RESEARCH ARTICLE

Conservation and sex-specific splicing of the *doublesex* gene in the economically important pest species *Lucilia cuprina*

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Abstract

Genetic control of sex determination in insects has been best characterized in *Drosophila melanogaster*, where the master gene *Sxl* codes for RNA that is sex specifically spliced to produce a functional protein only in females. SXL regulates the sex-specific splicing of *transformer* (*tra*) RNA which, in turn, regulates the splicing of *dsx* RNA to produce functional male and female proteins. In the Australian sheep blowfly (*Lucilia cuprina*), the *tra* gene (*Lctra*) is required for female development and *Lctra* transcripts are sex-specifically spliced such that only female *Lctra* mRNA codes for functional protein. In males, a factor encoded by the Y-linked male determining gene is thought to prevent the female-mode of splicing of *Lctra* RNA. To further our understanding of the sex determination regulatory hierarchy in *L. cuprina*, we have isolated the *dsx* gene (*Lcdsx*) from this species. We found that the *Lcdsx* transcripts are sex-specifically spliced in a similar manner as their counterparts in *D. melanogaster*, housefly and tephritids. The LcDSX proteins are well conserved and the male form of DSX contains a motif encoded by a male-specific exon that is within the female-specific intron. This intron/exon arrangement had previously been found only in the housefly *dsx* gene, suggesting this may be a unique feature of *dsx* genes of Calyptratae species.

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Introduction

The Australian sheep blowfly, *Lucilia cuprina*, (Diptera: Calliphoridae) is a major pest for the sheep industries in Australia and New Zealand (Beck *et al*. 1985; Heath and Bishop 2006), where it causes economic damage by laying eggs in the flesh of living sheep. When the maggots hatch they parasitize the sheep, resulting in general poor health and, if left untreated, lead to severe wounds and infection, eventually causing death. Traditionally, industry has depended on chemical insecticides and good farming practices but with the increasing resistance to some commonly used insecticides, there is great interest in alternative control measures, including genetic approaches.

Over 20 years ago, Foster and colleagues developed a 'field female-killing' strain of *L. cuprina* that showed promise in a large field trial on Flinders Island near Australia (Davidson 1989; Foster *et al*. 1991). We have been working towards developing a transgenic strain of *L. cuprina* that would be ideal for a male-only SIT (sterile insect technique) programme (Scott *et al*. 2004). Knowledge of the genes that determine sex in *L. cuprina* would greatly facilitate the development of such a strain.

The insect sex-determination system that is most well understood at the genetic level is that of the vinegar fly *D. melanogaster* (Belote 1992; Cline 1993). In brief, the master regulator sex-lethal (*Sxl*) is initially activated only in female embryos in response to X-linked signal elements or counting factors. *Sxl* RNA splicing is then autoregulated such that only females produce a transcript that codes for full-length SXL protein (Bell *et al*. 1991). SXL also regulates the splicing of transcripts of the *transformer* (*tra*) pre-mRNA such that only females produce a RNA that codes for a full length

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and functional TRA protein (Sosnowski *et al*. 1989). TRA together with TRA2, an RNA binding protein that is constitutively expressed in both sexes, promotes the female-specific splicing of *doublesex* pre-mRNA (*dsx*), the last component of the regulatory hierarchy (Ryner and Baker 1991). In females, the TRA/TRA2 protein complex binds to several sites in the 3′ untranslated region (3′UTR) of *dsx* pre-mRNA and promote the use of a upstream weak splice acceptor site (Lynch and Maniatis 1996). In this way the female-specific exon 4 is joined to the common exon 3 (figure 1A). In males, which have no functional TRA protein, the weak splice acceptor site is ignored and instead exon 3 is joined to exon 5. The male and female DSX proteins, DSX^M and DSX^F, are DNA binding proteins that promote sexual development by regulating the transcription of sex-specific differentiation genes (Burtis and Baker 1989). DSX^M and DSX^F share a DNA binding domain that is encoded by the common exons (Cho and Wensink 1997) but they differ in their C-terminal domains that are encoded by the sex-specific exons. Presumably, the sex-specific domains interact with different proteins.

In *L. cuprina*, sex 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 is unknown. As in other diptera (Saccone *et al*. 2002), an ortholog of *Sxl* is present in *L. cuprina* but it is not sex-specifically spliced and appears to have no role in sex determination (P. Atkinson, personal communication). In contrast, the *L. cuprina tra* gene is required for female development and *Lctra* transcripts are sex-specifically spliced (Concha and Scott 2009). *Lctra* splicing appears to be autoregulated as several TRA/TRA2 sites are present within or near the female-specific first intron. Further, the female-specific splicing pattern appears to be established in embryos by maternally inherited LcTRA protein. Presumably, the male determining factor somehow prevents the establishment of the female pattern of *Lctra* splicing. This sex-determination system is very similar to that described in housefly and tephritid species (Pane *et al*. 2002; Lagos *et al*. 2007; Hediger *et al*. 2010). To further our understanding of the sex determination genetic hierarchy in *L. cuprina*, we have isolated the *L. cuprina dsx* gene (*Lcdsx*). Here we report that the *Lcdsx* gene is well conserved and sex-specifically spliced in a similar manner as in housefly.

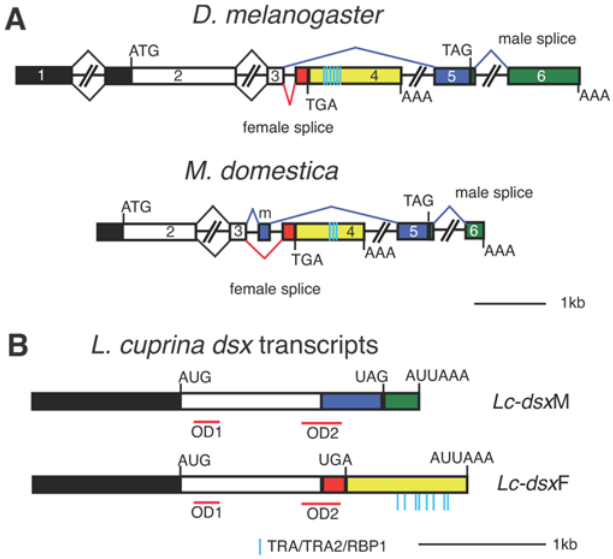

Figure 1. *dsx* gene organization in *D. melanogaster* and *M. domestica* and sex-specific transcripts of the *L. cuprina dsx* gene. (A) Schematic illustration of the organization of the *D. melanogaster* and *M. domestica dsx* genes. Exons are numbered and are drawn approximately to scale. With the exception of the female-specific intron, introns are large. Major male and female splicing patterns are shown above or below the introns respectively. The sex-specific male splices are shown in blue line and female splices are shown in red line. Common 5′UTR is in black, common coding region in white, male-specific coding region in blue, male-specific 3′UTR in green, female-specific coding region in red and female-specific 3′UTR in yellow. TRA/TRA2/RBP1 binding sites are shown as light blue lines in their approximate location within exon 4. (B) The male *L. cuprina dsx* transcript (*LcdsxM*) and female (*LcdsxF*) transcript of the *Lcdsx* gene. Location of start and stop codons, OD1 and OD2 motifs, TRA/TRA2/RBP1 binding sites and polyA signals are indicated. Colour coding of exons as in panel (A).

Materials and methods

L. cuprina adults were maintained in the laboratory at a constant temperature of 21°C under a 12/12 h light/dark cycle as described previously (Concha and Scott 2009). RNA isolation, cDNA preparation and PCR amplification were as described previously (Concha and Scott 2009). To isolate the *L. cuprina dsx* gene, we performed PCR on cDNA obtained from male and female *Lucilia* with Advantage 2 *TAQ* polymerase (Clontech, Mountain View, USA) using degenerate primers directed towards conserved amino acid blocks in the region that is common to both sexes. The primers were designated F1 and R1 for the first round and F2 and R2 for the second round of PCR. Cycling conditions for both PCR rounds were denaturation step at 95°C for 2 min, then 30 cycles (denaturation at 95°C for 25 s, annealing at 48°C for 30 s and extension at 68°C for 2 min), and lastly extension at 68°C for 5 min. Subcloning and sequencing of the candidate fragments were carried out by standard procedures. The degenerate primers were:

Dsx_deg_F1: 5′-TGTGAAAAATGTMGWTTRACHGCYGATMGWCA-3′
Dsx_deg_F2: 5′-CAAATDCATGAAGTTCCHCCHGTWGTWCA-3′
Dsx_deg_R1: 5′-CCRGCATCTTTYAAWATWACATACATYARDGGCAT-3′
Dsx_deg_R2: 5′-TCCCADGGATARCCRAATTTTTCWATYARTTTTTGACA-3′

To obtain full-length cDNA sequences, we used 5′ and 3′ RACE with *Lcdsx* gene specific primers (sequences available upon request) using the Smart RACE Kit and Advantage 2 *TAQ* DNA polymerase (Clontech, Mountain View, USA). Protein multiple sequence alignment was performed using CLUSTAL-W 1.83 software (http://www.ebi.ac.uk/Tools/clustalw2/) and analysis of the alignments was

performed using BOX SHADE (http://www.ch.embnet.org/software/BOX_form.html). The accession numbers for the genes reported in this study are: *L. cuprina doublesex* female transcript, GU784834; *L. cuprina doublesex* male transcript, GU784833; *L. cuprina doublesex* gene female-specific intron containing exon 'm', GU784832.

Results and discussion

The *D. melanogaster dsx* gene contains six exons (Burtis and Baker 1989). Exons from 1 to 3 are common to both male and female *dsx* transcripts whereas only the female transcript contains exon 4 and the male transcript contains exons between 5 and 6 (figure 1A). The *M. domestica dsx* gene (*Mddsx*) has a similar organization (figure 1A) but with the notable difference of an additional exon, 'm', which is spliced to exons 3 and 5 in males (Hediger *et al*. 2004). As the sheep blowfly is more closely related to housefly than to *Drosophila*, we anticipated that the *L. cuprina dsx* gene (*Lcdsx*) would likely have a similar organization as *Mddsx*. The *Lcdsx* gene transcripts were identified by PCR on cDNA of male and female adults using degenerate primers as described in Methods. From the assembled sequences, one female transcript (*Lc-dsxF*) of 3263 nucleotide and one male transcript (*Lc-dsxM*) of 3042 nucleotide were identified (figure 1). The female and male transcripts encode proteins of 396 and 532 amino acids, respectively. The amino terminal 367 amino acids are common to $LcDSX^F$ and $LcDSX^M$ proteins. The $LcDSX^F$ and $LcDSX^M$ proteins differ in the carboxyl-terminal motifs.

Multiple sequence alignments were performed using CLUSTALW with amino sequences of the DSX^F and DSX^M proteins from *L. cuprina*, *M. domestica* (GenBank AAR23813 and AAR23812) (Hediger *et al*. 2004), *C. capitata* (GenBank AAN63597 and AAN63598), *B. oleae* (GenBank CAD67986 and CAD67987) (Lagos *et al*. 2005), *A. bistrigata* (GenBank ABF50950 and ABF50960) (Ruiz *et al*. 2007) and *D. melanogaster* (GenBank NP_731197 and NP_731198) (Burtis and Baker 1989). The common amino terminal region of the $LcDSX^F$ and $LcDSX^M$ proteins is very similar to the corresponding regions of the DSX proteins from housefly, tephritids and *Drosophila* (figure 2). As expected, the strongest conservation is in the DNA binding domain (OD1) and in the dimerization domain (OD2) (An *et al*. 1996; Cho and Wensink 1997). The common part of the LcDSX proteins is most closely related to the homologous region of the housefly DSX proteins (74% identity, 79% similarity over 377 amino acids). This is most apparent in the region between the OD1 and OD2 domains. This region is very poorly conserved between housefly and fruit fly DSX proteins (Hediger *et al*. 2004). However, in the sheep blowfly and in housefly DSX proteins this region is quite similar, with 61% identity and 67% similarity over 89 amino acids. The short 30 amino acid female-specific carboxy-terminal motif of the $LcDSX^F$ protein is identical to the homologous region of $MdDSX^F$ (figure 3). Further, there are very few amino acid differences between the female-specific part of $LcDSX^F$ and that of tephritid or *Drosophila* DSX^F proteins. The female-specific region contains part of the OD2 dimerization domain, which could partly explain the high degree of conservation. The male-specific part of the $LcDSX^M$ protein is highly similar to the corresponding region of the housefly DSX^M protein (60% identity, 73% similarity over 146 amino acids). There is, however, very little similarity to the male-specific region of the DSX^M proteins from tephritid or *Drosophila* species (figure 3). The protein motifs of the male-specific region that are encoded by exon 'm' in both *L. cuprina* and *M. domestica* (see below) are quite similar (42%). It is somewhat surprising that the carboxyl-terminal male-specific region of the DSX^M proteins is not so well conserved as sex-specific transcription regulation by DSX proteins is thought to be due in part to interaction between the carboxyl-terminal motifs and co-regulators (Cho and Wensink 1997). It will be of interested to determine if the blowfly and housefly DSX^M proteins interact with the same co-regulators as tephritid and Drosophila DSX^M proteins.

In *D. melanogaster*, the phorid fly *Megaselia scalaris*, *M. domestica* and tephritid species (e.g. *B. tryoni*), the 3′UTR of *dsxF* transcripts contain multiple binding sites for TRA/TRA2 (Lynch and Maniatis 1996; Shearman and Frommer 1998; Kuhn *et al*. 2000; Hediger *et al*. 2004). In *Drosophila*, TRA/TRA2 bind to six copies of a 13-bp sequence in a complex with RBP1 and to a purine-rich element in a complex with dSRp30 (Lynch and Maniatis 1996). The female-specific-first intron of the *Lctra* gene contained six copies of a 13-bp sequence that closely matched the *Drosophila* TRA/TRA2/RBP1 binding site (Concha and Scott 2009). The consensus sequence for the *Lctra* TRA/TRA2/RBP1 sites was (A/U)(A/C)(A/U)(A/U)CAAUCAACA. We searched the *LcdsxF* 3′UTR and found eight sites that closely matched the consensus TRA/TRA2/RBP1 site from the *Lctra* gene (figure 1; table 1). With the exception of sites two and five, which differ only in the first nucleotide, the *Lcdsx* TRA/TRA2/RBP1 sites were a perfect match to the consensus TRA/TRA2/RBP1 site from the *Lctra* gene. We did

Table 1. Putative TRA/TRA2/RBP1 binding sites in the 3′UTR of *Lcdsx* exon 4.

Sequence	Distance from stop codon (nucleotide)
ACAACAAUCAACA	389
CCAACAAUCAACA	437
ACAACAAUCAACA	540
ACAACAAUCAACA	579
GCAACAAUCAACA	620
UCUUCAAUCAACA	680
UCUUCAAUCAACA	762
UCUUCAAUCAACA	796

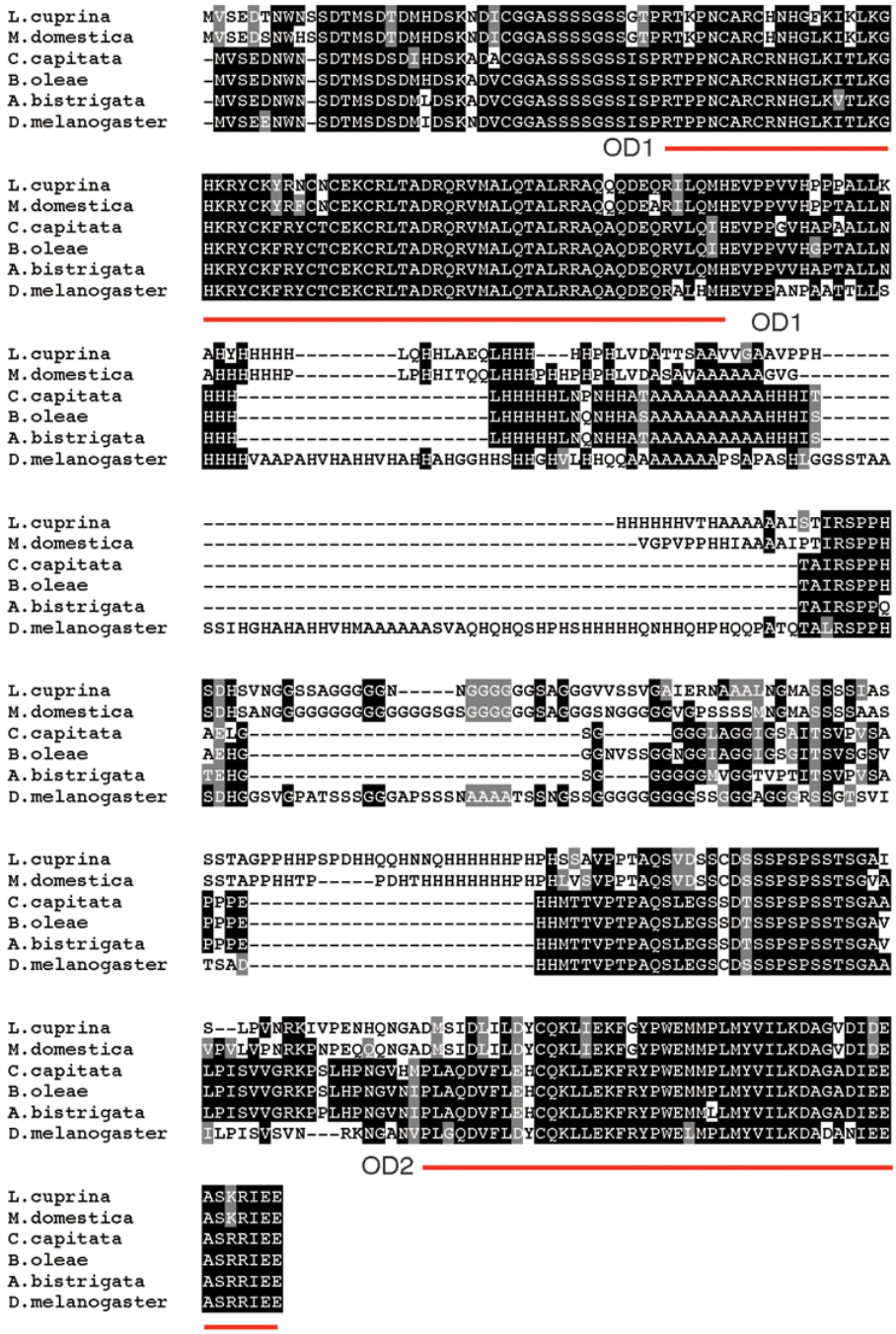

Figure 2. Multiple sequence alignment of the nonsex-specific regions of DSX proteins from *L. cuprina*, *M. domestica*, *C. capitata*, *B. oleae*, *A. bistrigata* and *D. melanogaster*. Identical amino acids are shaded in black while similar amino acids are shaded in grey. The OD1 and OD2 motifs are underlined in red.

not find a sequence in the exon 4, 3′UTR that matched the *Drosophila* purine-rich element.

Binding of TRA/TRA2 to the female *dsx* pre-RNA activates use of a weak upstream splice acceptor site. We examined the sequence of the *Lcdsx* female-specific intron and found that the splice acceptor site for the female intron is not a good match to the *Drosophila* consensus sequence (figure 4). The female splice acceptor site contains the highly conserved AG at the end of the intron. This is preceded by a short run of five uracils. However, further upstream is a purine-rich sequence that is a very poor match to the consensus splice acceptor sequence. With such a short polyprimidine stretch in the female splice acceptor site it would be predicted that splicing of exons 3 to 4 would be inefficient,

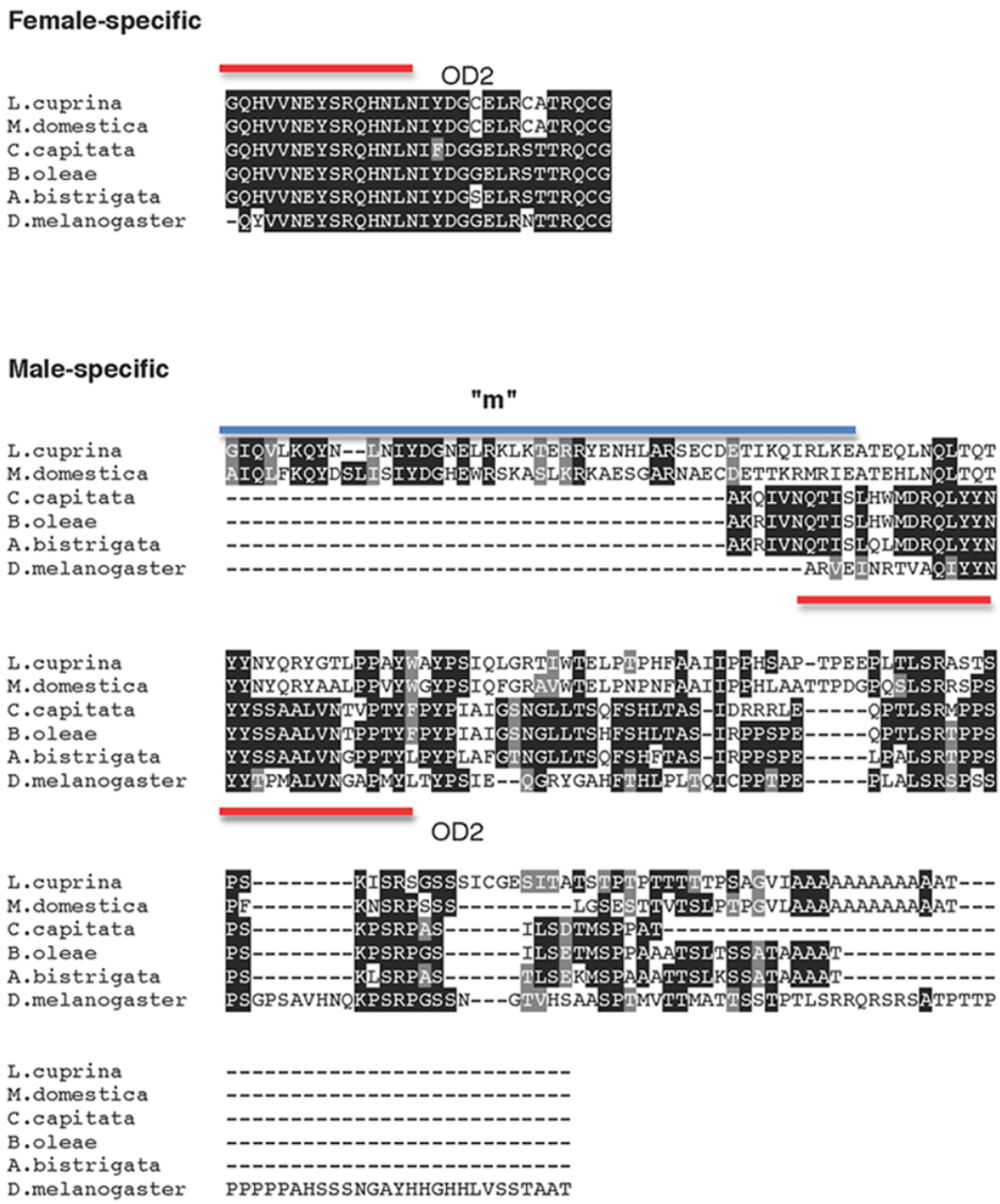

Figure 3. Multiple sequence alignments of the sex-specific regions of DSX proteins from *L. cuprina*, *M. domestica*, *C. capitata*, *B. oleae*, *A. bistrigata* and *D. melanogaster*. Identical amino acids are shaded in black while similar amino acids are shaded in grey. The OD2 motif is underlined in red. The blue line indicates the part of the *L. cuprina* and *M. domestica* proteins encoded by exon 'm'.

unless enhanced by TRA/TRA2. Indeed, injection of *Lctra* dsRNA into preblastoderm XX embryos led to the appearance of *Lc-dsxM* transcripts in sexually transformed adults (Concha and Scott 2009). Thus the mechanism of female splicing of *dsx* pre-RNAs in *L. cuprina* appears to be very similar to that of *D. melanogaster*, *M. scalaris* and several tephritid species.

A unique feature of the housefly *dsx* gene was the presence of a male-specific exon 'm' within the female-specific intron (figure 1A) (Hediger *et al*. 2004). As *L. cuprina* is more closely related to *M. domestica* than fruit fly species, we searched the nucleotide sequence of the *Lcdsx* female-specific intron for sequences that matched the *LcdsxM* male transcript. We found that, as in *M. domestica*, a 141-bp male-specific exon 'm' is present within the female-specific intron (figure 4). The splice donor and acceptor sites that flank exon 'm' are excellent matches to the *Drosophila* consensus sequences. Thus it is somewhat surprising that that female transcript does not contain exon 'm' spliced to exon 3. It remains possible that there are rare transcripts

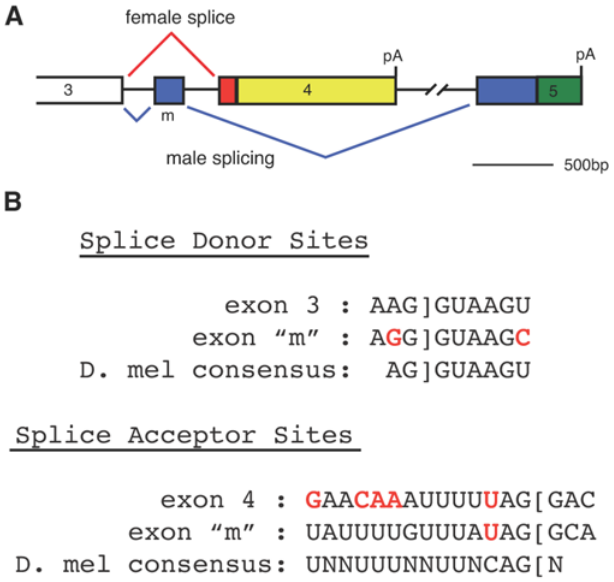

Figure 4. Sex-specific splicing of *Lcdsx* exons. (A) Schematic illustration of the female and male-specific slicing patterns of *Lcdsx* exons. Exons are numbered as in the *Md-dsx* gene. Exons are coloured as in figure 1. (B) Alignment of splice donor and acceptor sites with the consensus sequences for *Drosophila melanogaster* introns (Weir and Rice 2004). The splice sites used for splicing exons 3 to 4 in females are for splicing of exon 'm' in males are shown. Nucleotides that differ from the consensus are coloured in red. Exon/intron junctions are indicated by a square bracket. Note that the size of the intron between exons 4 and 5 is unknown.

containing exon 'm' in females that we did not detect, as a minor splice variant composed of exons 2-3-m-4 was detected in *M. domestica* in both sexes (Hediger *et al*. 2004). Presumably, the binding of TRA/TRA2/RBP1 to exon 4 in *L. cuprina* females causes the spliceasome to bypass exon 'm'. The *M. scalaris dsx* gene does not contain an exon 'm' in the female-specific intron (Kuhn *et al*. 2000). *M. scalaris* is in the section Aschiza whereas *Drosophila*, tephritids, housefly and blowfly are in the section Schizophora in the insect order Diptera. Thus it would appear that exon 'm' is unique to the *dsx* genes from insect species within the subsection Calyptratae of Schizophora. Attempts to isolate the other *Lcdsx* gene introns by PCR were unsuccessful, most likely because the introns are large as in the *Mddsx* gene (Hediger *et al*. 2004).

We conclude that the *Lcdsx* gene encodes well-conserved sex-specific proteins and *Lcdsx* transcripts are sex-specifically spliced in a similar manner as RNAs from the housefly *dsx* gene.

Acknowledgments

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