNA solution (10 nM) was injected laterally into the thorax of 2-day-old adult *Ae. albopictus* females under a microscope unless stated otherwise. In triple gene knockdown experiments, 600 ng of a 1:1:1 mixture of two kinds of dsRNA and siRNA (injection volume: 500 nl) for each targeted gene was injected into the thorax of 2-day-old adult *Ae. albopictus* females. The same amount of a *gfp* dsRNA and siRNA mix was also injected as a negative control. After injection, the adult mosquitoes were immediately transferred to small plastic cups (900 ml, 11 cm top diameter) and provided a 10% sucrose solution.

2.7 DsRNA soaking assay

First instar larvae (n=50 per group) were washed three times with Dulbecco’s phosphate-buffered saline (DPBS, ThermoFisher Scientific, USA) in a petri dish. After a final wash, the DPBS was removed, and the larvae were soaked in 350 μl of premixed dsRNA mixtures

diluted with DPBS (600 ng of a 1:1:1 mix of two kinds of dsRNA and siRNA). Larvae soaked in the same amount of *gfp* dsRNA and siRNA were used as a negative control group. After 24 hr of soaking, the larvae were transferred back to the pans and fed regularly. After 48 hr of regular feeding, the larvae were soaked again. This process was repeated three times. Half of the larvae in each group were collected when they developed into 4th instar larvae, and total RNA and genomic DNA were isolated from each individual using a Quick-DNA/RNA™ Microprep Plus Kit (Zymo Research, Irvine, CA, USA). Sex was determined by PCR amplification of the *Ae. albopictus* male-determining factor *Aalbnix* (our unpublished data), the homolog of *Ae. aegypti nix* (Hall et al., 2015). The RNA of larvae of the same sex were pooled together and then used for further *dsx* and *fru* analysis.

2.8 Ovarian measurements and fat body dissection

To explore the effect of *tra2* interference on ovarian development and the expression of *Ae. albopictus vitellogenin* (*Aalbvg*) mRNA and sex-specific transcripts of *dsx* and *fru*, the injected mosquitoes were mated with male mosquitoes and fed defibrinated sheep blood 48 hr post injection (p.i.). At 48 hr post blood meal (p.b.m.), the ovaries were dissected by removing the soft cuticle between the fifth and sixth abdominal sternites with a fine needle and then removing the terminal segments and placing them in a drop of suitable buffered mosquito saline (Hagedorn et al., 1977). The numbers of developed follicles were counted, and the individual follicle size was measured under a stereomicroscope (SMZ1000 with Digital Sight DS–U3, Nikon, Japan).

To explore the effect of *tra2* interference on sex-specific transcripts of mosquito *dsx* in the fat body p.b.m., 24 hr post emergence adult females were fully coupled with males and then injected with dsRNA/siRNA in the thorax, followed by routine rearing. After 36 hr of rearing, the surviving adult females were administered a second injection. The injected mosquitoes were fed with defibrinated sheep blood at 36 hr p.i.. The fat body tissues were dissected from surviving female mosquitoes (72 hr p.i., 36 hr p.b.m.) according to a protocol described previously (Chung et al., 2017). However, only the fat body and ovaries from individuals with obvious ovarian defects (left-right asymmetry, lack of development, etc.) were collected for *dsx* analysis (*Aalbtra2-α* interference group: ovarian defects n=16, total n=51; *Aalbtra2-γ* interference group: ovarian defects n=18, total n=45).

2.9 RT-PCR and qRT-PCR analysis

To analyze the temporal and spatial expression patterns of *Aalbtra2-α*, *Aalbtra2-β* and *Aalbtra2-γ*, total RNA was extracted from different developmental stages and different tissues of *Ae. albopictus* using TRIzol Reagent (Invitrogen, USA). Any residual DNA was removed using a TURBO DNA-free™ Kit (Ambion, USA). First-strand cDNA was synthesized from the total RNA using oligo-dT primers and a RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, USA). The *Ae. albopictus rpS7* gene was used as a control (Hall et al., 2015).

To analyze the effects of *Aalbtra2* downregulation on the sex-specific mRNA splicing patterns of the *dsx* and *fru* genes, the sex-specific transcripts of *dsx* and *fru* in RNAi females and males were detected by RT–PCR using specific primers designed to amplify the sex-

specific regions. The amplified RT-PCR products from female and male adults were separated on 1.0% or 2.0% agarose gels, depending on the size of the product, and purified using an E.Z.N.A. Gel Extraction Kit (Omega Bio–tek, Doraville, GA, USA). These cDNA fragments were cloned into the plasmid pGEM–T Easy Vector and sequenced.

The inhibition of *tra2* and the relative expression levels of the sex-specific transcripts of *dsx* and *fru* were quantified by qRT–PCR, using a SYBR Green kit (Roche, Basel, Switzerland) following the manufacturer's instructions. To analyze the effects of *Aalbtra2* downregulation on the mRNA expression of the major yolk protein precursor gene *Aalbvg* during vitellogenesis, ovaries were dissected from the mosquitoes in the *Aalbtra2-α*, *Aalbtra2-β* and *Aalbtra2-γ* triple interference groups, larvae soaking groups and control groups. At 48 hr p.b.m., total RNA was extracted from batches of 30 females from the *Aalbtra2-α*, *Aalbtra2-β* and *Aalbtra2-γ* interference groups and control groups using TRIzol (Molecular Research Center, Inc., OH, USA) according to the manufacturer's directions. Three *Ae. aegypti vitellogenin* orthologs were analyzed. *AalbvgA*, *AalbvgB*, and *AalbvgC* (AALF008766, AALF019930, and AALF008379, respectively [VectorBase]) shared 80.16% nucleotide identity, and primers were designed to match highly conserved regions of the *Aalbvg* mRNA sequences. To detect the transcript levels of *vg* in ovary samples from *Aalbtra2-α*- or *Aalbtra2-γ*-knockdown adults, only individuals with obvious ovarian defects, as described above, were selected for subsequent quantitative analysis. The transcript levels of *Vg* mRNA were measured using SYBR dye technology (Applied Biosystems, Foster, CA, USA).

For each treatment described above, three independent biological replicates were analyzed. *Ae. albopictus rpS7* was used as an endogenous reference gene for the normalization of the expression data. All quantification was performed using an Applied Biosystems™ 7500 Real-Time PCR System (Applied Biosystems, CA, USA). Gene expression levels were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). All of the primers used in this study are listed in Supplementary Table 3.

2.10 Statistical Analysis

All experiments were carried out with three biological replicates. Differences in the mean and standard error of the mean (SEM) among the groups in the RNAi study were analyzed by analysis of variance (ANOVA; GraphPad Prism Version 6.01). For one-to-one comparisons, the mean and SEM of the *dsx*, *fru*, and *vg* relative expression levels, and the ovarian ovariole counts were analyzed with GraphPad Prism Version 6.01, and differences between means were analyzed with Student's *t* test. The average ovarian follicle size was analyzed by ordinary one-way ANOVA, and the hatching of larvae was compared using Chi–square with Yates' correction. No statistical methods were used to predetermine sample size. The experiments were not randomized, and the investigators were not blinded to allocation during the experiments and outcome assessment. Statistical significance was assigned when P values were <0.05 using GraphPad Prism Version 6.01 and IBM SPSS Statistics 20.0.

3 Results

3.1 Isolation and molecular characterization of *Aalbtra2*

A tBLASTn search of the *Ae. albopictus* genome and transcripts using *D. melanogaster* Tra2 as a query was performed. Four draft sequences with e-values less than $1e^{-10}$, *AALF015404*, *AALF008878-RA*, *AALF008877-RA* and *AALF004560-RA*, were identified, which were located on scaffolds *JXUM01S000397, JXUM01S001946*, *JXUM01S001946*, and *JXUM01S001377,* respectively.

Molecular cloning of the three *Aalbtra2* genes was performed by combining traditional PCR amplification with 3' and 5' RACE. The three paralogs of *tra2* (*Aalbtra2-α*, *Aalbtra2-β* and *Aalbtra2-γ* corresponding to AALF008877–RA, AALF015404–RA and AALF008878-RA, respectively) with different supercontig locations were cloned and characterized, and their sequences were deposited in GenBank. The sequences included *Aalbtra2-α* (*GenBank:* MF682534), *Aalbtra2-β* (*GenBank:* MF682535), *Aalbtra2-γ.1* (*GenBank:* MF682536), and *Aalbtra2-γ.2* (*GenBank:* MF682537). The two splice isoforms *Aalbtra2-γ.1* and *Aalbtra2-γ.2,* were cloned from *Aalbtra2-γ*. However, we failed to clone the full-length cDNA of *AALF004560-RA* (located on *JXUM01S001377*) using RT–PCR and RACE.

Alignment of the *Aalbtra2* cDNA sequences with the corresponding genomic sequence was performed to determine the exon/intron organization and the alternative splicing events (Figure 1). Figure 1 shows the molecular organization of the *Aalbtra2* RNA molecules. *Aalbtra2-α* spans a 2933 bp genomic region located in supercontig *JXUM01S001946* with 6 exons and an ORF encoding 275 amino acids. The transcription start site is located at 147 bp upstream of the initiation codon AUG of the ORF. *Aalbtra2-β* was found in supercontig *JXUM01S000397* and consisted of four exons and three introns; the ORF encodes a protein of 248 amino acids (Figure 1). *Aalbtra2-γ* consists of 6 exons distributed over a 1.2 kb region on supercontig *JXUM01S001946* and contains an 897 bp ORF encoding a protein of 298 amino acids. The *Aalbtra2-γ*.1 isoform differs from *Aalbtra2-γ*.2 at the splice site used at the 3' end of noncoding exon 1.

3.2 Comparison of the molecular organization and protein sequences of Culicidae *tra2* genes with those of other species

The deduced protein products of all three *Aalbtra2* genes are aligned to Tra2 proteins from a few species of mosquitoes (Supplemental Figure 1), other insects and a few representative vertebrate species (Figure 2). The three *Ae. albopictus* Tra2 proteins contained an RRM flanked by two RS domains (RS1 and RS2), which are characteristics of SR splicing factors. Comparison of these RRMs and linker regions with those of other dipteran insects and vertebrates revealed that the regions are conserved (74 and 19 amino acids, respectively) in all examined species (Figure 2). The highest degree of similarity was observed for the last 7 amino acids of the linker region. In fact, the RRM-linker junction region is not similar to a region in any other known protein and is more likely than other regions to perform conserved functions that are specific to Tra2 (Dauwalder et al., 1996). A conserved RVDY motif was also found downstream of the RRM (Figure 2) (Dauwalder et al., 1996). RVDY (invertebrates) or RVDF (vertebrates) is a conserved motif in nearly all Tra2 sequences that

is implicated in protein phosphatase 1 (PP1) binding and is involved in the dephosphorylation of splicing factors (Meiselbach et al., 2006; Novoyatleva et al., 2008; Stamm, 2008). In addition to the motifs previously described in Tra2, a glycine–rich motif (GRM) proposed to be involved in protein-protein interactions (Bocca et al., 2005) was identified in a subgroup (see below) of the mosquito Tra2 proteins. This GRM, which is also found in vertebrate Tra2 (Rodriguez et al., 2016), is absent in the known Tra2 proteins of other dipteran insects.

3.3 Phylogeny and molecular evolution of the Tra2 proteins

For phylogenetic analysis, the Tra2 proteins encoded by the three *Ae. albopictus tra2* genes were aligned with homologous sequences from other species in the Culicidae family, representatives from other dipteran families and a few non-dipteran insects. We used the Tra2 sequences from *T. castaneum* (Coleoptera), *A. mellifera* (Hymenoptera) and *B. mori* (Lepidoptera) as outgroups to root the tree. The topology of the Tra2 protein phylogenetic tree (Figure 3) showed that all mosquito Tra2 sequences originated from the same common ancestor, forming a monophyletic group with 72% bootstrap support. Further groupings suggested that the three *Ae. albopictus* paralogs resulted from two duplication events. The first occurred in the common ancestor of Culicidae, giving rise to the *tra2-α* and *tra2-β* clades that are found across divergent mosquito genera, including *Aedes*, *Culex*, and *Anopheles*. The second duplication occurred within the *tra2-α* clade, giving rise to *tra2-γ* in *Ae. albopictus*. The most parsimonious explanation is that this second duplication occurred prior to the divergence between *Ae. albopictus* and *Ae. aegypti* and that *tra2-γ* was lost in the *Ae. aegypti* lineage. However, further analysis that includes all Tra2 sequences from additional *Aedes* species is needed to rule out the alternative hypothesis that the duplication occurred after the divergence between *Ae. albopictus* and *Ae. aegypti* and that *Aalbtra2-γ* underwent rapid evolutionary change after the duplication. A second duplication also occurred in *Culex quinquefasciatus*, giving rise to *Cquitra2-γ*. However, *Cquitra2-γ* and *Aalbtra2-γ* are not orthologs because *Cquitra2-γ* is a duplication within the *tra2-β* clade. Interestingly, the proteins in the mosquito *tra2-α* clade, including *Aalbtra2-γ*, contain a GRM that is not found in the *tra2-β* clade, and the proteins in the *tra2-α* clade show higher degrees of conservation, as indicated by shorter branch lengths, than the proteins in the *tra2-β* clade.

3.4 Expression patterns of the three *Aalbtra2* genes

To analyze the temporal and spatial expression patterns of the three *Aalbtra2* genes, both RT–PCR and qRT–PCR analyses were performed on total RNA extracted from various stages from embryo to adult using a series of gene-specific primers. Only *Aalbtra2-γ* produced splice isoforms, *Aalbtra2-γ*.1 and *Aalbtra2-γ*.2 (Figure 4A). Both *Aalbtra2-α* and *Aalbtra2-β* showed only one transcript (Supplementary Figure 2A). All PCR fragments were cloned and sequenced to confirm the expected sequences. To determine the transcription profiles of the three *Aalbtra2* genes, a qPCR assay was used with gene-specific primers. The results showed that the genes *Aalbtra2-α, Aalbtra2-β* and *Aalbtra2-γ* were expressed throughout development and during adulthood in both sexes. All three genes showed the highest relative transcript levels at the embryo stage, with *Aalbtra2-α* and *Aalbtra2-β* showing high levels throughout embryonic development, whereas *Aalbtra2-γ* showed a drop

in expression after 2–4 hr (Figure 5A). The spatial expression pattern of *tra2* in *Ae. albopictus* was also studied by performing RT–PCR and qPCR on total RNA separately extracted from heads, midguts, Malpighian tubules, ovaries, testes and carcasses. The two *Aalbtra2-γ* alternative splice products were detected in all tissues (Figure 4B), whereas no additional alternative splicing isoforms were found for either *Aalbtra2-α* or *Aalbtra2-β* (Supplementary Figure 2B). *Aalbtra2-α*, *Aalbtra2-β* and *Aalbtra2-γ* were expressed in most tissues in both sexes. Interestingly, *Aalbtra2-α*, *Aalbtra2-β* and *Aalbtra2-γ* all showed enriched expression in the ovary or testis (Figure 5B).

3.5 Effect of *tra2* downregulation on *dsx* and *fru* splicing in whole-body samples

In mosquitoes, *Aaegdsx* (Salvemini et al., 2011; Scali et al., 2005) and *Aaegfru* transcripts (Salvemini et al., 2013) exhibit sex-specific RNA splicing patterns. In *Ae. albopictus*, the sex-specific mRNA isoforms of *dsx* and *fru* have been identified (our unpublished work), and the full-length coding sequences (CDSs) have been deposited in GenBank under the accession numbers MF682531, MF682532, MF682533, and MG516586, MG516587, and MG516588. The molecular organization of *Aalbdsx* and *Aalbfru* is shown in Figure 6A, and the sex-specific splicing patterns are similar to those of orthologous genes in *Ae. aegypti* (Salvemini et al., 2013; Salvemini et al., 2011).

Having established the sex-specific alternative splicing patterns of *dsx* and *fru* mRNA, we tested whether silencing of any of the three *tra2* genes in adult *Ae. albopictus* by RNAi would affect the transcription levels or splicing patterns of *dsx* and *fru*. RT–PCR was used to display the splicing patterns of the *Aalbdsx* and *Aalbfru* genes with the primers dsx^{F}/dsx^{R} and fru^{F}/fru^{R} (Figure 6A), which were designed to produce sex-specific amplicons from the *Aalbdsx* and *Aalbfru* transcripts, respectively. In untreated males or females, the expected male- or female-specific *dsx* and *fru* cDNA products were detectable. However, changes in the splicing patterns of *Aalbdsx* and *Aalbfru* were not observed in either sex after silencing of any of the three *tra2* genes in *Ae. albopictus* (Figure 6B). Successful knockdown of these genes was confirmed by qRT–PCR with GFP RNAi controls (Figure 7A). Real-time PCR was used to detect possible changes in the expression levels of sex-specific isoforms of either *dsx* or *fru* using gene-specific primers (Supplementary Table 3) after knockdown of any of the three genes. Moreover, the percentages of $Aalbdsx^{F}$, $Aalbdsx^{M}$, $Aalbfru^{F}$ and $Aalbfru^{M}$ transcripts among all *dsx* and *fru* transcripts after *Aalbtra2* RNAi were determined. The results showed that there were no significant changes in the percentages of sex-specific isoforms among total *dsx* and *fru* transcripts in either males or females after knockdown of any of the three *tra2* genes or all *tra2* paralogs simultaneously at the earlier larval stage in development (Figure 7B row 4). Furthermore, no significant changes in the transcription levels of $Aalbdsx^{F}$, $Aalbdsx^{M}$, $Aalbfru^{F}$ and $Aalbfru^{M}$ were detected (Supplementary Figure 3).

3.6 Knockdown of *Aalbtra2-α* and *Aalbtra2-γ* suppresses ovarian development

We also investigated the effect of RNAi knockdown of the three *tra2* genes on ovarian development. Forty-eight hours after injection of interfering RNAs targeting one of the three *tra2* genes, mosquitoes were fed defibrinated sheep blood, and only freshly blood-fed female mosquitoes with obviously engorged, bright red abdomens were selected for subsequent

analysis. Mosquitoes in each group were dissected 36 hr p.b.m., and the knockdown efficiency was evaluated by using qRT–PCR with *gfp* RNAi as the control. The average follicle size (length of the long axis of follicle) was measured, and the ovarioles were also counted for each individual. The results showed that relative levels of *Aalbtra2-α*, *Aalbtra2-β* and *Aalbtra2-γ* mRNA, normalized using the reference gene *Aalb-Rps7*, were successfully depleted after injection to 40.44 ± 7.741% ($P<0.05$), 11.90 ± 1.609% ($P<0.001$) and 41.40 ± 5.205% ($P<0.05$), respectively, compared to the control groups. Furthermore, *Aalbtra2-α* downregulation significant decreased ($P<0.01$) the number of ovarioles in each individual (41.88 ± 2.434, n=24) compared with the control treatment (52.79 ± 2.563, n=24). Although *Aalbtra2-γ* RNAi did not significantly decrease ovariole numbers, the average follicle size was clearly reduced ($P<0.05$) (Figure 8A, B, C). *Aalbtra2-α* and *Aalbtra2-γ* interference also led to a significant reduction in the average ovarian follicle size compared to the control treatment, and some individuals showed marked left-right asymmetry and growth arrest and even a lack of ovarian development (Figure 8D). There were no statistically significant differences between the control and *Aalbtra2-β*-downregulated groups with regards to the ovariole number or average follicle size.

Vitellogenin (Vg) is a large phospholipoglycoprotein that is the precursor of major yolk proteins in oviparous vertebrates and invertebrates, including mosquitoes (Robinson, 2008). Vg is synthesized in the fat body, secreted into the hemolymph, and transported to the ovary. Vitellogenin transcription increases rapidly after a blood meal (Clements and Clements, 1992). *Ae. albopictus vg (Aalbvg)* displayed the expected expression pattern and reached maximum expression levels at 24 hr p.b.m. (Supplementary Figure 4). To explore whether the repressive effects of *Aalbtra2* downregulation on ovarian development may be associated with the downregulation of *vg* expression, the relative transcript levels of *Aalbvg* in the *Aalbtra2* interference groups were compared to those in the control groups at 36 hr p.b.m., and the silencing efficiency of *Aalbtra2* was also analyzed by qRT–PCR. The results showed that *Aalbvg* mRNA transcript levels were significantly lower in the *Aalbtra2-α* and *Aalbtra2-γ* knockdown groups (down to 40.31 ± 8.873% and 20.19 ± 2.203%, respectively; $P<0.05$) than in the control groups. No significant changes in *Aalbvg* expression were observed after *Aalbtra2-β* downregulation (Figure 9A, B row 1, 2, 3).

3.7 Effects of *Aalbtra2* interference on *dsx* transcription and splicing patterns in the fat body and ovary

Transcription of the *vg* gene is under the control of the Dsx protein in *T. castaneum* (Shukla and Palli, 2012), and the yolk protein genes are also directly regulated by the Dsx protein in *D. melanogaster* (Burtis et al., 1991; Coschigano and Wensink, 1993). Although we did not detect any changes in the sex-specific splicing patterns or transcript levels of *dsx* in whole-body RT–PCR and qRT–PCR analyses (Figures 6 and 7 and Supplementary Figure 3), it is possible that slight changes in *dsx* splicing or transcript levels may have occurred in the fat body due to *tra2* knockdown. Changes in *tra2* in the fat body, which could be masked in whole-body RT–PCR or qRT–PCR assays, could have a profound effect on ovarian development, as the fat body is the source of Vg synthesis. Therefore, we analyzed the sex-specific splicing patterns of *dsx* mRNA in adults in which *Aalbtra2-α* or *Aalbtra2-γ* was silenced by RNAi using fat body and ovary samples dissected from females 72 hr p.b.m..

Only individuals with obvious ovarian defects upon dissection (for *Aalbtra2-α*: n=16, total =51; for *Aalbtra2-γ*: n=18, total=45) were selected for subsequent quantitative analysis. The *Aalbtra2-α* and *Aalbtra2-γ* mRNA levels in the ovary, fat body and carcass were lower in the RNAi groups than in the control group (Figure 10A row 1, 2). For all three tissue types, no significant differences were observed in the relative transcript levels of $Aalbdsx^F$ in either the *Aalbtra2-α* or *Aalbtra2-γ* knockdown groups compared with the control group (Figure 10B row 1, 2). However, $Aalbdsx^M$ transcript levels in the fat body were significantly higher in both knockdown groups than in the control group (Figure 10B, row 1, 2). Notably, the transcript levels of $Aalbdsx^M$ were two orders of magnitude lower than those of $Aalbdsx^F$ in the fat body of adult females. Therefore, it is unclear whether the statistically significant change in $Aalbdsx^M$ expression in the fat body could induce changes in ovarian development. However, knockdown of transcripts of all three *tra2* genes either by adult injections or larvae soaking significantly reduced the female isoform of *dsx* mRNA and increased the male isoform of the *dsx* mRNA in both the ovary and the fat body in adult females (Figure 10B, row 3 and row 4).

4 Discussion

In this study, we isolated and characterized the three *tra2* genes of *Ae. albopictus*. Although the Tra2 proteins were overall conserved between mosquitoes and other dipteran insects, including *D. melanogaster*, the mosquito *tra2* genes showed some unique features. First, for all mosquito species tested, the *tra2* gene has exhibited multiple paralogs, while in other dipterans *tra2* has been detected as a single-copy gene. Extensive phylogenetic analysis of Tra2 proteins from divergent species in the Culicidae family and a number of other insects showed that a gene duplication event in the common ancestor of Culicidae gave rise to two *tra2* clades, *tra2-α* and *tra2-β,* that were found across divergent mosquito genera including *Aedes*, *Culex*, and *Anopheles*. A later duplication gave rise to a third *tra2* gene in some mosquitoes, including *Ae. albopictus*. Only a single *tra2* mRNA has been isolated from these non-mosquito dipteran insects, with the exception of *D. melanogaster,* which produces three *tra2* isoforms through alternative splicing and with multiple promoters (Amrein et al., 1990; Mattox et al., 1990). In *Drosophila*, the *tra2* gene also behaves as a splicing inhibitor of the M1 intron in *tra2* pre-mRNA to form the male-specific *tra2* transcript in the germline of *Drosophila* males. However, no sex-specific *tra2* transcripts were identified in *Ae. albopictus*.

Like other Tra2 proteins, mosquito Tra2 contains an RRM and a conserved linker region adjacent to the RRM (Figure 1 and 2). There are also two RS domains flanking the RRM. Interestingly a GRM that has been found in human Tra2α (*hTra2α*; GenBank: AAC50658) (Sievert et al., 1997) was identified between the RRM and RS2 in the mosquito Tra2 proteins in the Tra2–α clade but was missing in the mosquito Tra2 proteins in the Tra2–β clade. The GRM has not been found in any non-mosquito dipteran Tra2. It is thought that GRM is required for multiple mechanisms of splicing regulation (Wang and Burge, 2008), which may suggest that the mosquito Tra2 proteins in the two clades have acquired different functions. In this regard, it is interesting to note that the proteins in the mosquito *tra2-α* clade, including *Aalbtra2-α* and *Aalbtra2-γ*, showed higher degrees of conservation than the

protein sequences in the *tra2-β* clade. This difference may indicate a different level of evolutionary constraint or even neofunctionalization after gene duplication.

The three *Aalbtra2* genes showed different levels of transcription at different developmental stages (Figure 5). All three genes were transcribed in both sexes, with relatively high levels in the embryonic stages and in the reproductive organs of adults. The results from a previous study demonstrated that knockdown of *tra2* in embryos of *B. mori* (Suzuki et al., 2012) or *A. mellifera* (Nissen et al., 2012) can cause abnormal testis formation in some larvae, even leading to embryonic lethality, which suggests that *tra2* has vital functions during early embryonic stages.

As a component of the Tra/Tra2 complex, the Tra2 protein has been shown to play an important role in the sex-specific splicing of *dsx* in several species in Diptera (Concha and Scott, 2009; Peng et al., 2015; Schetelig et al., 2012). In this study, RNAi-mediated knockdown of any of the three *Aalbtra2* genes in adult mosquitoes failed to produce any detectable sex-specific splicing changes in *dsx* and *fru* in whole body assays. Similar results were observed even in mosquitoes in which all *Aalbtra2* genes had been knocked down since the larval stage (Figure 7 row 4). Notably, knockdown of the *tra2* gene in *B. mori* also has no effect on the sex-specific splicing patterns of *dsx* pre-mRNA (Suzuki et al., 2012). However, knockdown of transcripts of all three *tra2* genes significantly reduced the level of the dsx^F mRNA and increased the level of the dsx^M mRNA in both ovary and fat body in adult females. In these cases, the reduction of the dsx^F, which is hundreds of fold more abundant than dsx^M, did not result in an equivalent increase in dsx^M. Therefore, the relationship between *tra2* and the sex-specific splicing of *dsx* is likely different between *Aedes* and *Drosophila*. In this regard, it is worth noting that the *Aedes dsx* has more than one female-specific introns, which is different from *Drosophila* (Figure 6, (Biedler and Tu, 2016)).

RNAi–mediated knockdown of *Aalbtra2-α* or *Aalbtra2-γ* significantly suppressed ovarian development in females, and the transcription of the major yolk protein gene *vitellogenin* was significantly reduced. Knockdown of *tra2* has also been previously shown to affect the development of the male reproductive system or germline (Amrein et al., 1990; Mattox and Baker, 1991) (Liu et al., 2015; Suzuki et al., 2012) and to affect female fecundity or gonads in several insects (Burghardt et al., 2005; Pomerantz and Hoy, 2015; Schetelig et al., 2012; Shukla and Palli, 2013). It is likely that these effects result from shifts in the alternative splicing of *dsx* because the Dsx protein has been shown to be involved in the modulation of signaling pathways in both the genital disc (Ahmad and Baker, 2002; Gorfinkiel et al., 2003; Keisman et al., 2001) and gonad (DeFalco et al., 2008; Oliver et al., 1993; Wawersik et al., 2005) and even to directly target yolk protein gene expression in *T. castaneum* (Shukla and Palli, 2012) and *D. melanogaster* (Clough et al., 2014).

It is not yet clear how the two *tra2* genes affect ovarian development and Vg synthesis in *Ae. albopictus*. In the current study, when either *Aalbtra2-α* or *Aalbtra2-γ* was silenced, a significant increase of dsx^M in the adult female fat body was observed, although the relative amount of dsx^M is hundreds of fold lower than dsx^F. We cannot rule out the possibility that the subtle but significant change in the level of dsx^M in the fact body may have an impact on ovarian development and/or Vg synthesis. *Vitellogenin* is involved in a complex genetic

regulatory network (Chen et al., 2004; Martin et al., 2001; Zhu et al., 2007) and is directly or indirectly influenced by multiple factors in mosquitoes, including 20-hydroxyecdysone (20E) (Provost-Javier and Rasgon, 2014), juvenile hormone (JH) (Wang et al., 2017), members of the amino acid/target of rapamycin (AA/TOR) and insulin pathways (Attardo et al., 2006; Boudko et al., 2015; Carpenter et al., 2012; Hansen et al., 2011) and even miRNAs (Bryant et al., 2010). Thus, we cannot rule out the possibility that Tra2 indirectly affects *vg* via these factors.

In *B. mori,* Tra2 is apparently not involved in the regulation of the sex-specific splicing of *dsx* pre-mRNA, but it is required for normal ovary and testis development. The fact that male fertility was not affected by dsRNA soaking or injection may be due to some kind of barrier in the testis (Fairchild et al., 2016; Miranda and Cavicchia, 1986; Toshimori et al., 1979). Further studies are needed to determine the functions and mechanisms of each *tra2* gene across different developmental stages in mosquitoes, including the Asian tiger mosquito, which is an important invasive vector of arboviruses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Two duplication events have resulted in three *tra2* genes in *Ae. albopictus*.
2. A glycine-rich region has been identified in Tra2-α and Tra2-γ.
3. Knockdown of *Aalbtra2* paralogs didn't shift dsx^F and dsx^M in the ovary and fat body.
4. Knockdown of *Aalbtra2-γ* and *Aalbtra2-α* in adult females reduced post-blood meal ovariole number and ovary size.

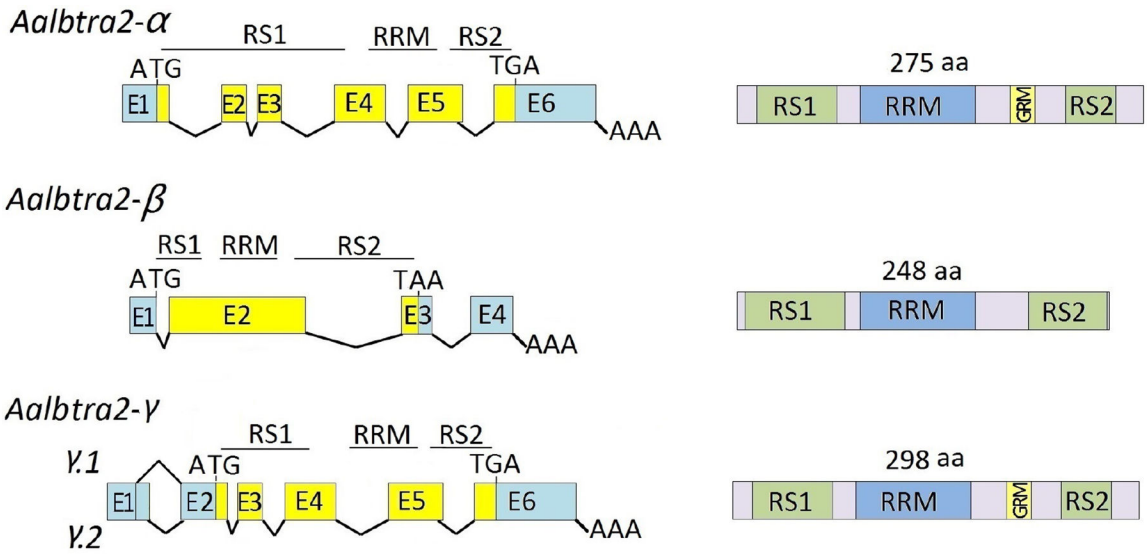

Figure 1. Molecular organization of the three *tra2* genes of *Aedes albopictus*.

The exons (boxes) and introns (dashed lines) are not drawn to scale. E1–E6 are the exon numbers. The start (ATG) and stop (TGA/TAA) codons and the poly (A) addition sites (AAA) are marked. In the left panel, the 5'– and 3'–untranslated regions (UTRs) are shown in light blue, and the protein-coding regions are shown in yellow. The arginine and serine-rich motifs (RS1 and RS2) and the RNA recognition motif (RRM) are indicated, and the glycine-rich motif (GRM) is also indicated in the schematics on the right showing the three Tra2 protein sequences.

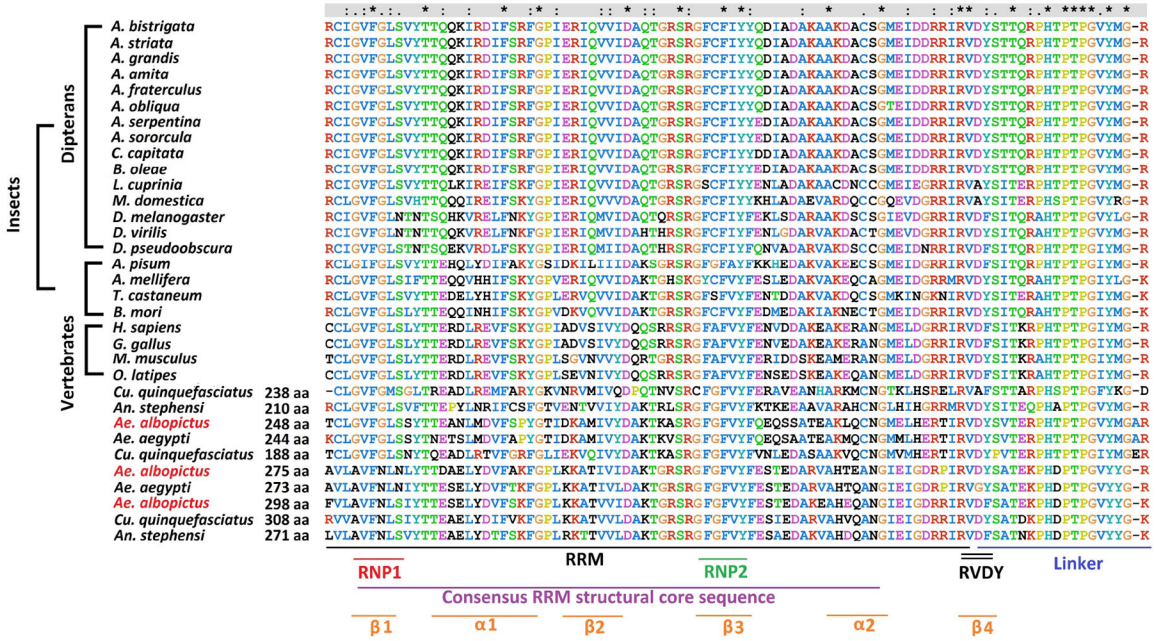

Figure 2. Amino acid sequence alignment of the RNA recognition motif (RRM) and linker region of the Tra2 homologs.

The asterisks (*) indicate amino acids that are identical in all species. A consensus RRM structural core sequence, putative α-helix or β–sheet regions, and a conserved RVDY motif are all indicated at the bottom of the alignment. Also indicated within the RRM are the positions of two ribonucleoprotein identifier sequences, RNP–1 and RNP–2.

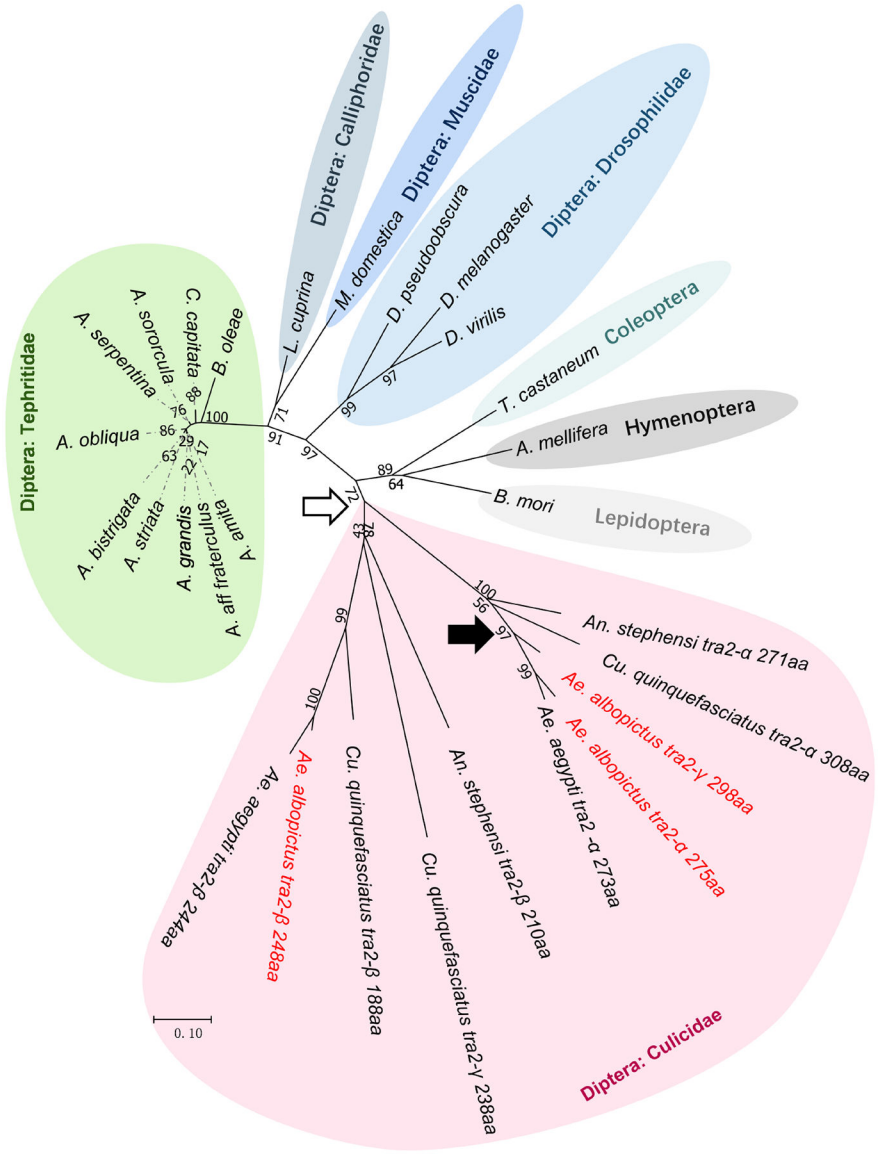

Figure 3. Phylogeny of the Transformer-2 (Tra2) protein in mosquitoes and other insects.

Bootstrap values (up to 100%) from 1000 replicate analyses are shown. The scale bar at the bottom indicates 0.10 differences per residue. The taxonomic relationships are indicated right next to the cluster of the tree. The tree was rooted with Tra2 sequences from *Tribolium castaneum* (Coleoptera), *Apis mellifera* (Hymenoptera), and *Bombyx mori* (Lepidoptera). The two duplication events that gave rise to the three *Aalbtra2* genes are indicated by an open and a solid arrow, respectively.

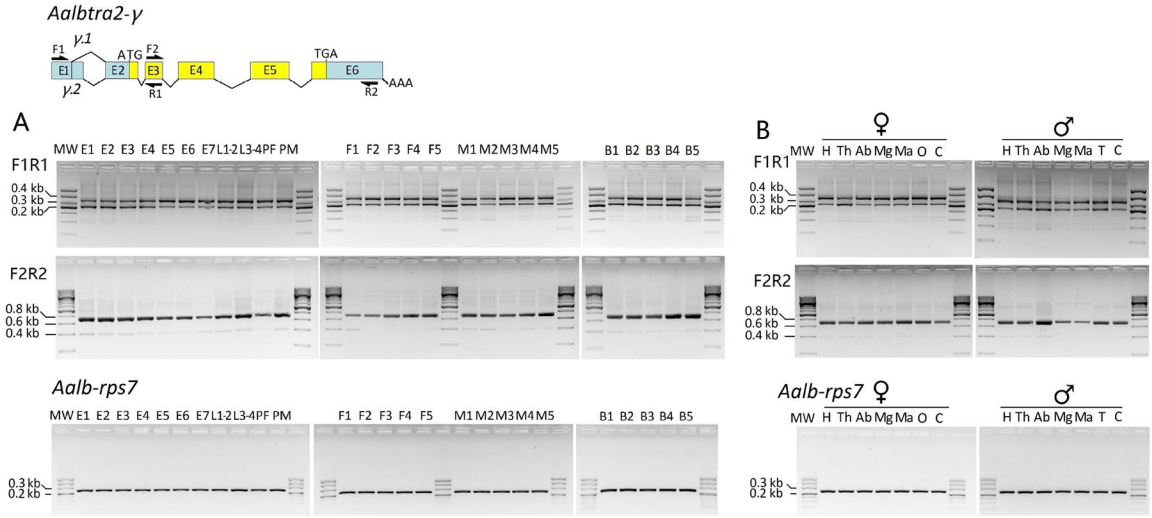

Figure 4. Developmental (A) and spatial (B) transcription patterns of the *Aalbtra2-γ* gene.

Overlapping sets of intron-spanning primers were used for RT–PCR to identify the splicing patterns of *Aalbtra2-γ* mRNA. The arrows denote the locations of the primers used. The F1/R1 primer combination amplified two slightly different cDNA fragments in all developmental samples of *Aalbtra2-γ*, corresponding to the two alternatively spliced isoforms of exon 1. *Aedes albopictus* ribosomal protein S7 gene (*Aalb-Rps7*) was used as an internal control. The analyses were performed on the following samples: E1 = 0–2 hr embryos; E2 = 2–4 hr embryos; E3 = 4–8 hr embryos; E4 = 8–12 hr embryos; E5 = 12–24 hr embryos; E6 = 24–36 hr embryos; E7 = 36–48 hr embryos; L1–2= 1st–2nd instar larvae; L3–4= 3rd-4th instar larvae; PF= female pupae; PM= male pupae; F1=0–6 hr emerged female adults; F2=6–12 hr emerged female adults; F3=12–24 hr emerged female adults; F4=24–48 hr emerged female adults; F5=48–72 hr emerged female adults; M1= 0–6 hr emerged male adults; M2= 6–12 hr emerged male adults; M3= 12–24 hr emerged male adults; M4= 24–48 hr emerged male adults; M5= 48–72 hr emerged male adults; B1= 0–6 hr post blood meal (p.b.m.) female adults; B2= 6–12 hr p.b.m. female adults; B3= 12–24 hr p.b.m. female adults; B4= 24–48 hr p.b.m. female adults; B5= 48–72 hr p.b.m. female adults; B4= 24–48 hr p.b.m. female adults; B5= 48–72 hr p.b.m. female adults; H=head; Th = thorax; Ab=abdomen; Mg = midgut; Ma= Malpighian tubule; O=ovary; T=testis; C= carcass; ♀= adult female; ♂=adult male. * The faint band appearing under the main product with the F2/R2 primer sets was a nonspecific amplicon.

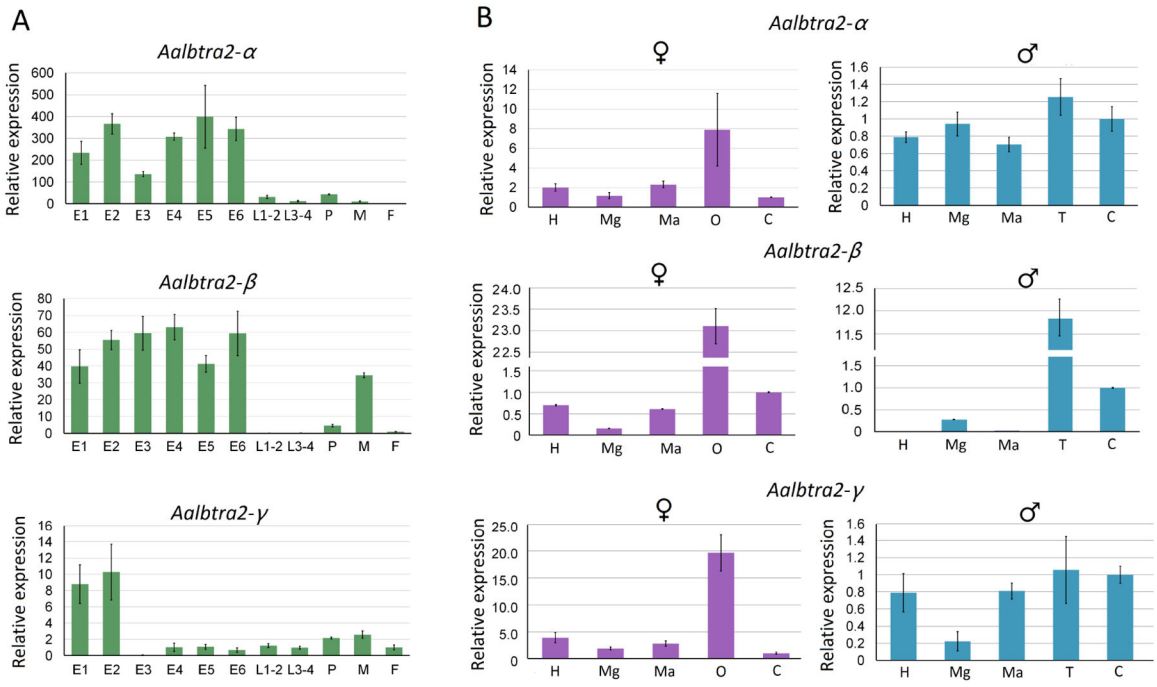

Figure 5. Temporal (A) and spatial (B) transcription profiles of the three *Aalbtra2* genes as measured by real-time RT-PCR.

(A) Temporal profiles at different developmental stages of *Aedes albopictus*. E1 = 0–2 hr embryos; E2 = 2–4 hr embryos; E3 = 4–8 hr embryos; E4 = 8–12 hr embryos; E5= 12–24 hr embryos; E6= 24–48 hr embryos; L1–2= 1st–2nd instar larvae; L3–4= 3rd–4th instar larvae; P= pupae; M=male; F=female. (B) Spatial transcription profiles in different tissues of *Ae. albopictus* adult females and males. H=head; Mg = midgut; Ma= Malpighian tubule; O=ovary; T=testis; C= remaining carcass; ♀= adult female; ♂=adult male. The x-axis indicates the sample ID, and the y-axis shows the relative expression value obtained by qPCR. The internal reference gene was *Ae. albopictus ribosomal protein 7 (Aalb-RpS7)*. The error bars represent the SEM.

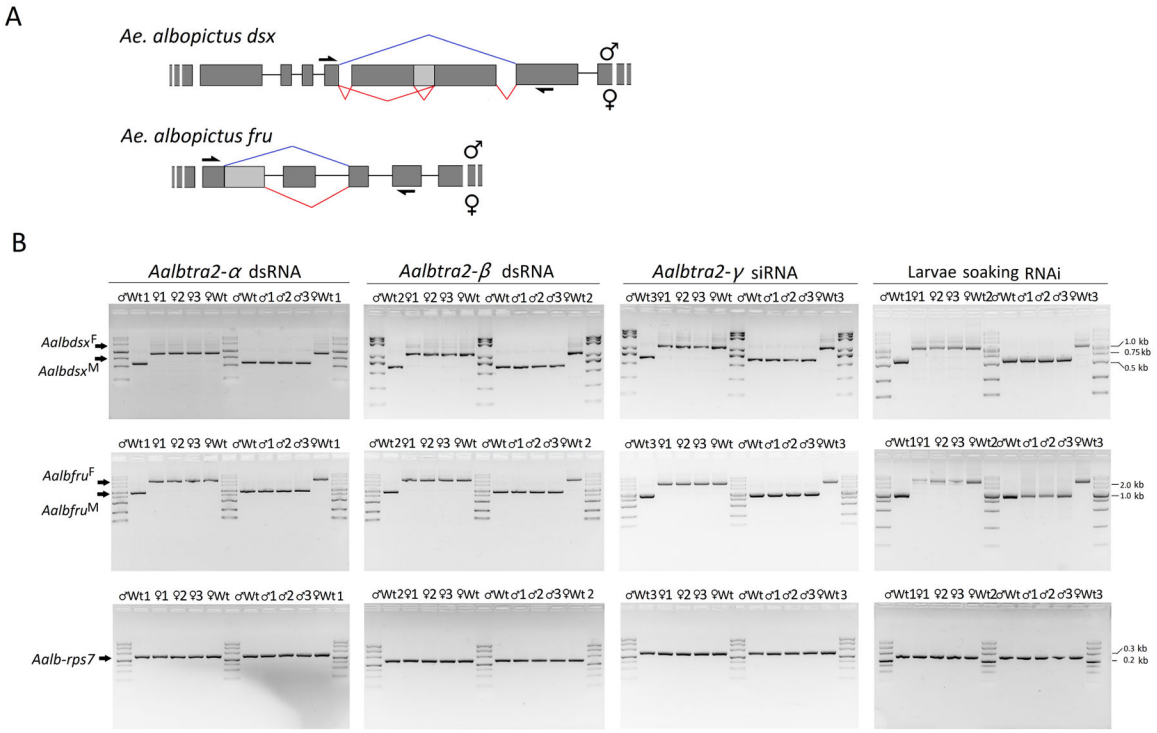

Figure 6. Sex-specific splicing patterns of the *dsx* and *fru* transcripts in *Aedes albopictus* adults after individual or triple RNAi knockdown of A*albtra2-α*, *Aalbtra2-β*, and *Aalbtra2-γ*.

(A) Genomic organization and splice variants of *dsx* and *fru* in *Ae. albopictus*. The male-specific and female-specific splicing patterns are marked in blue and red, respectively. The exons and introns are not drawn to scale (see Supplementary Table 3 for further details). The arrows denote the locations of the primers used to amplify the cDNA products of *dsx* and *fru*. The primer sets encompassed the alternative splicing junctions and thus produced sex-specific RT–PCR products. (B) RT–PCR products obtained using mRNA from individuals 48 hr after injection of siRNAs/dsRNA targeting one of the three *tra2* genes (columns 1–3) or from adult individuals in which all *Aalbtra2* genes had been knocked down since the larval stage through soaking with *Aalbtra2-α* and *Aalbtra2-β* dsRNA and *Aalbtra2-γ* siRNA mixtures (column 4).♂1–3 and ♀1–3 refer to the three replicate groups (n=15 adults per replicate) of *tra2* RNAi males and females, respectively. Wt♂1–3 and Wt♀1–3 indicate the three replicate groups (n=15 per replicate) of wild-type males and females. Fragments corresponding to the *fru* female (*Aalbfru*^{F}; 2010 bp) and male (*Aalbfru*^{M}; 987 bp) cDNA sequences and the dsx female (*Aalbdsx*^{F}; 1062 bp) and male (*Aalbdsx*^{M}; 620 bp) cDNA sequences were resolved by electrophoresis on a 1.5% agarose gel that was stained with ethidium bromide and photographed. The numbers with short lines on the right side indicate the molecular weights. *Ae. albopictus* ribosomal protein 7 *(rpS7)* mRNA was used as an internal control.

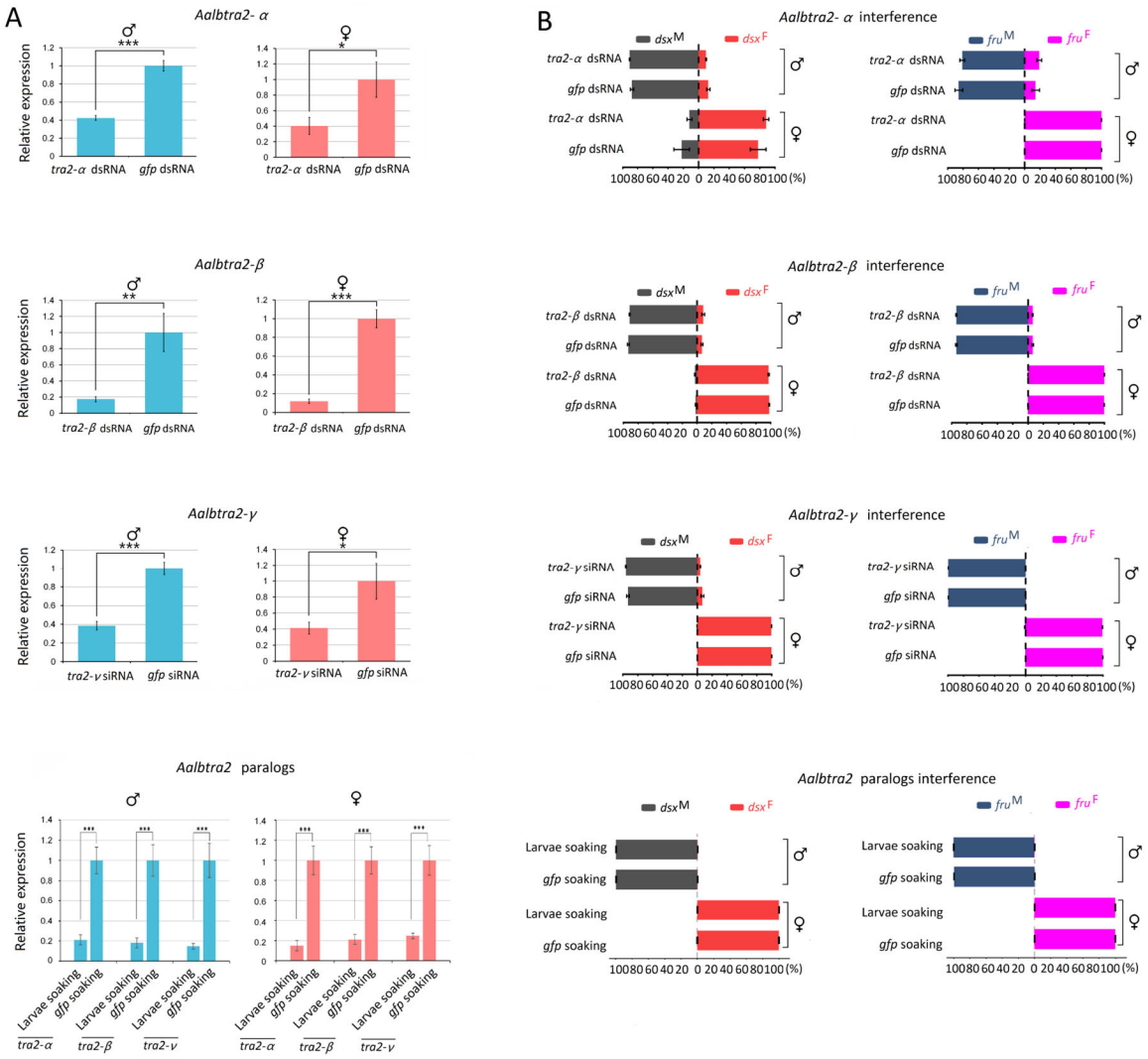

Figure 7. Expression ratios of male- and female-specific transcripts of *dsx* and *fru* in *Aedes albopictus* adults subjected to *Aalbtra2-α*, *Aalbtra2-β* and *Aalbtra2-γ* interference in adulthood or as larvae through soaking.

(A) Analysis of the effects of *Aalbtra2* interference in *Ae. albopictus* adults by qRT–PCR. The bars represent the SEM (n = 3). The x-axis indicates the groups. (B) Expression percentages of male- and female-specific *dsx* and *fru* transcripts in groups with *Aalbtra2* interference compared to control groups. The y–axis indicates the sample ID, and the x–axis shows the relative percentage of male- or female-specific transcripts among total *dsx* and *fru* transcripts based on the relative quantitation (RQ) value obtained by qRT–PCR. The error bars represent the SEM. *Ae. albopictus* ribosomal protein 7 (*rpS7*) mRNA was used as an internal control (*P < 0.05, **P < 0.01, and ***P < 0.001).

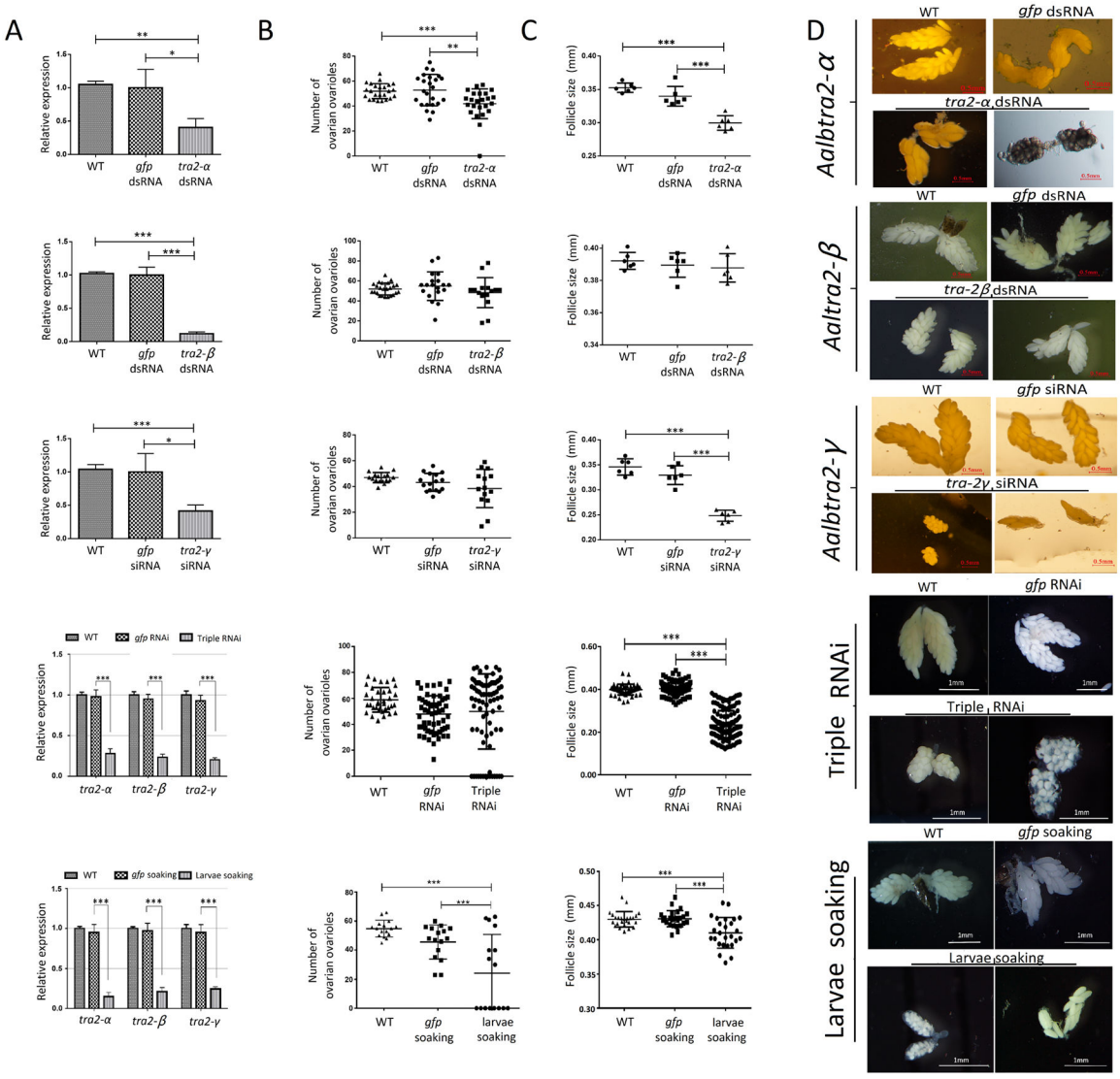

Figure 8. Effect of *Aalbtra2* knockdown on ovarian development in adult female *Aedes albopictus*.

(A) Relative expression of *Aalbtra2* in dsRNA or siRNA treatment groups (row 1–3), dsRNA and siRNA injection groups (row 4) or soaking knockdown groups (row 5) compared with GFP control groups. The error bars represent the SEM (n = 3). The x–axis indicates the groups; WT indicates wild-type mosquitoes without any treatment. The expression of each *Aalbtra2* gene in the GFP control group was set as 1 and used to normalize the *Aalbtra2* expression in the RNAi groups (*P < 0.05, **P < 0.01, and ***P < 0.001). (B) Number of ovarian ovarioles per female in the different treatment groups (single dsRNA or siRNA, triple interference and larvae soaking interference groups) compared with the GFP control groups. (C) Average follicle size (length of the long axis) in ovaries isolated from female mosquitoes in the *Aalbtra2-α*, *Aalbtra2-β* and *Aalbtra2-γ* treatment groups (single dsRNA or siRNA, triple interference and larvae soaking interference groups) and control groups. The data represent six biological replicates with 8–10 individuals in each replicate and are shown as the mean ± SEM (*P < 0.05, **P < 0.01, and ***P < 0.001). (D) Representative images of ovaries dissected from WT mosquitos and mosquitoes from the

Aalbtra2-α, *Aalbtra2-β* and *Aalbtra2-γ* treatment groups (single dsRNA or siRNA, triple interference and larvae soaking interference groups) and GFP control groups at 36 hr post blood meal (p.b.m.). The images were taken under a Nikon SMZ1000 stereomicroscope with Digital Sight DS–U3 (scale bar: marked in the picture).

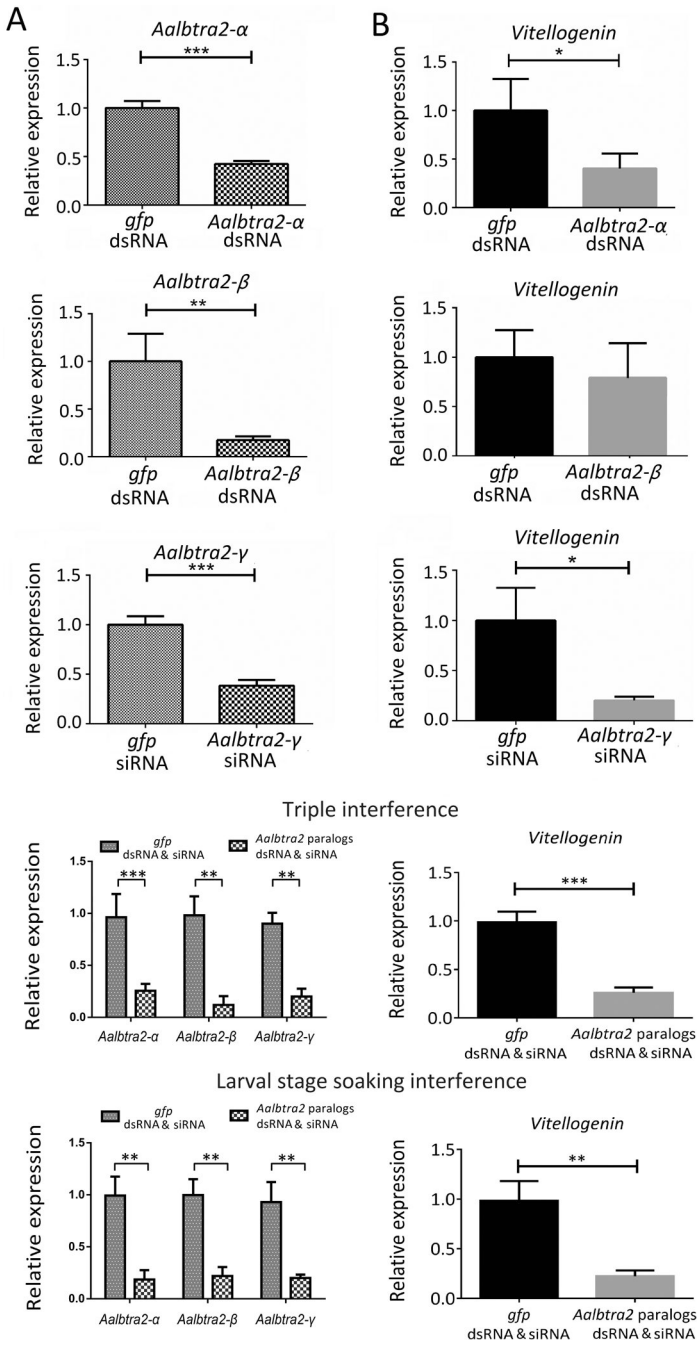

Figure 9. Effect of *Aalbtra2* knockdown on *vitellogenin* gene transcription in *Aedes albopictus* post blood meal (p.b.m.).

(A) Relative expression of *Aalbtra2* in various treatment groups (dsRNA or siRNA treatment, row 1–3; triple interference, row 4; and larvae soaking interference, row 5) compared with GFP control groups. The x–axis indicates the groups. (B) The relative expression of *vitellogenin* was quantified in female mosquitos from *Aalbtra2-α*, *Aalbtra2-β*, and *Aalbtra2-γ* treatment groups (dsRNA or siRNA treatment, row 1–3; triple interference, row 4; and larvae soaking interference, row 5) and control groups at 36 hr p.b.m.. The data are presented as the mean ± SEM (n=3; *P < 0.05, **P < 0.01, ***P < 0.001).

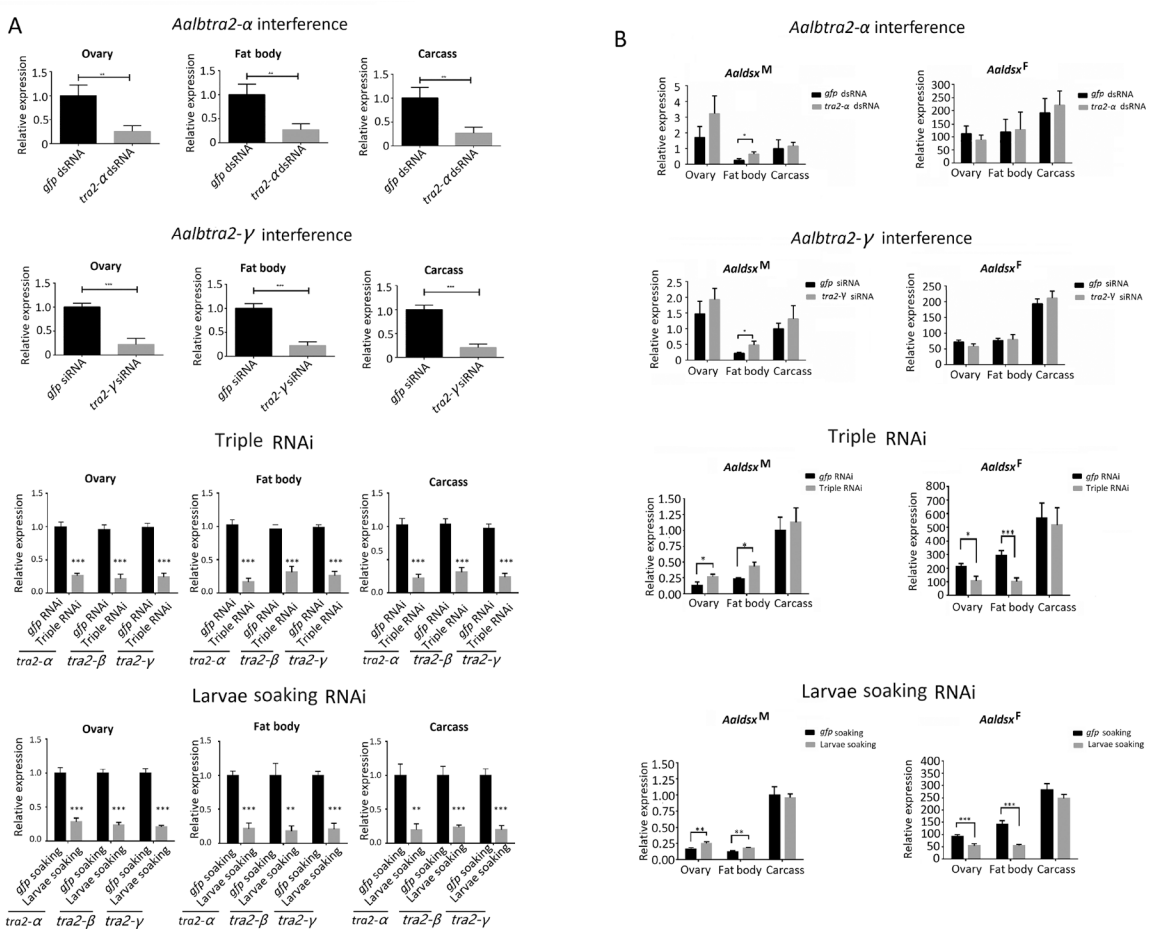

Figure 10. Effect of *Aalbtra2-α* or *Aalbtra2-γ* knockdown, triple interference and larvae soaking interference on the levels of sex-specific *dsx* transcripts in the ovary, fat body, and carcass.

For analysis of the sex-specific splicing patterns of *dsx* mRNA in adults subjected to *Aalbtra2-α* or *Aalbtra2-γ* interference or triple interference by RNAi, fat body and ovary samples were dissected from females at 72 hr post injection (p.i.) and 36 hr post blood meal (p.b.m.). For analysis of the sex-specific splicing patterns of *dsx* mRNA in adults that had been subjected to larvae soaking interference, fat body and ovary samples were dissected from females 36 hr p.b.m.. (A) Relative expression of *Aalbtra2* in dsRNA or siRNA treatment groups (row 1–2), triple interference groups (row 3) and larvae soaking interference groups (row 4) (gray) compared with GFP control groups(black). The error bars represent the SEM (n = 3). The x–axis in row 1–2 indicates the injected dsRNA or siRNA in the treatment and control groups, and the x-axis in row 3–4 indicates the gene. The expression of each *Aalbtra2* gene in the GFP control group was set as 1 and used to normalize *Aalbtra2* gene expression in the RNAi groups. (B) $Aalbdsx^{M}$ and $Aalbdsx^{F}$ levels in the ovary, fat body, and carcass were quantified with gene-specific primers in the *Aalbtra2-α* or *Aalbtra2-γ* interference groups, triple interference groups and larvae soaking interference groups compared with the control group, respectively. *Ae. albopictus* ribosomal protein 7 *(rpS7)* mRNA was used as an internal reference. The data are presented as the mean ± SEM (n = 3; *P < 0.05, **P < 0.01, and ***P < 0.001).