Sexual Development in *Lucilia cuprina* (Diptera, Calliphoridae) Is Controlled by the Transformer Gene

Carolina Concha and Maxwell J. Scott¹

Institute of Molecular BioSciences, Massey University, Palmerston North, 4442, New Zealand

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ABSTRACT

Insects use an amazing variety of genetic systems to control sexual development. A Y-linked male determining gene (*M*) controls sex in the Australian sheep blowfly *Lucilia cuprina*, an important pest insect. In this study, we isolated the *L. cuprina transformer* (*Lctra*) and *transformer2* (*Lctra2*) genes, which are potential targets of *M*. The LCTRA and LCTRA2 proteins are significantly more similar to homologs from tephritid insects than *Drosophila*. The *Lctra* transcript is alternatively spliced such that only females make a full-length protein and the presence of six TRA/TRA2 binding sites in the female first intron suggest that *Lctra* splicing is autoregulated as in tephritids. LCTRA is essential for female development as RNAi knockdown of *Lctra* mRNA leads to the development of male genitalia in XX adults. Analysis of *Lctra* expression during development shows that early and midstage male and female embryos express the female form of *Lctra* and males express only the male form by the first instar larval stage. Our results suggest that an autoregulatory loop sustains female development and that expression of *M* inhibits *Lctra* autoregulation, switching its splicing to the male form. The conservation of *tra* function and regulation in a Calliphorid insect shows that this sex determination system is not confined to Tephritidae. Isolation of these genes is an important step toward the development of a strain of *L. cuprina* suitable for a genetic control program.

INSECTS have developed a great variety of genetic systems to determine sex (MARIN and BAKER 1998; SCHÜTT and NÖTHIGER 2000; SACCONI *et al.* 2002; SHEARMAN 2002; SANCHEZ 2008). One of them consists of a Y-linked male determining factor whose activity represses female development and promotes the male phenotype. This system controls sex determination in the Mediterranean fruitfly *Ceratitis capitata*, the Olive fruitfly *Bactrocera oleae*, and the house fly *Musca domestica*. In the latter, a Y-linked dominant male factor *M*, which can be autosomal in some rare strains, represses *F*, the key gene for female sex determination, leading to male development. In the absence of *M*, *F* is activated, resulting in female development (DÜBENDORFER *et al.* 2002). The zygotic activation of *F* requires maternal activity of the *F* gene and is highly dose sensitive. Indeed, zygotes that are heterozygous for *F* develop as normal females if they derive from a mother with two functional *F* alleles, while those derived from heterozygous mothers cannot sustain female development. Thus, *F* appears to be autoregulated (DÜBENDORFER and HEDIGER 1998). The Australian sheep blowfly *Lucilia cuprina*, is an economically important pest insect belonging to the

Caliptratae subsection of dipterans and thus closely related to the house fly *M. domestica* (BECK *et al.* 1985; HEATH and BISHOP 2006). Sex in *L. cuprina* is determined by a male determining region that is located near the Y chromosome centromere (BEDO and FOSTER 1985). However, the nature of the male determining factor as well as the subordinate genes that compose the sex determination cascade in this species are unknown.

The insect sex determination system that has been best characterized is that of the fruitfly *Drosophila melanogaster*. Until recently, it was thought that the ratio of X chromosomes to sets of autosomes constitutes the primary signal for sex determination in this insect (CLINE 1993; PENALVA and SANCHEZ 2003). However, recent evidence points to the number of X chromosomes rather than the X:A ratio as the primary signal (ERICKSON and QUINTERO 2007). According to this view, the male or female dose of X chromosomes is defined by the collective concentrations of four X-linked signal element (XSE) proteins in the zygote, which function to activate the *Sex-lethal* gene (*SXL-F*) in females, thereby promoting female development through a short cascade of downstream genes. *SXL-F* regulates the splicing of *tra* pre-mRNA such that only females produce an RNA that codes for a full-length and functional TRA protein (SOSNOWSKI *et al.* 1989). TRA forms a complex with TRA2, a cofactor that is constitutively expressed in both sexes and promotes the female-specific splicing of *doublesex* pre-mRNA (*dsx*), the last

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¹Corresponding author: Institute of Molecular BioSciences, Massey University, Private Bag 11222, Palmerston North, 4442 New Zealand.
E-mail: M.J.Scott@massey.ac.nz

component of the regulatory hierarchy. Interestingly, a feedback loop has been described to maintain SXL-F activity in *Drosophila*, where the SXL-F protein is capable of activating the splicing of its own pre-mRNA as well as that of *tra*, and reciprocally, female TRA from either maternal or zygotic expression stimulates *Sxl*-positive autoregulation (SIEIRA and CLINE 2008). In the absence of functional SXL protein, male-specific splicing of *tra* occurs by default, and hence, male-specific splicing of *dsx*, resulting in the development of the male phenotype. The male and female DSX proteins, DSX^M and DSX^F, are transcription factors that promote sexual development by activating the transcription of sex-specific differentiation genes.

WILKINS (1995) proposed that the sex determination gene hierarchy evolved from the bottom up. Consistent with this model, orthologs of *Drosophila Sxl* have been found in several dipterans including *Megaselia scalaris* (SIEVERT *et al.* 1997), *C. capitata* (SACCONE *et al.* 1998), *B. oleae* (LAGOS *et al.* 2005), *M. domestica* (MEISE *et al.* 1998), and the Calliphoridae species *Chrysomya rufifacies* (MULLER-HOLTKAMP 1995) and *L. cuprina* (P. Atkinson, personal communication). However, *Sxl* isn't sex-specifically spliced in these species and does not appear to have a role in sex determination. In contrast, at the bottom of the sex determination hierarchy, *dsx* is sex-specifically spliced in *Apis mellifera* (CHO *et al.* 2007), *Bombyx mori* (SUZUKI *et al.* 2001), *Anopheles gambiae* (SCALI *et al.* 2005), *M. scalaris* (KUHN *et al.* 2000), *M. domestica* (HEDIGER *et al.* 2004), *Anastrepha obliqua* (RUIZ *et al.* 2005), *C. capitata* (SACCONE *et al.* 2002), *B. tryoni* (SHEARMAN and FROMMER 1998), and *B. oleae* (LAGOS *et al.* 2005).

Outside of the genus *Drosophila*, the *transformer* gene has been isolated from the tephritids *C. capitata* (*Cctra*) (PANE *et al.* 2002), *B. oleae* (*Botra*) (LAGOS *et al.* 2007), and from several species from the genus *Anastrepha* (e.g., the West Indian fruit fly *A. obliqua*) (RUIZ *et al.* 2007). The genomic organization and sex-specific splicing of *tra* is similar in all of these tephritid species. As in *Drosophila*, only females produce an RNA that codes for a full-length TRA protein. Further, TRA is essential for female development in *C. capitata* and *B. oleae* as was shown by RNAi experiments (PANE *et al.* 2002; LAGOS *et al.* 2007). Interestingly, the tephritid TRA genes contain several putative TRA/TRA2 binding sites within the male-specific exons and their flanking introns. These findings suggested an autoregulatory mechanism for the maintenance of female-specific expression of *tra* in these species. Moreover, a recent study shows that *C. capitata tra2* is also required for maintaining the positive feedback regulation of *Cctra* during development and is therefore necessary for establishing female sex determination in female embryos (SALVEMINI *et al.* 2009). A proposed model for *tra* autoregulation in tephritids suggests that the binding of the TRA/TRA2 complex to male-specific exon sequences in *tra* mRNA causes a

blockage of the male-specific splice acceptor sites to the general splicing machinery preventing the incorporation of the male exons into the mature *tra* mRNA (PANE *et al.* 2002). However, since overexpression of *C. capitata tra* in XY *Drosophila* leads to the female-specific splicing pattern of *dsx* (PANE *et al.* 2005), it is likely that medfly TRA has also retained the splicing enhancer function described for *Drosophila* TRA.

In this article we have isolated and characterized the *transformer* and *transformer2* genes of *L. cuprina* with a long-term aim of understanding the genetic mechanism controlling sex determination in this important pest species and its evolution from that of other Diptera. We have found that *Lctra* shares several common features with tephritid *tra* genes, such as the presence of six TRA/TRA2 binding sites in its pre-mRNA and a unique N-terminal domain, which is absent in the *tra* homologs of all the *Drosophila* species. The function of *Lctra* in selecting and maintaining the female pathway of development is conserved, showing that the tephritid sex determination system is present in a broader group of insects including the Calliphoridae family. The isolation of these genes will be useful for the development of modified strains of *L. cuprina* that can be employed in a genetic control program.

MATERIALS AND METHODS

Rearing of *L. cuprina* strains: *L. cuprina* adults were maintained in the laboratory at a constant temperature of 21° under a 12/12 hr light/dark cycle. The flies were fed water and a protein-rich cookie and given lamb liver every 4 days for egg laying. The larvae were grown in commercial jelly meat pet food at 27° until the wandering third instar larval stage. The pet food was then removed and the pupae incubated at 27° until eclosion. The HS14 transgenic line of *L. cuprina* carries the *Lchsp83-ZsGreen* marker on the X chromosome and is maintained in a stable manner under the same conditions.

PCR, RACE, and recombinant DNA: To isolate the *L. cuprina* sex determination genes our general strategy was to perform two rounds of PCR using nested primers with cDNA templates. The degenerate primers were designed against conserved amino acid blocks and were designated F1 and R1 for the first round and F2 and R2 for the second round of PCR. To make the cDNA template, total RNA was extracted with TRIZOL reagent (Invitrogen) following the manufacturer's instructions. The RNA was Turbo-DNAse treated (Ambion), phenol/chloroform extracted, ethanol precipitated, and resuspended in nuclease-free water to be used directly for RT-PCR. Adult male and female RNA was further purified by affinity chromatography with oligo (dT) cellulose (Sigma). First strand cDNA synthesis was performed using an oligo (dT) primer and Expand Reverse Transcriptase (Roche). Cycling conditions for both PCR rounds were denaturation 95° for 2 min, then 30 cycles (denaturation 95° for 25 sec, annealing at 48° for 30 sec, and extension at 68° for 2 min), and finally extension at 68° for 5 min. Subcloning and sequencing of the candidate fragments were carried out by standard procedures.

The degenerate primers were:

Tra-F1 5' TTYCAA MGW GAT GATATW GTD GTD AAT CC 3'.
Tra-F2 5' GAA AAA RTT CCH TAT TTY RTT GAT GAA RTT
MGW GAA 3'.

Tra-R1 5' GG WAC DGG AAC DGG AAT WGT AAT WAT TTG DGG 3'.
 Tra-R2 5' GG TTG DGG DGG YAA ACC ATA DGG DGG 3'.
 Tra2-F1 5' TGT ATW GGT GTD TTY GGT TTR AAT ACH AAT AC 3'.
 Tra2-F2 5' TAT GGT CCH ATW GAA CGU ATW CAA GTD GTD 3'.
 Tra2-R1 5' TA HAC ACC DGG DGT DGG DGT ATG TGC ACG TTG 3'.
 Tra2-R2 5' TA AKC HAC ACG WAT ACG ACG ACC ATC HAC 3'.

To obtain full-length cDNA sequences, we extended the cDNA fragment on both sides by 5' and 3' RACE using the Smart RACE Kit and Advantage 2 taq DNA polymerase (Clontech). Two rounds of PCR were performed on the 5' and 3' RACE libraries with specific primers directed to the library's adaptors and gene-specific primers. The cycling conditions were denaturation 95° for 2 min, then 30 cycles (denaturation 95° for 25 sec, annealing at 65° for 30 sec, and extension at 72° for 2 min), and finally extension at 72° for 5 min. The gene-specific primers used were:

Tra-F1 5'-ACC TAT CGT CAT CAT CGT CGT CGT CAA CTG C-3'.
 Tra-R1 5'-GTG GAG AAC GAG TTC GTG AAC GTG TTC TAG-3'.
 Tra-F2 5'-CGG CGA AGA CGT TCA ACC AGT AGA GAT CGT-3'.
 Tra-R2 5'-GAA CGA CGG CTT CTG TAG TCT CTT CTT ACG C-3'.
 Luc-tra2-RV1 5'-CTG GTC GTC CCA TAT AAA CAC CAG GCG TTG-3'.
 Luc-tra2-FW1 5'-CTC AAA CCG GAC GTT CTC GGG GTT TTT GTC-3'.
 Luc-tra2-FW2 5'-GCA GCC TGT GAT AAT TGC TGT GGC ATG GAA-3'.

To analyze the expression pattern of *Lctra* and *Lctra2* over development, cDNA templates were prepared from total RNA isolated from various developmental stages. Thermal cycling conditions were those used for RACE. Gene-specific primers were designed to different exons so that amplification products were significantly smaller than from any contaminating genomic DNA. Further, negative control templates were prepared by omitting reverse transcriptase from the first strand cDNA synthesis reaction. The gene-specific primers used were:

Luc-Tra-FW 5'-ATG GAC TCC ATT ACA ACA GGA TTG GCA GCA-3'.
 Luc-Tra-RV 5'-CTA ATG TTG TGG GGG TAA ACC ACC ATA AGA CGC-3'.
 Tra2-FW 5'-ATG AGT CCA CGT TCA CAT AGT CGT TCT GTT ACA CCA-3'.
 Tra2-RV 5'-TTA ATG GTATCG ATA ACG ATA ACG ACG TGG TGA-3'.

qRT-PCR analysis was performed with adult male or female total RNA template as described previously (Li *et al.* 2008). The primers used were:

M2 for 5'-CAACGCAGATTTGCTAAATATTTGCAATG-3'.
 M1 for 5'-TAAGCTACTTTTAAAGCTAAATATTCGAATGG-3'.
 Mrev 5'-TATCACGGGCATCTAGGGTTGTTTG-3'.
 α -tub for 5'-GTGATTTGGCCAAGGTACAACGTG-3'.
 α -tubrev 5'-CGACGTACCAGTGGACGAAAGC-3'.

RNAi: A cDNA fragment of the female *Lctra* gene was amplified from cDNA template with primers that introduced a

T7 promoter sequence at each of the product ends. The resulting 920-bp fragment, comprising part of exon 1, exons 2 and 3, and part of exon 4, was used to produce dsRNA fragments by *in vitro* transcription performed with the Megascript kit (Ambion). The dsRNA was ethanol precipitated and resuspended in injection buffer (0.1 mM sodium phosphate (pH 6.8), 5 mM KCl) to a final concentration of 1 μ g/ μ l. Embryos of a cross between males of the HS14 line and wild-type females were collected within 30 min of egg laying, microinjected, and allowed to develop at 21° until the stage of first instar larvae. The larvae were then observed under the fluorescent microscope and separated into fluorescent green larvae (XX individuals) and nonfluorescent larvae (XY individuals) and grown separately in pet food at 27°. After eclosion the adult flies were observed under the microscope for sexually dimorphic traits.

Sequence analysis: Protein multiple sequence alignment was performed using CLUSTAL-W 1.83 software and analysis of the alignments was performed using BOX SHADE. Phylogenetic analysis was carried out using a basic neighbor-joining algorithm in the Geneious software package and FigTree v1.1.1 was used to draw the tree. The putative TRA-TRA2 binding sites were identified in *Lctra* gene sequence using MACVECTOR software for Macintosh. The accession numbers for the genes reported in this study are:

L. cuprina transformer gene, FJ461621.
L. cuprina transformer female transcript, FJ461619.
L. cuprina transformer major male M1 transcript, FJ462786.
L. cuprina transformer minor male M2 transcript, FJ462785.
L. cuprina transformer 2 transcript, FJ461620.

RESULTS

Isolation of *Lctra*: We initially attempted two different strategies to isolate the *L. cuprina* homolog of *tra*. One approach exploited the very close linkage of the highly conserved gene *l(3)73Ah* with the *tra* gene in *Drosophila* species, *C. capitata*, and *B. oleae*. Indeed, this was the strategy that was used to isolate *Cctra* and *Botra* (PANE *et al.* 2002; LAGOS *et al.* 2007). Although we were able to isolate the *L. cuprina* homolog of *l(3)73Ah* and flanking sequences, the *tra* gene does not appear to be closely linked with this gene (data not shown). The second approach involved designing degenerate PCR primers on the basis of the few amino acid motifs that were conserved in the *tra* homologs of several *Drosophila* species and *C. capitata tra* (PANE *et al.* 2002). PCR reactions were performed with cDNA templates prepared from adult female poly(A)⁺ RNA using several different primer combinations. However, none of the PCR reactions produced a fragment that had any sequence similarity to *Cctra* or *Dmtra*. We then modified the design of degenerate primers on the basis of conserved amino acid blocks in the TRA proteins of the two tephritid species but not *Drosophila*. The rationale was that since both these species and *L. cuprina* use a Y-linked male determining gene to determine sex, *Lctra* might be more similar to *Cctra* and *Botra* than to *Dmtra*. With new primer combinations, a 416-bp amplification product was obtained from female cDNA

templates. Sequencing of the subcloned DNA fragment confirmed that we had isolated *Lctra*. To obtain full-length cDNA sequences, 5' and 3' RACE-PCR were performed with adult male and female RNA templates. From the assembled sequences, one female transcript of 1577 nt and two male transcripts of 1888 nt (male 1) and 1734 nt (male 2) were identified. The female transcript comprises a long open reading frame encoding a predicted protein of 377 amino acids. The LcTRA protein contains a high proportion of serine and arginine amino acids, characteristic of the SR family of splicing regulators. The male 1 and 2 transcripts encode short proteins of 88 and 76 amino acids, respectively, which are presumably nonfunctional as they lack the SR motifs involved in splicing regulation. Male 2 appeared to be a minor transcript as it was only detected in ~1 in 10 cloned PCR products. qRT-PCR analysis with primers that are specific for the M1 or M2 transcript confirmed that there is 8.4 times more of the M1 than the M2 transcript in adult males. Our results show that as in *Drosophila* and some tephritid insects, *Lctra* is sex-specifically spliced and the female TRA protein is likely to have a splicing regulator function.

Characterization of the *Lctra* gene: The genomic organization of *Lctra* was revealed by PCR amplification of genomic DNA using exon-specific primers. An alignment of genomic and cDNA sequences showed that *Lctra* consists of six exons and three introns that comprise 5427 bp of genomic DNA (Figure 1). The exons designated as one, two, three, and four are included in the mature transcripts of both sexes, while the exons M1 and M2 are male specific. The splicing patterns of the female and the major male 1 transcript are identical except that the splice donor sites in the first intron are different. In females the first donor site is used in splicing of exon 1 to exon 2. The major male 1 transcript arises from the use of a downstream splice donor site that is joined to the same splice acceptor as used in females. This results in the incorporation of the M1 and M2 sequences, which contain multiple in-frame translation stop codons (Figure 1). An additional splicing event excises the M1 sequence and gives rise to the minor male 2 transcript. This splicing event uses the same splice donor site as is used in female splicing of intron 1.

The position of the three introns in the female pre-mRNA is well conserved between *Lctra* and *Cctra* but has no correlation with the pattern shown for the *tra* gene in *Drosophila*. The first and third introns occur at identical positions in *Lctra*, *Cctra*, *Botra*, and *Aotra* (Figure 2). The second intron in *Lctra* is located near the position of the second intron in *Cctra*, *Botra*, and *Aotra*. However, in the region of the exon 2/exon 3 junction the LcTRA protein does not align well with the tephritid TRA proteins, making it difficult to precisely compare the relative locations of intron 2. Thus in general, the organization of the *Lctra* gene is very similar to the

tephritid *tra* genes (supporting information, Figure S1). However, the splicing patterns are somewhat simpler in *L. cuprina* as the female form and major male form of *Lctra* transcripts differ only in the choice of splice donor site for intron 1.

A ClustalW multiple sequence alignment was performed using the amino acid sequences for *L. cuprina* TRA, *C. capitata* TRA (GenBank AAM88673), *B. oleae* TRA (GenBank CAG29243), *A. obliqua* TRA (GenBank ABW04165), and *D. melanogaster* TRA (GenBank P11596). The alignment shows that LcTRA is more similar to TRA from the tephritid species than to *Drosophila* TRA (Figure 2). LcTRA is 30–33% identical and 13–14% similar to the tephritid TRA proteins but only 17% identical and 8% similar to *Drosophila* TRA. Most strikingly, the LcTRA amino terminal domain (aa 18–70) is very similar to the amino terminal domains of the tephritid TRA proteins but has no homology to *Drosophila* TRA. Indeed, *Drosophila* TRA, which is considerably shorter than LcTRA, doesn't appear to contain a region corresponding to the LcTRA amino terminal domain. LcTRA also contains a 16-amino-acid arginine-rich motif (PYRDEQREKDRIRRL) and an 11-amino-acid proline-rich motif (PQIIPVPVP), which are well conserved in tephritid and *Drosophila* TRA proteins. A phylogenetic analysis was performed using the TRA protein sequences from the species mentioned above. As anticipated the results showed that the three tephritid TRA proteins form a cluster and are more closely related to LcTRA than DmTRA (Figure S2).

Developmental expression of *Lctra*: To determine when the sex-specific splicing patterns are established during *Lucilia* development, RT-PCR was performed with primers that amplify across the first intron of *Lctra*, generating products of different size for the female and male transcripts (Figure 3A). For this experiment, individuals from a cross of line HS14 transgenic males with wild-type virgin females were used. Line HS14 contains a single X-linked insertion of the constitutively expressed *Lchsp83-ZsGreen* marker gene (M. C. Concha, E. J. Belikoff and M. J. Scott, unpublished results). Fluorescence from the ZsGreen marker is detected from midembryogenesis onward in XX individuals (9 hr at 21°), while XY individuals do not show fluorescence due to the absence of the ZsGreen gene. Total RNA was isolated from males and females at different stages of development and from early embryos (4 hr at 21°), which were mixed sex. As shown in Figure 3B, only the female form of *Lctra* was detected in early embryos of mixed sex as well as in midstage embryos from both sexes. The main male splice form of *Lctra* was not detectable until the first instar larvae stage, in which males made only the male form of *Lctra* RNA. From this stage onward differential sex-specific forms of *tra* RNA are found in males and females of *L. cuprina*. To determine if there was a significant maternal contribution of female *Lctra* transcript, RNA was isolated from

FIGURE 1.—Schematic drawing of the genomic organization and the structure of the sex-specific splice variants of *Lctra*. (A) The top diagram represents the genomic DNA comprising the *Lctra* locus (to scale). The position of the exons is shown as square boxes, with exons 1, 2, 3, and 4 in red representing common exons to both female and male mRNAs. Exons M1 and M2 in blue represent male specific exons. Introns are represented by solid lines and the 5'- and 3'-untranslated regions are represented by black boxes. Exon and intron sizes are indicated and the translational start and stop sites are marked, but for clarity not all stop sites are shown. The position of putative TRA/TRA2 binding sites within the M2 exon and the first intron is represented by red vertical lines. The splicing patterns of the male and females transcripts are shown below the gene organization diagram (introns are not to scale). (B) Sequence of the six TRA/TRA2 binding sites found in the *Lctra* genomic DNA sequence and comparison with the *D. melanogaster* and *L. cuprina* consensus. (C) Splice donor and acceptor sites for all *Lctra* introns. The intron 1 "female" donor site is used to make both the female and male 2 transcripts whereas the intron 1 "male" donor site is used only in males. The intron 1 "male" acceptor site is used only to produce the minor male 2 transcript. Both sexes use the intron 1 "common" acceptor site to produce the major transcripts.

unfertilized eggs laid by virgin females and from precellular embryos (<1 hr at 21°). Only the female form of *Lctra* was detected in both samples (Figure 3C). These results demonstrate that embryos of both sexes contain maternal RNA pools coding for the female TRA

protein and that in XY individuals the male form of *Lctra* appears sometime between midembryogenesis and the beginning of the first instar larvae stage. This finding raises the question of how the male pathway is then selected in the XY embryo and suggests that the

FIGURE 2.—Multiple sequence alignment of TRA proteins from *L. cuprina*, *C. capitata*, *B. oleae*, *A. obliqua*, and *D. melanogaster*. Identical amino acids are shaded in black while similar amino acids are shaded in gray. Vertical red lines indicate the corresponding locations of the exon/intron boundaries in the *L. cuprina* and tephritid *tra* genes. Arrows indicate the conserved motifs that were the basis of the degenerate primers that were used to amplify *Lctra* cDNA sequence.

function of the Y-linked male determining factor is essential in early male sex determination.

The *Lctra* gene is essential for female development: In tephritid species and in *Drosophila*, *tra* is an essential gene for female development. Injection of *tra* dsRNA into early *C. capitata* and *B. oleae* embryos led to the development of masculinized XX individuals (PANE *et al.* 2002; LAGOS *et al.* 2007). In these species TRA is proposed to have an autoregulatory function, which could explain why a transient reduction in RNA levels so effectively blocked female development. Therefore, we next investigated whether the function of *tra* was conserved in *L. cuprina*. To test this we compromised

Lctra gene expression during early development using the RNA interference technique. A 950-bp *Lctra* dsRNA was injected into the posterior end of preblastoderm embryos obtained from a cross of line HS14 transgenic males with wild-type virgin females. In this way, XX first instar larvae that developed from injected embryos were identified by fluorescence and readily separated from nonfluorescent XY siblings. In a control experiment, 116 fluorescent larvae were selected from uninjected embryos. One hundred six of them developed into adults and all were female. Of the 89 XX adults that developed from injected embryos, 68 showed evidence of sex reversal (Figure 4 and Table 1). Seventy-two

FIGURE 3.—Analysis of the expression of *Lctra* over development by RT-PCR. (A) Position of primers in exons 1 and 4 of *Lctra*, designed to amplify products of different sizes for female and male transcripts. (B and C) RT-PCR amplification of *Lctra* on total RNA obtained from different developmental stages of *L. cuprina*. Male transcripts are 1.5 kb whereas female transcripts are 1.1 kb in size. Stages in B are: E4 early embryos of mixed sexes at 4 hr of development, EF9 midstage female embryos at 9 hr, EM9 midstage male embryos at 9 hr, 1IF female first instar larvae, 1IM male first instar larvae, 3IF female third instar larvae, 3IM male third instar larvae, AF female adults, and AM male adults. In panel C, RNA was isolated from unfertilized eggs (UF) or fertilized precellular embryos at 30–60 min of development (E1) and cDNA prepared either with (+) or without (–) reverse transcriptase.

percent developed with external male genitalia but with female interocular width. Two percent had both male genitalia and male interocular width. Twenty-six percent appeared to be phenotypically normal females. Of the 64 XY adults that developed from injected embryos, 100% showed a normal male phenotype. It is of note that PANE *et al.* (2002) observed that injections of *Cctra* dsRNA into the posterior end of embryos led to the development of some adult XX individuals with male genitalia but heads with a female bristle pattern. As a control of the RNAi technique, dsRNA directed against ZsGreen was injected into the posterior end of preblastoderm embryos of a cross between line 56 males and wild-type virgin females (Figure 5A). Line 56 contains an autosomal single insertion of the *Lchsp83-ZsGreen* marker. Fluorescence from the ZsGreen marker is observed in both males and females from midembryogenesis onward. Figure 5A shows loss of fluorescence in the posterior end but not in the anterior end of injected late embryos and first instar larvae. The knockdown of ZsGreen is only transient as injected individuals show complete fluorescence by the stage of third instar larvae (data not shown). The situation observed for ZsGreen RNAi correlates with that observed for *tra* RNAi, where the effect of gene knockdown is mostly observed in the

posterior end but not in the anterior end of the animal. This effect can be attributed to the failure of the dsRNA solution to diffuse to the opposite end of the long *Lucilia* embryo, causing a knockdown of gene expression only in the rear. The control RNAi experiment also suggests that the strong transformation of XX individuals into males is caused by a transient knockdown of *tra* during early development.

Some of the XX flies with external male genitalia were dissected and found to have testes of normal morphology (Figure 4). Since the *L. cuprina* Y chromosome appears to be devoid of genes that encode fertility factors (BEDO and FOSTER 1985), we wondered whether any of the transformed XX flies were fertile. Out of 10 crosses between single XX transformed males and wild-type virgin females, 1 was fertile. As anticipated, all of the offspring from this cross developed into females. These results show that *tra* is essential for female development in *L. cuprina* and that the Y chromosome is not essential for fertility. Since a transient reduction in female *Lctra* RNA in *Lucilia* embryos causes a strong sex reversal in adult flies, it is likely that the role of *Lctra* in selecting the sexual fate occurs early in development.

Regulation of *Lctra* activity: A feature of the tephritid *tra* genes is the presence of multiple putative TRA/TRA2 binding sites within the male-specific exons and flanking introns (RUIZ *et al.* 2007) (Figure S1). A bioinformatics search of the *Lctra* gene sequence identified 6 putative TRA/TRA2 sites that were a perfect match to the consensus (T/A)(C/A)(A/T)(A/T)CAATCAACA (Figure 1). Five of the sites were clustered over a 233-bp region that is 2063 bp downstream of the female splice donor site but 341 bp upstream of the intron 1 splice acceptor site. The sixth TRA/TRA2 site is located within the M2 male-specific exon sequence, 70 nt upstream of the male-specific splice donor site. That only one TRA/TRA2 site is in a male exon is in contrast to the *Cctra*, *Botra*, and *Aotra* genes where most of the TRA/TRA2 sites are in the male-specific exons (Figure S1). The presence of these multiple TRA/TRA2 sites in *Lctra* unspliced transcripts suggests a potential for autoregulation of *Lctra* RNA splicing.

To investigate how *tra* activity is regulated in *L. cuprina* we tested for the presence of sex-specific transcripts of *tra* and its downstream target gene *dsx* in transformed XX males. Transcripts from the *Lcdsx* gene are sex-specifically spliced in a similar fashion as the *M. domestica* *dsx* RNAs (M. C. Concha and M. J. Scott, unpublished results). Total RNA was isolated from dissected heads and remaining bodies (thorax plus abdomen) of transformed XX individuals (XXM1–4). Primers were designed that amplified different size PCR products for the male and female forms of the *Lctra* and *Lcdsx* RNAs. The results of the RT-PCR analysis are shown in Figure 5, B–D. Both male and female forms of *Lctra* were detected in RNA from bodies but a majority of the female form was detected in head RNA. XXM1,

FIGURE 4.—Injection of *Lctra* dsRNA into the posterior end of preblastoderm embryos causes female-to-male sex reversal. Phenotypically wild-type females (XX female, A and B) can be recognized from males by a wider interocular distance in the head and by the presence of an ovipositor in the genitalia, whereas males (XY male, A and B) have a pigmented copulatory apparatus with characteristic claspers. Internally, adult females present two ovaries with a large number of eggs (XX female, C) and males present red pigmented testes (XY male, C). Only XX individuals carry the X-linked ZsGreen marker gene. Most frequently, transformed XX males show male genitalia and gonads but conserve the characteristic female head (XX male 1, A, B, C, and D) while a small percentage of the injected XX individuals develop as completely transformed males (XX male 4, A, B, C, and D).

XXM2, and XXM3 presented female interocular width and male external genitalia, while XXM4 was a completely transformed XX male. In this individual, head RNA seems to contain a slightly more abundant fraction of male *tra* than in the other tested flies. In the case of the *dsx* gene, both male and female forms of *Lcdsx* were detected in bodies but only the female form was detected in heads of all tested individuals. In XXM4, female *Lcdsx* is present in scarce amounts in both head and body RNA. These results show that knockdown of

female *Lctra* expression in early development causes the activation of the male mode of splicing of *Lctra* and a change from the female to the male mode of splicing in *Lcdsx*.

Isolation and characterization of the *L. cuprina tra2* homolog: To obtain a more complete understanding of sex determination in *L. cuprina* we isolated the *tra2* homolog. Using a similar strategy to that used to isolate *Lctra*, an amplification product of the expected size was obtained from cDNA templates and sequence compar-

TABLE 1
Injection of *Lctra* dsRNA into embryos blocks female development

Genotype	Percentage (N)	Interocular width	Genitalia	Gonads
XX	72 (64)	F	M	Testes
XX	2 (2)	M	M	Testes
XX	26 (23)	F	F	Ovaries
XY	100 (65)	M	M	Testes

isons confirmed that we had isolated the *L. cuprina* homolog of *tra2*, which was designated *Lctra2*. To obtain full-length cDNA sequences 5' and 3' RACE was performed with adult male and female RNA templates. The sequences were assembled into a unique 1499-nt transcript.

In *D. melanogaster* the *tra2* gene encodes a protein with an RNA-recognition motif (RRM), flanked by two arginine-rich/serine-rich regions (RS domains), which mediate protein-protein interactions. Three *tra2* transcripts arise due to alternative splicing and transcription start sites (AMREIN *et al.* 1990; MATTOX *et al.* 1990). In the medfly *C. capitata* and in the housefly *M. domestica* only a single *tra2* transcript is detected (BURGHARDT *et al.* 2005). As in the latter insects, only a single *Lctra2* transcript was detected in the RACE experiments and in RT-PCR performed on RNA isolated from different developmental stages (data not shown). A multiple sequence alignment was performed using CLUSTALW with amino sequences of the TRA2 proteins from *L. cuprina*, *C. capitata* (GenBank EU437408), *B. oleae* (GenBank AJ547623), *M. domestica* (GenBank AAW34233), and *D. melanogaster* (GenBank AAA62771). *LcTRA2* is very similar to TRA2 from other Diptera (Figure 6). As expected, the strongest conservation is in the RRM domain and in the linker motif that immediately follows the RRM domain. The linker motif is highly conserved among TRA2 homologs and is considered a signature motif of TRA2 proteins (DAUWALDER *et al.* 1996). With the notable exception of *Drosophila* TRA2, there are also regions of high sequence similarity in the arginine- and serine-rich amino and carboxyl terminal domains. To investigate the phylogenetic relationships of LCTRA2 to other insect TRA2 proteins a phylogenetic analysis was performed using the TRA2 sequences from the species mentioned above and additionally *D. virilis* (GenBank AAB58114), *D. pseudoobscura* (GenBank XP_001360605), *A. mellifera* (GenBank:XP_001121070), and *B. mori* (GenBank AAX47001). The results obtained using a neighbor-joining algorithm are shown in Figure 7. LCTRA2 clusters with *M. domestica* TRA2 as expected since both species belong to Calyptratae, a subsection of Schizophora in the insect order Diptera. *L. cuprina* and *M. domestica* TRA2 proteins are more closely related to the homologs from tephritid species than *Drosophila*

FIGURE 5.—Analysis of the splicing patterns of *Lctra* and *Lcdsx* in XX transformed males and wild-type individuals. (A) Control for the RNAi technique; embryos from a cross between *Lchsp83-ZsGreen* males and wild-type females were injected with dsRNA for ZsGreen. Fluorescence is lost in the posterior end of injected embryos and larvae but not in the anterior end. (B) RT-PCR amplification with *Lctra*-specific primers on total RNA isolated from heads (h) and bodies (thorax plus abdomen) (b) of transformed XX males presenting male genitalia and female head (XXM1, XXM2, and XXM3), a completely transformed XX male (XXM4) and of a wild-type female and male. The primers used amplify different size products for male (traM) and female transcripts (traF). (C and D) RT-PCR amplification with *Lcdsx* female and male specific primers, using the same total RNA samples as in B. The sex-specific amplification products are labeled dsxF for female and dsxM for male, respectively. (E) RT-PCR control using α -tubulin-specific primers. The RNAi knockdown of *Lctra* in XX males sets *Lctra* splicing in the male mode in the posterior end of injected individuals, which in turn changes the splicing pattern of *Lcdsx* from the female to the male form.

species, which is consistent with previous analysis (GOMULSKI *et al.* 2008). Both tephritid and *Drosophila* species belong to the Acalptrae subsection of Schizophora. However, it has been hypothesized that Calliphoridae are more closely related to Tephritids than Drosophilids (CRAMPTON 1944).

DISCUSSION

In the present study we have isolated and characterized the *transformer* gene from the pest insect species *L. cuprina*. As in other Diptera, *Lctra* is alternatively spliced

FIGURE 6.—Multiple sequence alignment of TRA2 proteins in *L. cuprina*, *M. domestica*, *C. capitata*, *B. oleae*, and *D. melanogaster*. Identical amino acids are shaded in black while similar amino acids are shaded in gray. Arrows indicate the conserved motifs that were the basis of the degenerate primers that were used to obtain *Lctra2* DNA sequence. The RNA recognition motif (RRM) is underlined in red. RNP-1 and RNP-2 are the highly conserved ribonucleoprotein identifier sequences found in RRM motifs. The TRA2 proteins of all these insects are highly conserved in the RRM as well as in the flanking RS domains.

such that only the female transcript codes for a full-length protein. Interestingly, LcTRA presents little sequence similarity with the TRA proteins of the *Drosophila* species and shows a high degree of homology with TRA of Tephritidae. Indeed, as in tephritid insects, *Lctra* is essential for female development and its activity is autoregulated, suggesting that in *L. cuprina* *tra* is likely the top switch of the female development cascade. Since *L. cuprina* is a member of the Calliphoridae family of insects, this regulatory system in which *tra* acts as the female master switch, is not confined to Tephritidae but appears to have a much wider distribution among Diptera. We have also isolated the *transformer2* gene and found that it is not alternatively spliced, giving rise to a single transcript in males and females, as is the case in *M. domestica tra2* and *C. capitata tra2*, and to the contrary of *D. melanogaster*, which presents several forms of *tra2* in somatic and germline cells. Overall, both *Lctra* and *Lctra2* resemble more closely its homologs in the tephritid species of insects than in *D. melanogaster*.

Regulation of the *Lctra* gene: The organization of the *Lctra* gene is similar to that of the *C. capitata*, *B. oleae*, and

A. obliqua tra genes but is simpler. In the tephritid species, the *tra* gene contains three or more male exons and the male splicing patterns are complex, particularly in *A. obliqua*. In *L. cuprina*, the difference between the female and major male splicing patterns is simply that females chose the first donor site in intron 1 and males chose a downstream site. Both sexes splice to the same acceptor site. The presence of six putative TRA/TRA2 binding sites in the regulated first intron suggests that *Lctra* RNA splicing is autoregulated. This hypothesis is supported by the results from the RNAi experiments as injection of *Lctra* dsRNA lead to XX individuals switching from the female to male form of *Lctra* splicing.

Regulation of *Lctra* RNA splicing could be achieved by the binding of TRA/TRA2 to the female primary transcript to either block the use of the male splice donor site or activate the use of the female donor site. As the female splice donor site (UG|GUAUUU) is a better match to the *Drosophila* consensus (AG|GUAAGU) (WEIR and RICE 2004) than the male donor site (GU|GUGAGU) and most of the TRA/TRA2 binding sites are closer to the male donor site than the female site, it is more likely that LcTRA could regulate its own

FIGURE 7.—Neighbor-joining tree of insect TRA2 amino acid sequences. The numbers represent bootstrap support values from 1000 replicates. The scale represents the mean character distance.

splicing by inhibiting the use of the male splice donor site. This explanation is consistent with the study on *C. capitata tra* presented by PANE *et al.* (2002), who proposed that TRA negatively regulates the male mode of splicing in females. The fact that the amino terminal domain of TRA is well conserved in *L. cuprina* and the tephritid species but absent in *Drosophila* TRA is consistent with the suggestion that this domain is involved in TRA autoregulation.

RUIZ *et al.* (2007) identified putative RBP1 and TRA2-ISS binding sites in the regulated first female intron of the *C. capitata*, *B. oleae*, and *A. obliqua tra* genes. They proposed that RBP1 and TRA2 somehow combine with the TRA/TRA2 complex to repress the male-splicing pattern. We searched the *Lctra* gene sequence but found only one putative RBP1 type A site (TCAACTTTTA) and one TRA2-ISS site (CAAGA) in the female first intron. Although there were several matches to the RBP1 type B site (ATCYNNA), there were no more than expected for a short AT-rich sequence in the 2728-bp female first intron that is 71% AT. Thus it would seem unlikely that this model could explain how splicing of the *Lctra* gene is regulated.

Clearly a high priority is to develop a splicing assay system to determine if sequence elements other than the identified putative TRA/TRA2 sites are required for sex-specific splicing. It would also be of interest to determine if the spatial arrangement of the TRA/TRA2 sites relative to the major male splice donor site is important for regulated splicing. For example, if TRA/TRA2

bound to the male exon interacted with TRA/TRA2 bound to sites in the intron this could lead to looping out of the intervening sequence. As a consequence the male donor site would be inaccessible to the splicing apparatus or the female donor site might be brought into close spatial proximity with the acceptor site.

***Lctra* function and expression:** The RNAi knockdown of female *Lctra* mRNA in early embryos caused the selection of the male splicing mode of *Lctra* and *Lcdsx*, particularly in the bodies of adults derived from embryos injected in the posterior end. The female splice variants of these genes were also found in the heads and to a lesser extent in the bodies of adult transformed XX males. The presence of the female forms in bodies is most likely because this included thoracic tissue derived from cells from the anterior half of the injected embryos. Female *Lctra* and *Lcdsx* transcripts in heads could be because the relatively rapid development and long length of *L. cuprina* embryos (GREGOR *et al.* 2005) would have limited diffusion of the dsRNA to the anterior end of the embryo, furthest from the site of injection. Consequently, it would be anticipated that LcTRA protein levels would be low at the posterior end and high at the anterior end of injected embryos, as is observed in the ZsGreen control experiments. These observations suggest that there may be a threshold of LcTRA protein level to be attained to activate the female splicing of *Lctra* and *Lcdsx*.

The availability of a transgenic line expressing a strong X-linked ZsGreen marker gene allowed the

identification of females and males from midembryogenesis onward. Using this system we found that both sexes contain female *Lctra* mRNA during embryonic stages and that the male form of *Lctra* is expressed in males from the first instar larvae stage onward. Moreover, the presence of female *Lctra* mRNA in unfertilized eggs and in very early precellular embryos of mixed sex showed that there is maternal inheritance of female *Lctra* RNA in embryos of both sexes. These results raise the question of how the female pathway of development is initiated in XX individuals and how it is prevented in XY individuals. Our results suggest that female development is established during embryogenesis by a maternal pool of female *Lctra* mRNA that is translated into protein in zygotes to initiate an autoregulatory loop, thus promoting its own splicing and activity. This system allows LcTRA to accumulate in female embryos over a certain threshold required for its maintenance and for the activation of the female RNA splicing mode of its target gene *dsx*, thereby promoting female differentiation. It should be noted that it remains to be shown that maternal *Lctra* RNA is translated into protein in developing embryos. In males, this autoregulation would be blocked by the activity of M, thereby selecting the male pathway of development by default. PANE *et al.* (2002) proposed that in *C. capitata* males, M blocks *Cctra* autoregulation by directly inhibiting splicing of *Cctra* transcripts or by acting on the CcTRA protein. Alternatively, M could transiently inhibit *Cctra* transcription or translation of *Cctra* transcripts. Similar models could explain how M blocks *Lctra* autoregulation in male *L. cuprina* embryos. However, it would seem unlikely that M acts to inhibit the translation of maternal *Lctra* RNA, thereby preventing the initiation of the autoregulatory loop. Only the female form of *Lctra* RNA was detected in males at midembryogenesis, well after the onset of general zygotic transcription. If M acts solely to block translation of maternal *Lctra* transcripts, it would be anticipated that some male *Lctra* transcripts would be detectable by the midembryo stage. Thus, while it is possible that M could inhibit translation of *Lctra* RNA, it is more likely that M regulates *Lctra* at the splicing or, temporarily, at the transcriptional level thus preventing the accumulation of female LcTRA protein over a threshold required for the maintenance of the autoregulatory loop.

The genetic control of sex determination in *L. cuprina* presents many similarities to the system used by *C. capitata*. Both species of flies use a dominant Y-linked male determining gene to control sexual development and in both the *tra* gene functions as a master switch that controls the female fate by establishing an autoregulatory loop (PANE *et al.* 2002). Moreover, both species present maternal inheritance of female *tra* transcripts in embryos of both sexes further supporting its conserved role in the initiation of *tra* autoregulation. The use of alternative splice donor sites and exon

skipping in *Lctra* and *Cctra* is in contrast to the *Drosophila tra* 3' alternative splicing mechanism. Furthermore, in both species of insects RNAi knockdown of *tra* results in fertile XX males, indicating that in contrast to *Drosophila*, the Y chromosome is not essential for fertility in these flies. Sex determination in *L. cuprina* also resembles that of its closer relative *M. domestica*. In the latter, female sex determination is controlled by the *F* gene, whose activity is maintained by an autoregulatory loop and is strongly dependent on maternally inherited *F* product (DÜBENDORFER and HEDIGER 1998). Although the isolation of the *M. domestica tra* gene has not yet been published it has been proposed that *F* corresponds to *tra* (BURGHARDT *et al.* 2005). Moreover, RNAi mediated knockdown of *tra2* in *Musca* embryos results in transformed XX males that are fertile. Consequently, this sex determination system where *tra* acts as a master switch may be more widely spread to include other groups such as the Muscidae and Calliphoridae.

Interestingly, while transient RNAi knockdown of *Lctra* resulted in a high proportion of XX individuals developing male genitalia, only 1 in 10 tested was fertile. A possible explanation for this low fertility rate is that although the *Lucilia* Y chromosome does not contain essential fertility genes, it may contain genes involved in male fitness and performance in mating. Alternatively, since the heads of transformed XX individuals contained mostly the female forms of *Lctra* and *Lcdsx* transcripts, the sexual courtship behavior may have been less than optimal. It is well documented that the *dsx* and *fruitless (fru)* genes are involved in sexual courtship behavior in *Drosophila* and that the control of this behavior resides in specific areas of the brain (SHIRANGI *et al.* 2006). Splicing of *fru* transcripts is also regulated by TRA/TRA2 in *Drosophila* (HEINRICHS *et al.* 1998) and *C. capitata* (SALVEMINI *et al.* 2009). It would be of interest to study courtship behavior and male fitness in a transgenic line of *Lucilia* expressing a stable inducible *tra* dsRNA construct.

Evolution of *Lctra* and sex determining systems:

From a detailed comparative morphological study of male terminalia, CRAMPTON (1944) found that within the Diptera order, the Calyptratae group, to which *L. cuprina* belongs, shared many features with some members of the Acalyptratae, including species from the superfamily Tephritoidea. That Calyptratae may be more similar to Tephritidae (Acalyptratae) than to Drosophilidae (Acalyptratae) has been supported by studies of the *white* (GOMULSKI *et al.* 2001), *glucose-6-phosphate dehydrogenase* (SOTO-ADAMES *et al.* 1994), *alcohol dehydrogenase* (BROGNA *et al.* 2001), and *tra2* genes (GOMULSKI *et al.* 2008). Our results strongly support this evolutionary hypothesis.

It will be of interest to determine whether *tra* acts as a master switch of sex determination in other important Calyptratae species such as *M. domestica*, tsetse flies, and the screwworm fly *Cochliomyia hominivorax*. The latter

species have been the subject of major control efforts using the sterile insect technique (KRAFSUR 1998). Since these species are more closely related to *L. cuprina* than tephritid species, we would predict *tra* function and regulation would be conserved in at least some other Calypttratae.

Genetic control of *L. cuprina*: *L. cuprina* is an economically important insect species that constitutes a major pest to the sheep industries in Australia and New Zealand (BECK *et al.* 1985; HEATH and BISHOP 2006). This insect has long been considered to be a good target for a genetic control program (SCOTT *et al.* 2004). Indeed, considerable effort was made to develop a “field female killing system” that was shown to be effective in reducing a *L. cuprina* island population in a large field trial (DAVIDSON 1989; FOSTER *et al.* 1991). The isolation of the *Lctra* gene will facilitate the development of genetically modified strains that would have certain advantages for genetic control programs. For example, induction of expression of *Lctra* double-stranded RNA could lead to the development of a male population of flies, which could be sterilized by radiation before field release. We have recently isolated *L. cuprina* heat inducible gene promoters for this purpose. Alternatively, the regulated first intron of *Lctra* could be used to control the sex-specific expression of a tetracycline-repressible lethal gene, as recently developed for *C. capitata* (FU *et al.* 2007).

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Supporting Information

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Sexual Development in *Lucilia cuprina* (Diptera, Calliphoridae) Is Controlled by the Transformer Gene

Carolina Concha and Maxwell J. Scott

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FIGURE S1.—Comparison of the organization of the *L. cuprina* *transformer* gene and the *transformer* genes from three tephritid species. Exons (boxes) and introns (lines) are drawn to scale and lengths in base pairs are indicated. 5'UTR and 3'UTR are indicated by black boxes. The positions of predicted TRA/TRA2 binding sites are indicated by vertical red lines. In females, the red numbered exons are joined to produce a single transcript. Exons included in male-specific transcripts are indicated with blue boxes. Two male-specific transcripts were detected in *L. cuprina*; the predominant transcript contains both m1 and m2 sequences, the minor transcript includes only the m2 sequence. In *C. capitata*, two male-specific transcripts were reported; one includes m1, m2 and m4 the second contained m2, m3 and m4 sequences (PANE *et al.*, 2002). Two male-specific transcripts were reported for *B. oleae* (LAGOS *et al.*, 2007), one includes the m2, m3 and m4 sequences, the other had m1, m2, m3 and m4 sequences. In *A. obliqua* there are three female transcripts that differ in the length of 3'UTR, only one of which is shown (RUIZ *et al.*, 2007). There are five male-specific transcripts in *A. obliqua*; the first has m1, m2 and m3 sequences, the second has m1 and m3 sequences, the third has the m1 sequence, the fourth has m1, m2 and m3 sequences and includes the intron between m2 and m3 and the fifth transcript has part of m1 and the m3 sequences.

FIGURE S2.—Neighbour-joining tree of dipteran TRA amino acid sequences. The numbers represent bootstrap support values from a 1000 replicates. The scale represents the mean character distance.