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Research article

Isolation and Molecular Characterization of the *Transformer* Gene From *Bactrocera cucurbitae* (Diptera: Tephritidae)

Ya Luo,[1,*] Santao Zhao,[1,*] Jiahui Li,[1] Peizheng Li,[1] and Rihui Yan[1,2,3]

[1]Department of Plant Protection, Institute of Tropical Agriculture and Forestry, Hainan University, Haikou, Hainan Province, China (707837080@qq.com; jiahui.li1984@qq.com; 1807504292@qq.com; 13756124@qq.com; ryan1@hainu.edu.cn), [2]Corresponding author, e-mail: ryan1@hainu.edu.cn, and [3]Hainan Key Laboratory for Sustainable Utilization of Tropical Bioresources, Hainan University, Haikou, Hainan Province, China (ryan1@hainu.edu.cn)

*These authors contributed equally to this work.
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Abstract

transformer (*tra*) is a switch gene of sex determination in many insects, particularly in Dipterans. However, the sex determination pathway in *Bactrocera cucurbitae* (Coquillett), a very destructive pest on earth, remains largely uncharacterized. In this study, we have isolated and characterized one female-specific and two male-specific transcripts of the *tra* gene (*Bcutra*) of *B. cucurbitae*. The genomic structure of *Bcutra* has been determined and the presence of multiple conserved Transformer (TRA)/TRA-2 binding sites in *Bcutra* has been found. BcuTRA is highly conservative with its homologues in other tephritid fruit flies. Gene expression analysis of *Bcutra* at different developmental stages demonstrates that the female transcript of *Bcutra* appears earlier than the male counterparts, indicating that the maternal TRA is inherited in eggs and might play a role in the regulation of TRA expression. The conservation of protein sequence and sex-specific splicing of *Bcutra* and its expression patterns during development suggest that *Bcutra* is probably the master gene of sex determination of *B. cucurbitae.* Isolation of *Bcutra* will facilitate the development of a genetic sexing strain for its biological control.

Key words: *Bactrocera cucurbitae* (Coquillett), transformer, Bcutra, sex determination, sex-specific splicing

Sex determination that generates female and male dimorphism is a fundamental characteristic of animals and is an essential component of sexual reproduction that is important for the continuity of life. The sex determination of insects has been heavily studied and has been one of the best understood systems (Sanchez 2008, Bachtrog et al. 2014, Bopp et al. 2014). There are several genetic mechanisms that determine the sexual fate among different insects (Saccone et al. 2002, Sanchez 2008, Gempe et al. 2009, Hediger et al. 2010, Shukla and Palli 2012, Verhulst et al. 2013, Kiuchi et al. 2014, Morrow et al. 2014). In general, the primary signals that initiate the sexual fate are perceived by early effectors and thereafter such genetic information are conveyed in early embryonic stages to determine sex and sustained for the sexual identity in the whole life of an organism.

The genetic mechanism of sex determination in *Drosophila melanogaster* has been extensively examined. In *D. melanogaster*, the primary signal has been considered to be the ratio of X chromosomes to autosomes (X:A), which actually represents the interaction of the proteins encoded by X-linked elements with the proteins encoded by autosomes (Erickson and Quintero 2007). *Sex lethal* (*Sxl*) that acts as a binary switch gene can be switched on or off by the primary signals (Nagoshi et al. 1988). When the X:A ratio is 1:1 (XX:AA), the early female-specific promoter of *Sxl* is activated, producing an early female-specific SXL protein. This early protein enables the generation of the late SXL protein that can maintain the female-specific splicing of its own pre-mRNA by autoregulation. The late SXL protein also directs the female-specific splicing of its subordinate gene *tra*, generating a functional TRA protein (Boggs et al. 1987, Nagoshi et al. 1988, Belote et al. 1989, Inoue et al. 1990). TRA and the nonsex-specific Transformer-2 (TRA-2) form a complex to regulate the female-specific splicing of the pre-mRNAs of *double sex* (*dsx*) and *fruitless* (*fru*), leading to the generation of non-functional FRU^F peptide and functional DSX^F protein that stimulates the downstream genes to adopt the female pathway of development. On the contrary, when the ratio of X:A is 0.5 (XY:AA, or XO:AA), the early promoter of *Sxl* is not activated and no early SXL protein is synthesized. Consequently the pre-mRNA of *Sxl* adopts the male-specific splicing way by default and yields a male-specific transcript with premature in-frame stop codons, resulting in a truncated nonfunctional SXL peptide. The absence of functional SXL protein leads to a cascade of its downstream genes with the

male-specific splicing mode, including the generation of nonfunctional TRA and functional male-specific DSX^M and FRU^M proteins that promote male sexual development (Burtis and Baker 1989, Hoshijima et al. 1991, Tian and Maniatis 1993).

In contrast to *D. melanogaster*, the primary signal of sex determination in *Ceratitis capitata* (Wiedemann), a tephritid fruit fly, is an uncharacterized *male determining factor* (*M* factor) on Y chromosome (Willhoeft and Franz 1996). It has been proposed that the Y-linked *M* factor regulates the sex-specific expression of the gene *tra* by suppressing maternal TRA (Pane et al. 2002). *Sxl*, however, is expressed in both sexes regardless of the existence of the *M* factor (Saccone et al. 1998), suggesting that *Sxl* probably does not have the switch function as in *D. melanogaster*. In XX females where the *M* factor is absent, the splicing of the *tra* homologue of *C. capitata* (*Cctra*) is female specific and can produce a functional TRA protein, and CcTRA in turn facilitates its own female-specific splicing through forming an autoregulatory loop (Pane et al. 2002, Gabrieli et al. 2010). The CcTRA/CcTRA-2 complex has been postulated to bind to the putative TRA/TRA-2 binding sites only existed in the male-specific exons and to inhibit the default male-specific splicing mode. Therefore, the female-specific splicing of *dsx* pre-mRNA is activated, producing a functional female-specific DSX^F protein and promoting female sexual development and differentiation. In XY males, however, the presence of the *M* factor leads to the male-specific splicing of *Cctra* that generates a truncated non-functional TRA peptide. Without functional TRA, the downstream gene *dsx* is expressed as functional male-specific DSX^M (Pane et al. 2002, Salvemini et al. 2009, Gabrieli et al. 2010).

Although the primary signals of sex determination are different among species, the downstream genes are relatively conserved, such as *tra*, *tra-2*, and *dsx*. *tra* has also been isolated and characterized from other Dipteran flies, such as *Bactrocera dorsalis* (Liu et al. 2015, Peng et al. 2015, Laohakieat et al. 2016), *B. oleae* (Lagos et al. 2007), *B. tryoni*, *B. jarvisi* (Morrow et al. 2014), *Anastrepha suspensa* (Schetelig et al. 2012), *A. obliqua* (Ruiz et al. 2007), *Lucilia cuprina* (Concha and Scott 2009), *L. sericata*, *Cochliomyia hominivorax*, and *C. macellaria* (Li et al. 2013). Their molecular organizations and sex-specific splicing patterns are similar to those found in *C. capitata*. In females, the male-specific exons of those *tra* genes are spliced out and the resulting transcript is female-specific and can be translated into a functional TRA protein; however, in males, the final transcript includes the male-specific exons with in-frame stop codons and therefore only truncated nonfunctional TRA peptides are translated. The putative TRA/TRA-2 binding sites in the male-specific exons have been found in the *tra* genes of all the species mentioned above suggest that the regulation of their sex-specific splicing is also similar to the mode in *C. capitata*, i.e., an autoregulatory mechanism may also occur in those *tra* genes.

The master role of *tra* in determining sexual dimorphism makes *tra* a potential target gene for genetic control of pests. Previous studies have shown that the XX embryos that are supposed to develop into females were reversed to XX pseudomales when the expression of *tra* was transiently knocked down by microinjecting dsRNA of *tra* into the embryos of many Dipteran flies, such as *C. capitata* (Pane et al. 2002), *B. dorsalis* (Liu et al. 2015, Peng et al. 2015, Laohakieat et al. 2016), *B. oleae* (Belote et al. 1989), *B. tryoni* (Raphael et al. 2014), *C. macellaria*, *L. sericata* (Li et al. 2013), *A. suspensa* (Schetelig et al. 2012), and *L. cuprina* (Concha and Scott 2009). This ability to reverse XX females to pseudomales can be highly advantageous for the application of sterile insect techniques (SIT) in which the male-only release is much more efficient than the release of both sterile males and females (Rendon et al. 2004). Moreover, the region of male-specific exons of *tra* genes, which acts as an intron in females, has been applied in the control strategy of female-specific lethality in some species and the technology has shown its great potential in genetic control of pests. For example, a female-specific autocidal genetic system has been established in *C. capitata* through the insertion of a *Cctra* intron into the gene of a heterologous tetracycline-repressible transactivator (tTAV). In this system, the tTAV transcript is disrupted in males as the *Cctra* intron is not spliced out while the female tTAV transcript is complete due to the removal of the *Cctra* intron, and therefore generating a functional tTAV protein that acts as a transactivator and is toxic to cells when it is overexpressed (Fu et al. 2007). Similar systems have established for *L. cuprina* (Li et al. 2014) and *C. hominivorax* (Concha et al. 2016).

The melon fly *Bactrocera cucurbitae* (Diptera: Tephritidae: Dacini) is widely distributed in temperate, subtropical, and tropical regions of the world, causing severe damages in many countries, particularly in China and India. It has been reported that *B. cucurbitae* damages over 81 host plants and is a very destructive pest of cucurbitaceous vegetables, such as bitter gourd, cucumber, and pumpkin (Dhillon et al. 2005). Many management methods have been applied for its control and one of them is SIT. SIT can be improved by the male-only release that can be achieved by the introduction of female-specific lethality obtained from molecular and genetic modification methods (Rendon et al. 2004, Fu et al. 2007, Li et al. 2014, Concha et al. 2016). However, the mechanism of sex determination and differentiation in *B. cucurbitae* is still obscure, particularly, the *transformer* gene of *B. cucurbitae* has not been identified yet and the role of *transformer* in sex determination of *B. cucurbitae* remains unclear.

In this article, we isolate and characterize the *transformer* gene of *B. cucurbitae* and examine its expressions at different developmental stages. Phylogenetic tree analysis of BcuTRA demonstrates that it is very similar to the TRA proteins from other tephritid insects. Our results will be beneficial to the understanding of sex determination of *B. cucurbitae*, the expansion of our knowledge about insect sex determination and the establishment of the theoretical basis for its genetic control in future.

Materials and Methods

Rearing of *B. cucurbitae*

The melon flies used in this study were collected on City West campus of Hainan University and maintained in the laboratory at 26°C, 70% RH, and a photoperiod of 14:10 (L:D) h. The larvae were reared on artificial diet (Zhou et al. 2016), and the grown larvae were transferred into small plastic boxes with sand before pupation. After 7 d, the pupae were transferred into insect cultivation cages for eclosion. The adults were fed water and a protein-rich food consisting of 1:2 brewer yeast powder/sugar (w/w).

Isolation of *Bcutra*

To isolate *Bcutra*, total RNA of *B. cucurbitae* female adults was prepared using TRI Reagent (Sigma-Aldrich) following the manufacturer's instructions. The total RNA was treated with DNase I (TaKaRa, Dalian, China), extracted with phenol/chloroform, precipitated with ethanol, and resuspended in nuclease-free water to be used directly for RT-PCR reverse transcription polymerase chain reaction. The first-strand of cDNA was synthesized with an oligo(dT) primer using TaKaRa Prime Script RT-PCR Kit (TaKaRa, Dalian, China). *Bcutra* cDNA was amplified by PCR with the primers F311+ and R2793- (Supp Table 1 [online only]). These primers were designed on the basis of the alignment of cDNA sequences and protein sequences from *B. dorsalis*, *B. oleae*, *B. jarvisi*, *B. tryoni*,

and *B. correcta*. This procedure yielded a specific amplification product of 1,165 bp in length. To identify the 5′ and 3′ ends of the *Bcutra* transcript, 5′ and 3′ RACE rapid amplification of cDNA ends reactions were performed on the 5′ and 3′ RACE cDNA libraries that were made from *B. cucurbitae* females using SMARTer RACE 5′/3′Kit (Clontech). Two rounds of PCR were performed with the specific primers complimentary to the RACE adaptors and gene-specific primers. For the 3′ RACE, the primer pairs for the first and second (nest) rounds of PCR were F2715+/UPM and F2765+/NUP, respectively. For the 5′ RACE, the primer pairs for the first and second (nest) rounds of PCR were R1625-/UPM and R338-/NUP, respectively.

To determine the male-specific transcripts of *Bcutra*, similar RT-PCR using male total RNA was carried out with the primers F11+ and R2865-.

To determine the genomic structure of *Bcutra*, genomic DNA was prepared from adults of *B. cucurbitae* using Wizard Genomic DNA Purification Kit (Promega), the *Bcutra* gene was amplified by PCR with the primers F11+ and R2865-.

All PCR products were purified with TaKaRa Mini BEST Agarose Gel DNA Extraction Kit (TaKaRa, Dalian, China) and sequenced by outsourcing (Huada Gene, Shenzhen, China).

Sequence Analysis

Sequence similarity and alignment analysis were performed using BLAST program from NCBI website. Multiple sequence alignment of TRA proteins from different Dipterans was performed using the software DNAMAN (Lynnon Corp.). A neighbor-joining distance tree of TRA proteins was constructed using MEGA6 (Tamura et al. 2013), and the reliability was assessed by the bootstrap method.

Expression of *Bcutra* Over Development by Semiquantitative RT-PCR

To analyze the temporal expression of *Bcutra*, total RNAs from different developmental stages (eggs at 0–0.5, 0.5–1, 1–3, 3–6, 6–12, 12–24, and 24–48 h after egg laying; the mixed sexes of the first, second, and third instar larvae, the mixed sexes of pupae; immediately eclosed females and males) were prepared using TRI Reagent (Sigma-Aldrich) and treated with DNase I (TaKaRa, Dalian, China), the synthesis of the first strand of cDNA was performed using TaKaRa Prime Script RT-PCR Kit (TaKaRa, Dalian, China) following the manufacturer's instructions. Semiquantitative RT-PCRs were performed using the primer pairs F11+/R1573- and F311+/R1063-. The RT-PCR product of the reference gene *α-tubulin* with the primer pair of α-tubF and α-tubR was used as a control (Liu et al. 2015).

All the primers used in this article are listed in Supp Table 1 [online only].

Results

Gene Structure and Splicing Pattern of *Bcutra*

To obtain the *Bcutra* transcription units, we carried out RT-PCR and RACE experiments using total RNA from females and males to obtain full-length cDNAs. Those results revealed that *Bcutra* is sex-specific splicing (Fig. 1A), similar to *tra* from other *Bactrocera* species (Lagos et al. 2007, Morrow et al. 2014, Liu et al. 2015, Laohakieat et al. 2016) and other Dipterans (Verhulst et al. 2010, Geuverink and Beukeboom 2014). *B. cucurbitae* females contain only one transcript of 1,685 nt (GenBank: KY616908) that comprises a long open reading frame. However, males contain 2 different transcripts (male 1 and male 2) of 2,041 nt (GenBank: KY616909) and 2,424 nt (GenBank: KY616910), both of which are prematurely terminated in that multiple in-frame stop codons exist in the male-specific exons. The full-length *Bcutra* gene (GenBank: KY616911) is 3,038 bp. The female and male transcripts share the five exons that are designated as exons 1A, 1B, 2, 3, and 4, whereas the exons MS1, MS2, and MS3 are male specific (Fig. 1A).

The translation start codon is located on the second exon. The TRA protein in females consists of 420 amino acids with a predicted molecular weight of 48.85 kDa and PI value of 11.38. No transmembrane structure was found based on the bioinformatics method TMHMM (Krogh et al. 2001). Both of the male transcripts start translation at the same position with the female transcript but only produce a prematurely truncated and nonfunctional peptide with 66 amino acids as the first stop codon (TAA) exists in MS1 exon at the position of 371 bp (Fig. 1A). Based on the data from similarity analysis of D. *melanogaster* (Qi et al. 2007), B. *dorsalis* (Liu et al. 2015, Laohakieat et al. 2016), C. *capitata* (Pane et al. 2002), *Anastrepha* spp. (Ruiz et al. 2007), six TRA/TRA-2 binding sites, one TRA-2 intronic splicing silencer (ISS) sequence (Qi et al. 2007) and two type B of RBP1 binding sites (Heinrichs and Baker 1995, Qi et al. 2007) were found in the *Bcutra* gene (Fig. 1B). Moreover, BcuTRA contains 10 highly conserved amino acids at the region of 284–294, characteristic of the Serine-arginine dipeptides (SR) family that functions in the regulation of protein interactions and specific splicing site recognition (Manley and Tacke 1996, Lagos et al. 2007).

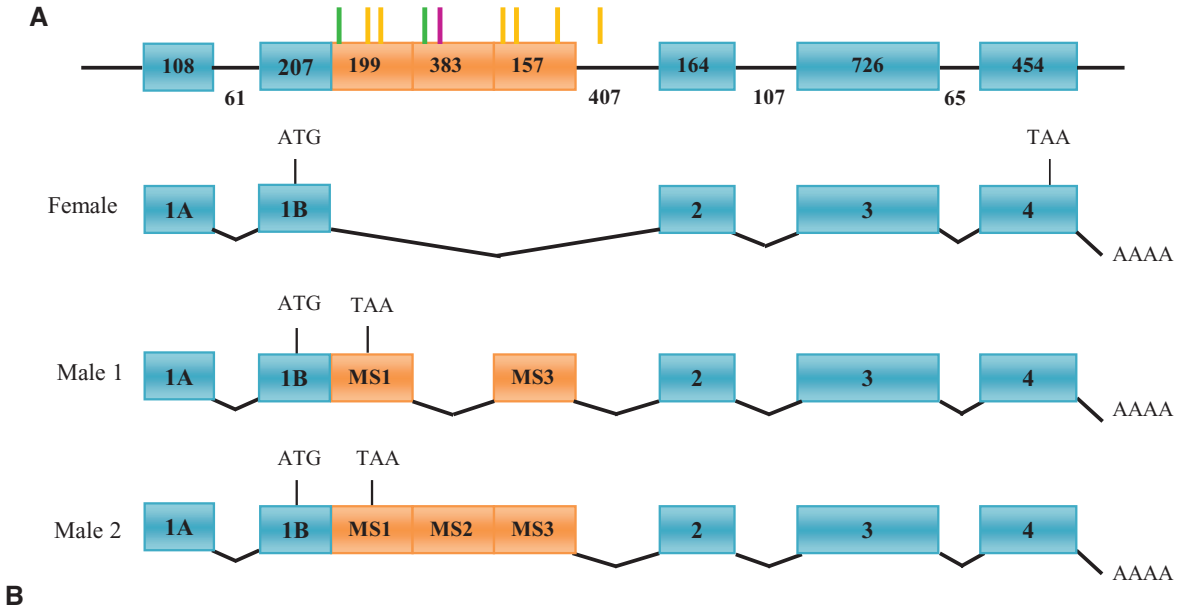
Amino Acid Sequence Alignment and Phylogenetic Analysis of *Bcutra*

In order to explore the phylogenetic relationship of TRA proteins in Dipterans, including *B. cucurbitae*, *B. dorsalis*, *B. oleae*, *B. correcta*, *B. tryoni*, *B. jarvisi*, *A. suspensa*, *C. capitata*, *D. melanogaster*, multiple sequence alignment and phylogenetic analysis were performed. The results showed that these TRA proteins from Dipteran insects are very similar, exhibiting 71% amino acid identity (Fig. 2A). BcuTRA exhibits the highest degree of similarity with TRA from *B. oleae*, 74% identical to *B. oleae* TRA. The secondary similarity occurs between BcuTRA and the TRA protein of *B. dorsalis*, which is 71% identical. On the other hand, BcuTRA is only 37% identical to the TRA of *D. melanogaster*. Indeed, Drosophila TRA is considerably shorter than BcuTRA, only 197 amino acids and lack of the amino terminal domain of tephritid TRA.

The phylogenetic tree of 15 Dipteran TRA proteins was reconstructed using neighbor-joining method replicated 1,000 times with bootstrap resampling. The results revealed that the phylogenetic relationship for TRA proteins from different Dipteran species is in high agreement with the taxonomic relationship (Fig. 2B). The species of genus *Bactrocera*, including *B. correcta*, *B. cucurbitae*, *B. dorsalis*, *B. jarvisi*, *B. tryoni*, and *B. oleae*, form one cluster, while *Anastrepha* species are clustered into the *Anastrepha* group. The genera of *Bactrocera*, *Anastrepha*, and *Ceratitis* are grouped into the branch of Tephritidae. As expected, TRA from *B. cucurbitae* and other *Bactrocera* species are more close to the TRA protein from other tephritid insects than that of Muscidae, Calliphoridae, and Drosophilidae.

Developmental Expression of *Bcutra*

To determine the expression pattern of *Bcutra* at various developmental stages of *B. cucurbitae*, RT-PCR was performed with the primers that amplify a region across the shared first and third exons of *Bcutra*, generating products of different sizes from the transcripts in females and males (Fig. 3A). As shown in Figure 3B, the female *Bcutra* transcript appeared from early embryonic stages to pupae as there was always an amplification product of 366 bp detected at these stages. Moreover, the female transcript appeared earlier than



B

TRA-2 ISS sequences

D.melanogaster	B.dorsalis	C.capitata	Anastrepha spp.
G **CAAG** A	G **CAAG** A	G **CAAG** A	G **CAAG** A

TRA/TRA2 binding sites

D.melanogaster	B.dorsalis	C.capitata	Anastrepha spp.
AA A TC C **ATCAACA** TT G	GTAC C CT **CAAT** AA ACTA T AA	CAA CAACA N T **CAAT** T ATT TGTAG	A A C A A C N **CATCAAC** G T A G T

RBP1 binding sites

D.melanogaster	B.dorsalis	C.capitata	Anastrepha spp.
type A	type A	type A	type A
T G **ACATCTTTA** G A	None	None	T A **CA CTTTA** A G
type B	type B	type B	type B
C **ATC NNA** T	C **ATC NNA** T	TA **ATC NA** CG	AA **ATCTT A** CT

B.*cucurbitae*
TRA/TRA2 binding sites

GCAT**CAAT**CAACA

ACTA**CAAT**CAACA

ACTA**CAAT**CAACA

ACAA**CAAT**CAACA

ACAA**CAAT**CAACT

CAGT**CAAT**TTAAT

B.*cucurbitae*
TRA-2 ISS sequences

CAAGG

B.*cucurbitae*
RBP1 binding sites

ATCTTA**A**

ATCCTTA

Fig. 1. (A) Genomic structure and transcripts of *Bcutra*. (B) The consensus sequences of the putative TRA/TRA-2 binding sites, TRA-2 ISS and RBP1 binding sites. (A) Exons and introns of *Bcutra* are represented by boxes and lines, respectively. Exon and intron sizes are represented by the numbers in the boxes and under the lines, respectively. Exons 1A, 1B, 2, 3, and 4 (blue) are common in both females and males. Exons MS1, 2, and 3 are male specific. The start codon and the first stop codon in exons are labeled by black vertical bars. The positions of the putative TRA/TRA-2 binding sites, TRA-2 ISS, and RBP1 binding sites are labeled by purple, yellow, and green vertical bars, respectively. The diagram is not drawn to scale. (B) The conserved sequences are bolded.

the male transcripts as the amplification products of 722 and 1,105 bp from the male transcripts were only detected from the embryos 2 h after egg laying (Fig. 3B). In order to determine the expression of *Bcutra* in males more precisely, the male-specific transcripts were amplified with the primers of F311+ that is common for the transcripts of males and females and R1063- that is located on the male-specific exon. The results revealed that the male-specific transcripts were able to be detected from the embryos 1 h after egg laying although the amplification bands were a little faint.

Discussion

In this study, the *transformer* gene of the tephritid pest *B. cucurbitae* was isolated using RT-PCR and RACE and characterized using multiple methods. Our data show that the *transformer* gene *Bcutra* in *B. cucurbitae* is sex-specific alternative splicing, similar to that of other tephritid fruit flies, such as *B. oleae*, *C. capitata*, *A. suspensa*, *Bcutra* in females produces a female-specific transcript that encodes a functional TRA protein, whereas *Bcutra* in males generates two male-specific transcripts that are translated as a nonfunctional truncated

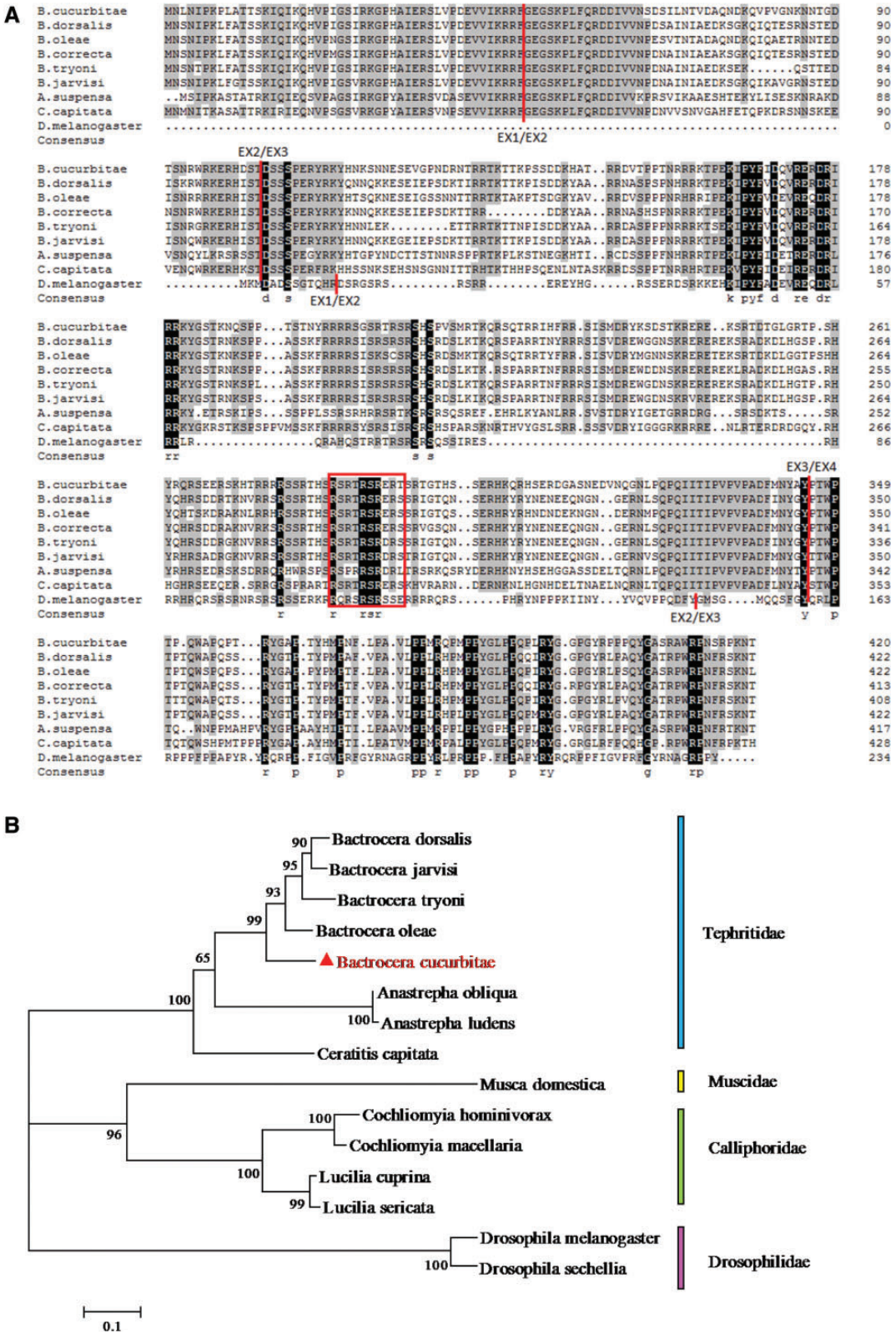


Fig. 2. Multiple sequence alignment and phylogenetic tree of TRA proteins. (A) Multiple sequence alignment of TRA proteins in *B. cucurbitae*, *B. dorsalis*, *B. oleae*, *B. jarvisi*, *B. tryoni*, *B. correcta*, *A. suspensa*, *C. capitata*, and *D. melanogaster*. The black-shaded represent the amino acids that are identical in all species, and the gray-shaded represent that the amino acid similarity between species is higher than 75%. The boundary of exons is shown by red vertical lines. SR are within the red box. (B) The neighbor-joining distance tree of Dipteran TRA proteins. The numbers next to the branches represent bootstrap support values from 1,000 replicates. The scale represents the mean character distance. The families are exhibited by color bars on the right. *B. cucurbitae* (this study) is highlighted with red and with the red triangle. *A. obliqua* (GenBank: ABW04165.1), *A. ludens* (GenBank; ABW04176.1), *B. dorsalis* (GenBank: AME17913.1), *B. oleae* (GenBank: CAG29241.1), *B. tryoni* (GenBank: AIK25402.1), *B. jarvisi* (GenBank: AIK25400.1), *C. capitata* (GenBank: AAM88673.1), *C. macellaria* (GenBank: AGE31794.1), *C. hominivorax* (GenBank: AGE31793.1), *D. melanogaster* (GenBank: AAB59226.1), *D. sechellia* (GenBank: AAO38911.1), *L. sericata* (GenBank: AGE31795.1), *L. cuprina* (GenBank: ACS34687.2), *M. domestica* (GenBank: ACY40709.1).

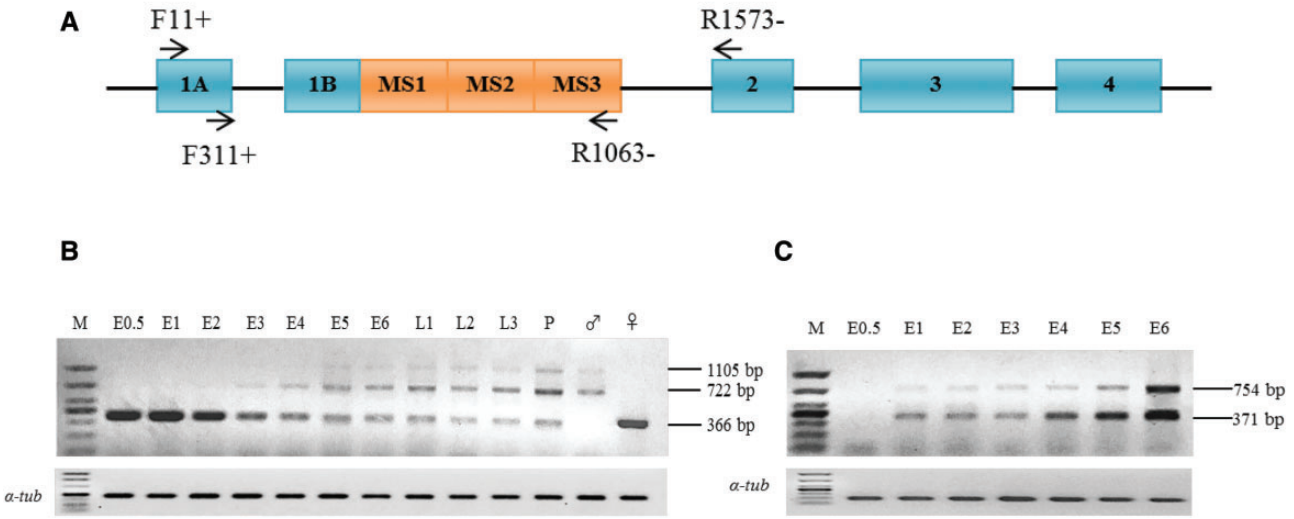


Fig. 3. Expression of *Bcutra* at different developmental stages. (A) The exon and intron structure of *Bcutra*. The primers used in (B) and (C) are labeled. (B) The male and female transcripts are amplified using RT-PCR with the primers F11+ and R1573-. (C) The male-specific transcripts are amplified using RT-PCR with the primers F311+ and R1063-. M: DL1000 DNA marker; E0.5-E8: embryos of mixed sexes at 0–0.5, 0.5–1, 1–3, 3–6, 6–12, 12–24, and 24–48 h after egg laying; L1–L3: the first, second and third instar larvae of mixed sexes; P: pupae of mixed sexes; ♂: male adults; ♀: female adults. *α-tub*: the internal control gene *α-tubulin*.

peptide in that there are multiple in-frame stop codons in the male transcripts.

The structure of *Bcutra* and the length of its transcripts in this work are significantly different from the previous transcriptome assembly by Geib and his colleagues in which one female-specific and two male-specific transcripts are predicted (Sim et al. 2015). The first exon predicted by them includes all the sequence of the first exon identified by our work except for the 10 bp (ACATATCTAT) at the very 5′ end. Moreover, the 3′ end of *Bcutra* in our work is 760 bp less than the one in the previous study. The polyadenylation signal sequence AATAAA exists at the 3′ end of *Bcutra* isolated from our work and a stretch of poly A is present at the 3′ end of the transcripts from 3′ RACE experiments, suggesting that our results are reliable. Although the female-specific transcripts from two studies are different, the two predicted TRA proteins based on the transcripts have the same sequence of amino acids. In addition, the second exon of the male-specific transcripts are different in size in that the second exon of male 1 and male 2 transcripts in our work are 406 and 946 bp, respectively, their sizes in the previous study, however, are 1,291 and 1,517 bp, respectively.

Our study demonstrates that BcuTRA protein contains a region of Serine-arginine dipeptides (Fig. 2A), suggesting BcuTRA belongs to the SR protein family that is likely to play an important role in protein–protein interactions and splicing control. Although no RNA-binding protein domain exists in BcuTRA, there are TRA/TRA-2 binding sites, TRA-2 ISS sequence, RBP1 binding sites in the male-specific exon region that belongs to the second intron in the female-specific pre-mRNA. The study on TRA-2 of *B. cucurbitae* has shown that TRA-2 has no sex-specific alternative splicing and belongs to the SR protein family with RNA binding domain (Liu and Wan 2015). These results indicate that BcuTRA may interact with TRA-2 to maintain the splicing of *Bcutra*, i.e., to autoregulate its own splicing, similar to the splicing of *tra* in other tephritid fruit flies. In previous studies from *B. dorsalis*, *C. capitata*, *A. suspensa*, et al., TRA has been found to interact with TRA-2 to from TRA/TRA-2 complex to bind the female-specific exon of the downstream gene *dsx* (Salvemini et al. 2009, Schetelig et al. 2012, Liu et al. 2015). The phylogenetic tree analysis of Dipteran TRA proteins demonstrates that BcuTRA clusters with the TRA proteins from genus *Bactrocera* and is genetically very close to the genera *Ceratitis* and *Anastrepha*. As discussed below, the female-specific *Bcutra* mRNA appears earlier than the male-specific counterparts (Fig. 3), indicating the existence of maternal *Bcutra* mRNA in embryos of *B. cucurbitae*. However, the sex determination of XY embryos is not disturbed by the maternal *Bcutra* mRNA, suggesting that its function is inhibited. Taken together, we propose the sex determination pathway in *B. cucurbitae* as shown in Figure 4. In *B. cucurbitae*, the existence of maternal *Bcutra* mRNA in XX embryos leads to the translation of functional BcuTRA to initiate the autoregulatory loop of the female-specific splicing of zygotic *Bcutra* pre-mRNA transcript. The newly translated zygotic BcuTRA and TRA-2 form BcuTRA/TRA-2 complex to maintain the autoregulation of *Bcutra* and control the female-specific splicing of the downstream gene *dsx*, which activates female sexual development. In contrast, in XY embryos a Y-linked M factor probably also exists in *B. cucurbitae* and prevents the expression or disrupts the functions of maternal *Bcutra* mRNA. Therefore, the initiation of the autoregulatory loop is suppressed and no functional BcuTRA is translated, resulting in the male-specific splicing of zygotic *tra* and *dsx*, and consequently male development.

The expression patterns of *Bcutra* at different developmental stages demonstrate that the female-specific transcript appears at very early embryonic stages. However, the male-specific transcripts appear at approximately 1 h later after egg laying, which is also later than the female counterpart. This pattern is similar to that of *B. dorsalis* (Liu et al. 2015). It is actually common as previous studies have shown that the appearance of male-specific transcripts is different in various species. For example, the male-specific transcripts are only detected 4–5 h after egg laying in *C. capitata* (Pane et al. 2002, Gabrieli et al. 2010) and are detected until the first larval stage in *Lucilia cuprina* (Concha and Scott 2009). Our research suggests that the upstream regulators of *Bcutra* may work in the sex determination pathway earlier in *B. cucurbitae* than in *C. capitata* and *L. cuprina*.

The isolation and characterization of *Bcutra* that reveal some interesting properties of *Bcutra* have made it become a potential target for genetic control of *B. cucurbitae*. The conservation of TRA/TRA-2 binding sites, TRA-2 ISS sequence, RBP1 binding sites, and SR region of tephritid *tra*, including *C. capitata* and *B. cucurbitae*, suggests that it

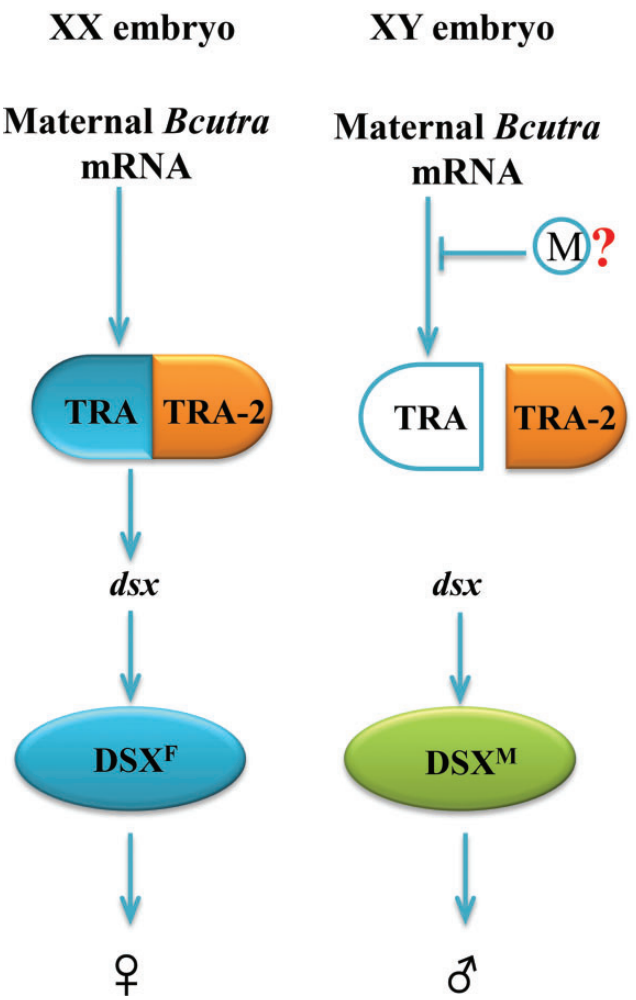


Fig. 4. Model for sex determination in *B. cucurbitae*. The functional proteins are represented with color, and the nonfunctional proteins are colorless. Female-specific, male-specific, and common proteins are labeled with blue, green, and orange, respectively.

is likely to use the strategy of female-specific lethality to genetically control *B. cucurbitae*. Although RNAi of knocking down *Bcutra* is not carried out yet, the high conservation of *Bcutra* indicates that a very similar result of the reversal from XX genotypic females to XX phenotypic males will probably occur when *Bcutra* is knocked down by RNAi techniques. And therefore, the genetic manipulation of *Bcutra* can be applied to improve the efficiency of SIT of the pest, or to control the pest by combining other genetic targets, such as the genes that can cause the pest lethal or sterile at a specific developmental stage when they are mutated or their expression is disrupted.

The research about sex determination of tephritid insects has been carried out heavily, however, most studies focus on the downstream regulated genes, such as *tra*, *tra-2* and *dsx*, the upstream regulators of sex determination have been not very clear. It will be intriguing to study the primary signal of sex determination and its downstream target genes to better understand the whole picture of sex-determining pathway, and therefore providing substantial theoretical basis for the research and applications of sex determination of insects.

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Supplementary Data

Supplementary data is available at *Journal of Insect Science* online.

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