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Masculinization of XX *Drosophila* transgenic flies expressing the *Ceratitis capitata* Doublesex^{M} isoform

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ABSTRACT The Doublesex (DSX) transcription factor regulates somatic sexual differentiation in *Drosophila melanogaster*. Female and male isoforms (DSX^{F} and DSX^{M}) are produced due to sex-specific RNA splicing. Here we show that in the distantly related dipteran *Ceratitis capitata*, the DSX^{M} male-specific isoform is conserved and able to induce masculinization of both somatic and germline tissues when ectopically expressed in XX *Drosophila* transgenic individuals.

KEY WORDS: *Ceratitis, sex determination, DSX^{M} isoform*

Introduction

In the genetic model system *Drosophila melanogaster*, the *doublesex* gene (*dsx*) constitutes the terminal effector of the sex determination cascade in which the *transformer* (*tra*) and *Sex-lethal* genes (*Sxl*) are the main upstream regulators, and the XSEs (X-linked signalling elements) are the primary signal for sex determination (Erickson and Quintero, 2007). This sex determining regulatory cascade (XSE>*Sxl*>*tra*>*dsx*) is based mainly on sex-specific alternative splicing: the protein product of a gene controls the sex-specific splicing of the pre-mRNA produced from a downstream gene in the genetic cascade (Nagoshi *et al.*, 1988). *Sxl* plays the key role of maintaining female sex determination by a positive autoregulation (Cline, 1984). The *transformer* gene occupies an intermediate position in this hierarchy and is necessary for all aspects of female somatic sexual differentiation. The *dsx* gene regulates most aspects of somatic sexual differentiation and encodes two protein isoforms, DSX^{M} and DSX^{F}, through sex-specific splicing of its primary transcript (Baker and Wolfner, 1988). The processing of the *dsx* pre-mRNA in females requires the activity of the *tra* gene and of *transformer-2* gene (*tra-2*), which encodes a non-sex-specific auxiliary factor with similarity to the SR family of RNA-binding proteins (Amrein *et al.*, 1988, Hoshijima *et al.*, 1991). The two DSX isoforms are responsible to promote male and female sexual development, respectively, by activating or repressing the transcription of a series of target genes in the two sexes (Burtis and Baker, 1989).

The *doublesex* gene is known to act in concert with other regulatory genes to control the development of the sexual dimorphic structures. For instance, in the *Drosophila* genital disc, which gives rise to the terminalia and which is composed of two genital plus the anal primordia (Sanchez and Guerrero, 2001), *dsx* acts together with the homeotic gene *Abdominal-B (Abd-B)* to determine which of the two genital primordia will develop and which will be repressed (Keisman and Baker, 2001, Sanchez and Guerrero, 2001). The *bric-a-brac* gene (*bab*) is involved in the sexually dimorphic pigmentation of the 5th and 6th abdominal tergites by integrating inputs from *dsx* and *Abd-B* (Kopp *et al.*, 2000). Another integrated genetic input made up by *dsx* and the homeotic gene *Sex combs reduced* (*Scr*) has been invoked for sex-specific differentiation of the basitarsus of the prothoracic leg, developing sex combs in males (Jursnich and Burtis, 1993).

We have previously reported that similar to *Drosophila*, the *Ceratitis capitata transformer* homologue (*Cctra*) is required for the female-specific splicing of the *Ceratitis dsx* pre-mRNA (Graham *et al.*, 2003, Pane *et al.*, 2002). The evolutionary conservation of the *tra*>*dsx* genetic module was recently revealed also in other related Tephritidae as *Bactrocera oleae* (Lagos *et al.*, 2007) and various *Anastrepha* species (Ruiz *et al.*, 2007b). The molecular mechanism of *dsx* regulation seems to be fairly conserved in insects since *tra-2* homologues have been identified in *Musca domestica* (Burghardt *et al.*, 2005) and in *Ceratitis capitata* (Salvemini *et al.*, submitted) and are required in both these

Abbreviations used in this paper: DSX, doublesex; PRE, purine rich element.

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species for female-specific *dsx* splicing.

This study reports the structure, the regulation and the evolutionary functional analysis of the *Ceratitis dsx* orthologue (*Ccdsx*), performed by expressing the male-specific *Ccdsx*[M] isoform in transgenic *Drosophila* flies.

Results

To isolate the *Ceratitis dsx* orthologue a radioactive probe was prepared from the *Drosophila* female-specific *dsx* cDNA, which is highly conserved in other Tephritidae species, such as *Bactrocera tryoni* (Shearman and Frommer, 1998) and *Bactrocera oleae* (Lagos *et al.*, 2005, Shearman and Frommer, 1998). The probe was used to screen two *Ceratitis* cDNA libraries (prepared respectively from female adults and dissected ovaries). Two clones, named F1 (1.1 Kb, from female adults) and Ov1 (1.6 Kb, from ovaries), were isolated and sequenced. Their sequences match a putative *dsx* ORF truncated in the amino-terminus. RT-PCR analysis on sexed adult flies confirmed that the two cDNA clones correspond to part of female-specific *Ccdsx* mRNAs. A PCR-based screening of a genomic medfly library led to the isolation of two additional non overlapping clones corresponding to the 5' and internal regions of the gene. RT-PCR analyses were performed to isolate the full-length *Ccdsx* ORFs encoding the CcDSX[M] and CcDSX[F] proteins. A 1.3 Kb male-specific cDNA fragment (M1) was amplified by using the *BtdsxM* reversed primer (designed on the *Bactrocera oleae dsx* male-specific se-

A **Common region**

```
                                                   OD1
Dm 1 MVSEE-NWN-SDTMSDSDMIDSKNDVCGGASSSSGSSISPRTPPNCARCRNHGLKITLKGHKRYCKFRYCTCEKCRLTADRQR
Cc 1 MVSED-NWN-SDTMSDSDIHDSKADACGGASSSSGSSISPRTPPNCARCRNHGLKITLKGHKRYCKFRYCTCEKCRLTADRQR
Ao 1 MVSED-NWN-SDTMSDSDMLDSKADVCGGASSSSGSSISPRTPPNCARCRNHGLKITLKGHKRYCKFRYCTCEKCRLTADRQR
Bo 1 MVSED-NWN-SDTMSDSDMHDSKADVCGGASSSSGSSISPRTPPNCARCRNHGLKITLKGHKRYCKFRYCTCEKCRLTADRQR
Bt 1 MVSED-SWN-SDTIADSDMRDSKADVCGGASSSSGSSISPRTPPNCARCRNHGLKITLKGHKRYCKFRFCTCEKCRLTADRQR
Md 1 MVSEDSNWNSSDTMSDTDMHDSKADICGGASSSSGSSGTPRTKPNCARCRNHGLKITLKGHKRYCKYRFCNCEKCRLTADRQR

Dm VMALQTALRRAQAQDEQRALHMHEVPPANPAATTLLSHHHHVAAPAHVHAHHVHAHHAHGGHHSHHGHVLHHQQAAAAAAAAPSA
Cc VMALQTALRRAQAQDEQRVLQIHEVPPGVHAPAALLNHHH--------------------LHHHHHLNPNHHATAAAAAAAA---
Ao VMALQTALRRAQAQDEQRVLQMHEVPPVVHAPTALLDHHH--------------------LRHHHPLNQNHHATAAAAAAAA---
Bo VMALQTALRRAQAQDEQRVLQIHEVPPVVHGPTALLNHHH--------------------LHHHHHLNQNHHASAAAAAAAA---
Bt VMALQTALRRAQAQDEQRVLQIHEVPPVVHGPTALLNHHH--------------------LHHHHHLNQNHHASAAAAAAAA---
Md VMALQTALRRAQQQDEARILQMHEVPPVVHPPTALLNAHHHHHHPLP------HHITQQLHHHPHHPHPHLVDVSAVAAAAAAGV

Dm PASHLGGSSTAASSIHGHAHAHHVHMAAAAAASVAQHQHQSHPHSHHHHHQNHHQHPHQQPATQTALRSPPHSDHGGSVGPATSS
Cc --------------------------------------------AAHHHIT-------------TAIRSPPHAEL----------
Ao --------------------------------------------AAHHHIS-------------TAIRSPPQTEH----------
Bo --------------------------------------------AAHHHIS-------------TAIRSPPHAEH----------
Bt --------------------------------------------AAHHHIS-------------TAIRSPPHAEH----------
Md GVG----------------------------------------PVPPHHIA---------AAAIPTIRSPPHSDHSANGGGGGGG

Dm SGGGAPSSSNAAAATSSNGSSGGGGGGGGGSSG------GGAGGGRSSGTSVITSADH-----------------HMTTVPTPAQ
Cc -------------------------GSGGGGLA------GGIGSAITSVPVSAPPPEH-----------------HMTTVPTPAQ
Ao --------------------SG----GGGGGMV------GGTVPTITSVPVSAPPPEH-----------------HMTTVPTPAQ
Bo --------------------GGNVSSGGNGGIA------GGIGSGITSVSGSVPPPEH-----------------HMTTVPTPAQ
Bt --------------------GGNVSS--SGGIA------GGIGSAITSVPGSVPPPEH-----------------HMTTVPTPAQ
Md GGGGGGGSGSGGGG---GGSAGGGSNGGGGSVGPSSSSMNGMASSSAASTSSTAPPHHTPPDHTHHHHHHHPHPHLVSVPPTAQ

                                                      OD2
Dm SLEGSCDSSSPSPSSTSGAAILPISVSVNRK----NGANVPLGQDVFLDYCQKLLEKFRYPWELMPLMYVILKDADANIEEASRR
Cc SLEGSSDTSSPSPSSTSG-AALPISVVGRKPSLHPNGVHMPLAQDVFLEHCQKLLEKFRYPWEMMPLMYVILKDAGADIEEASRR
Ao SLEGSSDTSSPSPSSTSG-AVLPISVVGRKPPLHPNGVNIPLAQDVFLEHCQKLLEKFRYPWEMMPLMYVILKDAGADIEEASRR
Bo SLEGSSDTSSPSPSSTSG-AVLPISVVGRKPSLHPNGVNIPLAQDVFLEHCQKLLEKFRYPWEMMPLMYVILKDAGADIEEASRR
Bt SLEGSSDTSSPSPSSTSG-AVLPISVVGRKPSLHPNGVNIPLAQDVFLEHCQKLLEKFRYPWEMMPLMYVILKDAGADIEEASRR
Md SVDSSCDSSSPSPSSTSG-VAVPVLVPNRKPNPEQQQNGADMSIDLILDYCQKLIEKFGYPWEMMPLMYVILKDAGVDIDEASKR

Dm IEE 397
Cc IEE 286
Ao IEE 287
Bo IEE 291
Bt IEE 289
Md IEE 367
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B **Female-specific region**

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Dm 398 GQYVVNEYSRQHNLNIYDGGELRNTTRQCG 427
Cc 287 GQHVVNEYSRQHNLNIFDGGELRSTTRQCG 315
Ao 288 GQHVVNEYSRQHNLNIYDGGELRSTTRQCG 317
Bo 292 GQHVVNEYSRQHNLNIYDGGELRSTTRQCG 321
Bt 290 GQHVVNEYSRQHNLNIYDGGELRSTTRQCG 319
Md 368 GQHVVNEYSRQHNLNIYDGCELRCATRQCG 397
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C **Male-specific region**

```
Dm 398 -----------------------------------------ARVEINRTVA------QIYYNYYTPMALVNGAPMYLTYPS
Cc 287 -----------------------------------------AKRIVNQTISLHWMDRQLYYNYYSSAALVNTVPTYFPYP-
Ao 288 -----------------------------------------AKRIVNQTISLQLMDRQLYYNYYSSAALVNGPPTYLPYP-
Bo 292 -----------------------------------------AKRIVNQTISLHWMDRQLYYNYYSSAALVNTPPTYFPYP-
Bt 290 -----------------------------------------AKRIVNQTISLHWMDRQLYYNYYSSAALVNTPPTYFPYP-
Md 368 AIQLFKQYDSLIS--IYDGHEWRSKASLKRKAESGARNAECDETTKRMRIEATEHLNQLTQTYYNYQRYAALPPVYWGYPS

Dm IEQG---RYG---AHFTHLPLTQICPPTPE-PLALSRSPSSPSGPSAVHNQKPSRPGSSNGTVHSAASPTMVTTMATTSSTPTLS
Cc IAIG---SNGLLTSQFSHLTAS-MRPPSPE-QPTLSRMPPSPS--------KPSRPAS-----------ILSDTMSPPATATSLT
Ao LAFG---TNGLLTSQFSHFTAS-IRPPSPE-LPALSRTPPSPS--------KLSRPAS-----------TLSETMSPVAATTSLK
Bo IAIG---SNGLLTSHFSHLTAS-IRPPSPE-QPTLSRTPPSPS--------KPSRPGS-----------ILSETMSPPAAATSLT
Bt IAIG---SNGLLTSHFSHLTAS-MRPPSPE-QPTLSRTPPSPS--------KPSRPGS-----------ILSETMSPPAAATNLP
Md IQFGRAVWTELPNPNFAALIPPHLAATTPDGPQSLSRRSPSPF--------KNSRPSS-----------SLGSESTTVTSLPTPG

Dm RRQRSRSATPTTPPPPPPAHSSSNGAYHHGHHLVSSTAAT 549
Cc S-----AAT--------------------------ATAAT 394
Ao S-----SAT--------------------------AAAAT 396
Bo S-----SAT--------------------------AAAAT 400
Bt S-----SAT--------------------------AAAAT 398
Md VLAAAAAAA-------------------------AAAAAT 527
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Fig. 1. Comparison of the DSX predicted polypeptides in Dipteran species. Drosophila melanogaster (Dm) *(Burtis and Baker, 1989)*, Ceratitis capitata (Cc) *(this work)*, Anastrepha. obliqua (Ao) *(Ruiz* et al., *2007a, Ruiz* et al., *2005)*, Bactrocera oleae (Bo) *(Lagos* et al., *2005)*, Bactrocera tryoni (Bt) *(Shearman and Frommer, 1998) and* Musca domestica *(housefly)* (Md) *(Hediger* et al., *2004).* **(A)** *Sequences common to both sexes;* **(B)** *female-specific sequences;* **(C)** *male-specific sequences. The DNA binding domain OD1 and the oligomerization domain OD2 are shaded in grey. Gaps were introduced in the alignments to maximize similarity.*

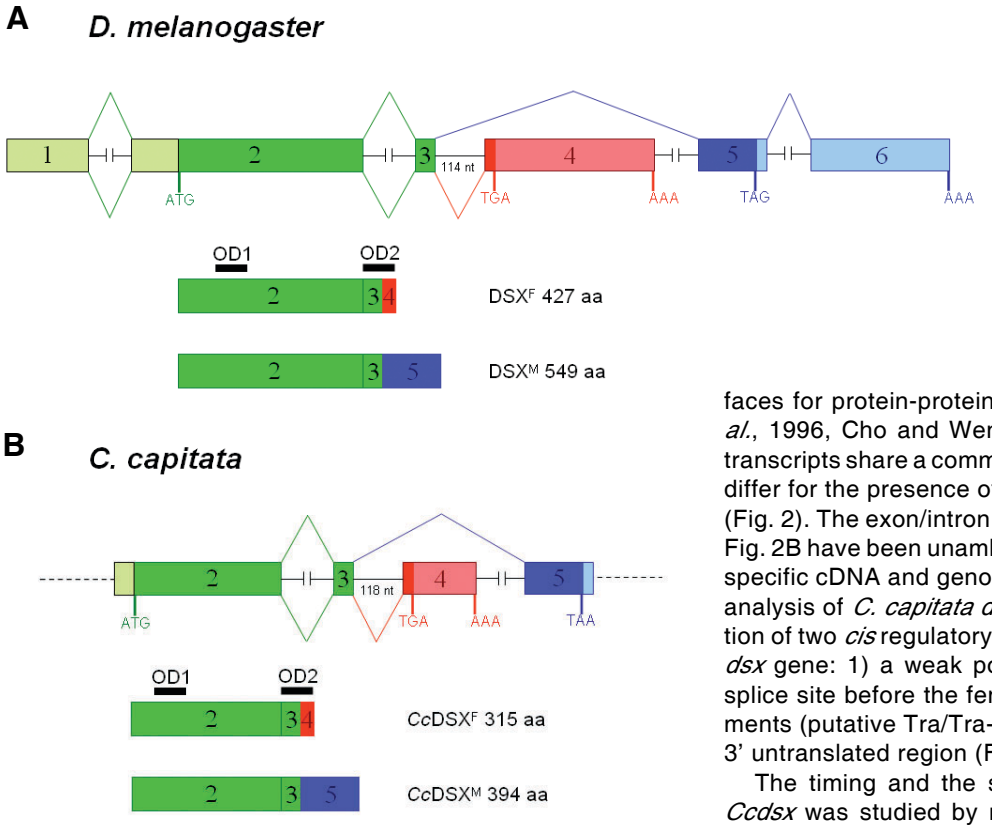

Fig. 2. Molecular organization of (A) the *Ceratitis capitata dsx* gene and its comparison with *dsx* of (B) *D. melanogaster*. *Exons (boxes) and introns (lines - when broken indicate that the length of the corresponding intron is unknown) are not drawn to scale. The numbers inside the boxes indicate the number of the exon. The coding sequence of sex-specific transcripts and open reading frame (ORF) are shown in the figure. The beginning and the end of the ORF are indicated by ATG and TGA, TAG or TAA, respectively.*

quence) and *Ccdsx*P+ primer (from the *Ccdsx* 5'UTR - see supplemental data). A 1.1 Kb female-specific cDNA fragment (F2) was amplified by using the *Ccdsx*P+ and the *Ccdsx*F2000- primers. The conceptual translation of the male and female amplicons indicates that they encode a polypeptide of 394 (*Cc*DSX^{M} acc. n°: AF434935) and 315 amino acids (*Cc*DSX^{F} acc. n°: AF435087), respectively.

An alignment of the two *Ceratitis* sex-specific CcDSX isoforms led us to define respectively a 285 aa long common region, a 30 aa long female-specific region and a 109 aa long male-specific region. BLAST analysis showed that the two CcDSX isoforms are homologous to the corresponding sex-specific isoforms of many other dipteran species (the degree of identity ranges between 40-91%), such as *Bactrocera* (Lagos *et al.*, 2005, Shearman and Frommer, 1998) and *Anastrepha* species (Ruiz *et al.*, 2007a, Ruiz *et al.*, 2005), *D. melanogaster* (Burtis and Baker, 1989), *Megaselia scalaris* (Kuhn *et al.*, 2000), *Musca domestica* (Hediger *et al.*, 2004), the mosquitoes *Anopheles gambiae* (Scali *et al.*, 2005) and *Aedes aegypti* (Mauro *et al.*, in preparation). Figure 1 shows a multiple Clustal-W alignment of *Ceratitis capitata* DSX proteins with other dipteran DSX homologues. The N-terminus of the *Drosophila* DSX^{F} protein appears to be longer due to the presence of an about 100 aa region enriched in stretches of specific aminoacids, which is absent in the *Ceratitis* and other Tephritidae DSX^{F} proteins. The most conserved regions are those corresponding to the *Drosophila* DSX protein OD1 (containing an atypical zinc finger DM domain) and OD2 domains (oligomerization domain that extends into the female-specific region), which serve as interfaces for protein-protein and protein-DNA interactions (An *et al.*, 1996, Cho and Wensink, 1997). The *Ccdsx* sex-specific transcripts share a common region composed of two exons, but differ for the presence of a female- and a male-specific exons (Fig. 2). The exon/intron junctions depicted in the schematic in Fig. 2B have been unambiguously determined, comparing sex-specific cDNA and genomic sequences. Nucleotide sequence analysis of *C. capitata dsx* led to identify sequence conservation of two *cis* regulatory elements described in the *Drosophila dsx* gene: 1) a weak polypyrimidine tract at the 3' acceptor splice site before the female-specific exon and 2) *dsx*RE elements (putative Tra/Tra-2 binding sites) in the female-specific 3' untranslated region (Fig. 3).

The timing and the sex-specific pattern of expression of *Ccdsx* was studied by northern blot and RT-PCR analyses. Northern blot on total RNA, extracted from adult males and females, revealed that a prominent 3,6 Kb long transcript is present in males (Probe C, derived from 5' end of the Ov1 clone; Fig. 4A) while a single 3 Kb long transcript is produced in females (identified by Probe C and Probe F, derived from 3' end of the Ov1 clone; Fig. 4A; the weaker signal by the probe F is due to different exposure time). RT-PCR experiments were performed with an oligonucleotide mix containing one single forward (1400+) and 2 reverse primers (the male-specific M1130- and the female-specific F2000-) on total RNA extracted from unfertilized eggs, embryos, larvae and pupae as well as from sexed adult flies. A single 0,6 Kb long cDNA product was observed in unfertilized eggs and embryonic stages (collected within 3-20h), which corresponds in size to the adult female-specific splicing variant, while a doublet of 0,6 and 0,3 Kb was observed in larval and pupal stages, which correspond in size to the female- and male-specific products respectively observed in adult sexed flies (Fig. 4B). RT-PCR was performed also also on single larvae (data not shown) and on adult somatic tissues (male and female dissected heads), showing again the presence of the expected *Ccdsx* sex-specifically spliced products (data not shown).

The *Ceratitis* male-specific DSX isoform showed 51% identity and 58% similarity when compared to DmDSX^{M}. To test whether the CcDSX^{M} and DmDSX^{M} proteins were functionally interchangeable, we examined the ability of the CcDSX^{M} protein to induce masculinization of *D. melanogaster* XX flies by competing with the endogenous DmDSX^{F}. We generated 20 *Drosophila* transgenic lines expressing a *Ccdsx^{M}* cDNA from the *hsp70* heat shock promoter (*hsp70::Cc*DSX^{M}). When raised at 25°C, males and females, showed wild type anal and genital

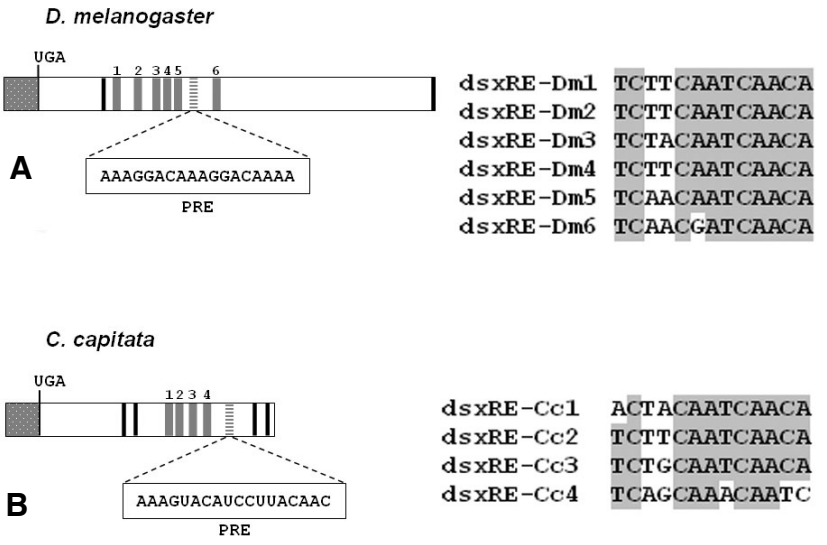

Fig. 3 (Above). Distribution of Tra/Tra-2 binding sites (*dsx*RE elements) and Purine Rich Element (PRE) in the 3′ untranslated region of *dsx* female-specific exons. (A) D. melanogaster *and* **(B)** C. capitata. *The* dsx*RE are marked in grey boxes. The* PRE *is marked as a lined box and the respective putative sequence is reported below. Putative polyadenylation signals are marked as black boxes and the stop codons are indicated. On the right of each diagram the* dsx*RE sequences present in the female-specific exon of the respective species are shown. The shading indicates identical nucleotides.*

regions, and no evident reduction in viability or fertility. However, females of all transgenic lines showed full male-like pigmentation of the sixth tergite and variable pigmentation also of the fifth tergite (Fig. 5A – as in dsxM12 line). More severe adult phenotypes, similar to those described by Jursnich and Burtis (1993), were observed when transgenic animals were reared at 29°C and exposed to two daily heat shock regimens, from first instar larvae till adult stages: 1) transformation of legs bristles towards a sex-combs-like morphology (Fig. 5B), and larval/pupal lethality, with only a low number of adult escapers (approx 70-90% lethality). Masculinized females showed 2) full male-like pigmentation of the fifth tergite (Fig. 5A – as in dsxM6 line), and 3) transformed genitalia, with reduction in the size of the vaginal plates and in the number of vaginal teeth (Fig. 5C). However, no pigmentation of dorsal spinules and ventral setae were observed in third-instar transgenic larvae. In two different transgenic lines, recovered after heat shock treatment, a few pseudomales (12 out of 70 survivors) showed strongly altered (7 flies) or even almost completely masculinized (5 flies) genitalia. Upon dissection these pseudomales displayed defective female or male germline tissues (Fig 5D). PCR-based analysis was used to asses the karyo-

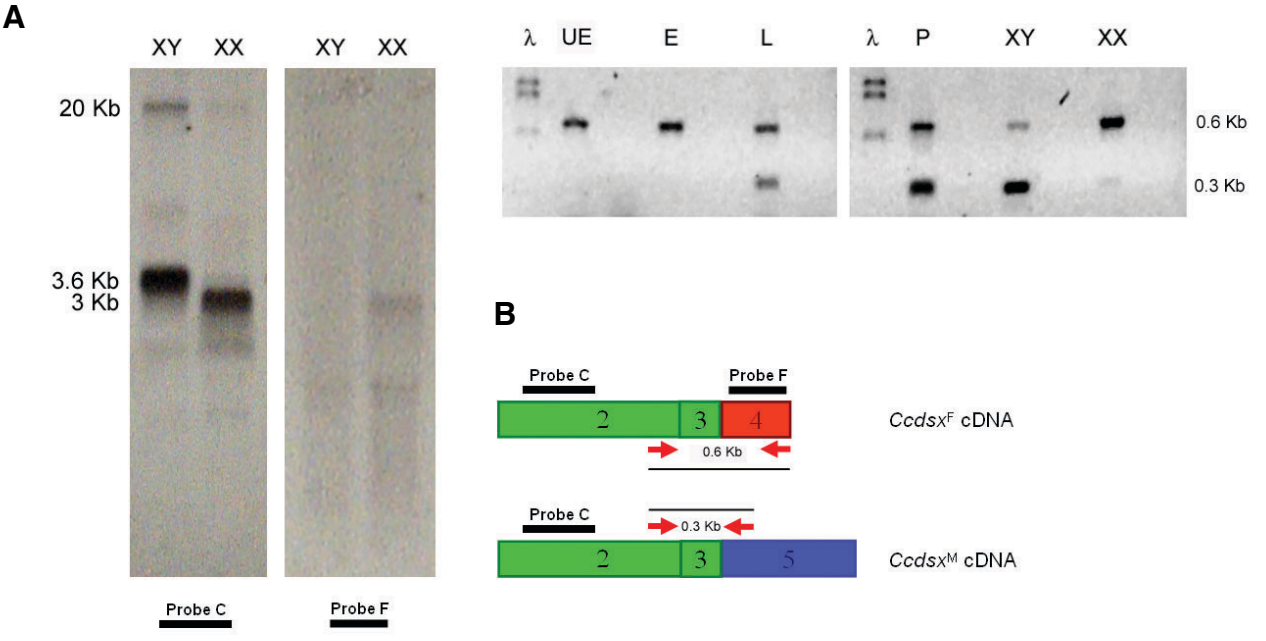

Fig. 4. Timing of expression of the *C. capitata dsx* gene. (A) *Northern blots of total RNA from adult males (XY) and females (XX). Northerns were hybridized with two DNA probes obtained from the* Ccdsx *female cDNA: probe C, corresponding to the common region of the* Ccdsx *gene, and probe F, corresponding to female-specific region.* **(B)** *RT-PCR analyses on total RNA of unfertilized eggs (UE), embryos 3-20 h old (E), larvae (L), pupae (P), male (XY) and female (XX) adults. At the bottom of (B), the molecular organization of the corresponding cDNAs and the localization of RT-PCR primers (red arrows) are shown.*

type of pseudomales from lines 2 and 11 using two Y-specific primers and, as positive internal control, two primers specific for the autosomic *Dmdsx* gene (Fig. 5E). The lack of Y-specific amplification in pseudomales samples confirm their XX karyotype.

Discussion

In this study we report the isolation and characterization of the *Ceratitis capitata dsx* gene (*Ccdsx*). As in *Drosophila*, *Musca* and in two Tephritidae species, the *Ccdsx* gene is transcribed in males and females individuals, from early stages of development till adulthood, and its primary transcript undergoes sex-specific splicing, producing the female CcDSX^F and male CcDSX^M proteins. The presence of conserved Tra/Tra-2 binding sites within the female-specific exon of *Ceratitis capitata dsx* gene further suggests that also in this species the *dsx* female-specific splicing may be regulated by the gene products of *Cctra* and *Cctra-2*. Hence *Ceratitis dsx* female-specific splicing appears to be regulated by a conserved alternative splicing mechanism in which, as in *Drosophila*, during development the male-specific mode is the default state (default: absence of CcTRA), while the female-specific mode is the regulated state, which requires the positive activity of the CcTRA/CcTRA-2 splicing complex, as also suggested by *in vivo* RNAi against *Cctra* and *Cctra-2* (Pane *et al.*, 2002; Salvemini *et al.*, submitted). While in unfertilized eggs and XX/XY embryos (3-20h) only the *Ccdsx* female-specific variant is amplified by RT-PCR, the male-specific variant seems to be present later on, from early larval stages. Hence we propose that *Ceratitis* male-sex differentiation is controlled by CcdsxM as Dm dsx^M in *Drosophila*, and it starts from larval developmental stages. It is presently thinkable that at embryonal stages sex determination of *Ceratitis* is set up by default in a female mode as suggested by the presence of maternal mRNAs corresponding to CcdsxF, but also *Cctra*^{F} and *Cctra*-2 (Salvemini *et al.,* submitted) and that later on only in XY embryos *Cctra* and consequently *Ccdsx* switch the splicing pattern to the male mode, because of the action of the Y-linked male determining factor, still to be molecularly identified.

We presented data showing that the male-specific CcDSX^M isoform induces a strong masculinization when expressed in *Drosophila* transgenic females. These results indicate that the CcDSX^M protein can efficiently outweigh the endogenous DmDSX^F protein causing a masculinization of the sexual dimorphic structures of the XX transgenic flies. These results further show the biochemical capacity of the CcDSX^M protein to interact with the

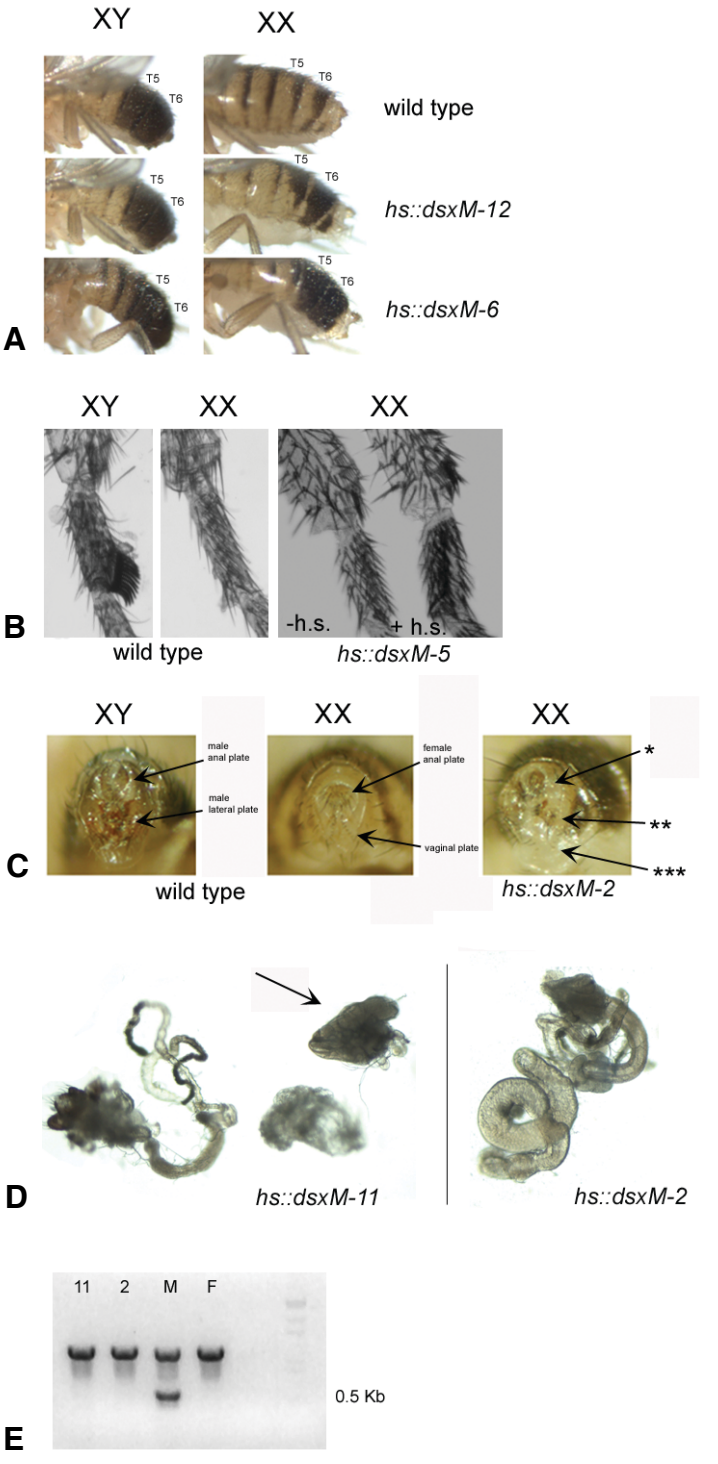

Fig. 5. Phenotypes of *Drosophila hs::CcdsxM* transgenic lines. (A) *Abdominal pigmentation. Abdomens from wild type males (XY), females (XX) and transgenic males display the characteristic dimorphic pigmentation patterns in the most posterior tergites, T5 and T6. In transgenic XX individuals male-like pigmentation of T5 and T6 tergites can be observed to a variable extent (lines dsxM-12 and dsxM-6).* **(B)** *Bristle modification. Prothoracic leg of wild type male (XY), wild type female (XX) and of transgenic female from line dsxM-5, without heat shock treatment (- h.s.; left) and after heath shock treatment (+ h.s.; right). After heat shock bristles of basitarsus region exhibited a slight bluntness and increased pigmentation.* **(C)** *External genitalia modification. XX transgenic female from dsxM-2 line, recovered after heat shock treatment, display intersexual external genitalia: arrow*, male like anal plate; arrow**, male like lateral plate; arrow***, reduced vaginal plate.* **(D)** *Dissected genitalia of pseudomales from line dsxM-11 and dsxM-2. We observed abnormal ovaries in flies from line 11 (indicated by arrow) and apparently normal testes in flies from line 2.* **(E)** *Y-specific PCR on carcasses of two pseudomales (from lines dsxM-11 and dsxM-2) and of a control male (M) and female (F) flies. The control amplification signal of 1 Kb is present in all samples while a Y-specific amplification signal of 0.5 Kb is observed only in XY male control sample.*

other regulatory partners of the *Drosophila* DSX protein and support the idea of its functional conservation in *Ceratitis* sex determination. Interestingly, in the case of *tra* which is a functional conserved key female-determining gene in both *Ceratitis* and *Drosophila*, Pane *et al.* (2005) showed that CcTRA protein, although very weakly conserved in its sequence (18% identity) and lenght (Cc 429 aa versus Dm 197 aa) is is able to efficiently feminize *Drosophila* XY transgenic flies. On the contrary in the case of Sxl which is a key female-determining gene in *Drosophila* but not in *Ceratitis*, Saccone *et al.* (1998) showed that the CcSXL protein, although very highly conserved is not able to efficiently feminize or kill (acting on dosage compensation) *Drosophila* XY transgenic flies. as expected in the case of conservation of its biochemical properties. These two previous studies in which an heterologous functional test for *Ceratitis* genes was successfully used, support the potential consistency of this "indirect" approach to evolutionary genetic functional conservation. Hence we suggest that not only the biochemical but also the developmental roles of the dsx^M homologues are similar in both *Ceratitis* and *Drosophila*.

A similar experiment using *Musca domestica* DSX^M transgene, driven by UAS/*hsp70*-GAL4 system (which usually permit higher expression levels with the respect of the simple *hsp70* fusion construct) in *Drosophila* lines, led only to male-like pigmentation of the posterior tergites (Hediger *et al.*, 2004). The extent of pigmentation was much less pronounced than the one observed with $CcDSX^M$, possibly because the higher sequence similarity of CcDSXM to DmDSXM (58% Cc/Dm versus 50% Md/Dm), and the closer phylogenetic relationship of *Ceratitis* to *Drosophila* (both belong to Acalyptratae), than to *Musca* (Calyptratae).

It has been shown that the sex of *Drosophila* germ line requires cell-autonomous and inductive signals from the gonadal soma (Hempel and Oliver, 2007, Nothiger *et al.*, 1989, Steinmann-Zwicky *et al.*, 1989). The underdeveloped gonads of XX pseudomales expressing the $CcDSX^M$ protein cannot be explained by the expression of this protein within the XX germ cells since the *dsx* gene is not cell-autonomously required for oogenesis: XX cells either lacking *dsx* function or expressing the DSX^M protein form normal oocytes when transplanted into a female embryo (Schupbach, 1982). Rather, the defective gonads are likely the consequence of masculinisation of the female gonadal soma caused by the $CcDSX^M$ protein so that no match exists between the cell-autonomous female signal of XX germ cells and the masculinised signal from their surrounding gonadal soma. It is hence conceivable that the transgene in some *Drosophila* transgenic lines can express $CcDSX^M$ at a level such that a strong masculinizing somatic signal is sent to the XX germ line cells, inducing them to become spermatogenetic.

In conclusion, *Ceratitis capitata* sexual differentiation seems to be controlled as in *Drosophila* by the binary switch gene *dsx*, encoding conserved sex-specific transcriptional factors. It will be of interest in future to identify the main gene targets of DSX in both species to evaluate the stability and the divergence of the downstream branching genetic networks which control the development of the sexual dimorphic traits.

Materials and Methods

Cloning of Ccdsx *gene*

The screening of *C. capitata* cDNA libraries was performed using a 300 bp PCR fragment amplified from *Drosophila* genomic DNA using primers for the female-specific region (Dm*dsx*2251+ and Dm*dsx*2541-). Hybridization and identification of positive clones were performed using standard protocols described in Maniatis *et al.* (1982). All PCR and RT-PCR products were sub-cloned in pUC18 cloning vector (Promega).

Northern blots and RT-PCR

Northern blots and hybridizations were performed according to standard protocols (Maniatis *et al.*, 1982). For RT-PCR analyses two micrograms of total RNA from each developmental stage were reverse transcribed with the Superscript II RNase H – reverse transcriptase (Invitrogen) using oligo dT primers and following the manufacturer's instructions. 1/20 v/v of the synthesised cDNA was amplified by PCR. RT-PCR products were analyzed by agarose gel electrophoresis. The primers used are: CcdsxC 1400+ located in *Ccdsx* common exon 3, CcdsxM 1130– located in *Ccdsx* male-specific exon 5 and CcdsxF 2000– located in *Ccdsx* female-specific exon 4. RT-PCR products were gel-purified, cloned using the pGEM-T Easy Vector Kit (Promega) and sequenced with Big Dye® Terminator v1.1 Sequencing Kit (Applied Biosystem).

Generation of $CcDSX^M$ Drosophila *transgenic lines*

Germline transformation experiments were performed as described by Rubin and Spradling (Rubin and Spradling, 1982). Preblastoderm embryos of *D. melanogaster* were injected with 500 ng/μl of pCaSpeR-hs-$Ccdsx^M$-act vector and 150 ng/μl of the P-element Δ2-3 helper vector and 20 transgenic lines were produced. To induce the expression of the $Ccdsx^M$ cDNA from the Hsp70 promoter, flies from each line were reared at 29°C and exposed twice a day to heat-shock regimen at 37°C for 1 hr, from embryonic stages until adulthood.

Karyotyping of Drosophila *pseudo-females*

Genomic DNA of pseudo-females from transgenic lines 2 and 11 was extracted using the *Drosophila* DNA/RNA Isolation protocol developed by Andres and Thummel (Andres and Thummel, 1994). Y-specific PCR was performed for each line on 50 ng of the extracted genomic DNA with 1 unit of Taq Polymerase (Amersham Pharmacia) according to the manufacturer's directions in the presence of the specific primers for *DmCCY* gene and of *Dmdsx* gene as internal control (CCY+, CCY-, DMF and DF primers).

Sequences of primers utilized in this paper

Dmdsx2251+	5'-AGCCATTTGCCGATCTC-3'
Dmdsx2541-	5'-TCATCCACATTGCCGCG-3'
BtdsxM	5'- TGTTGCTGTTACGTAGCTGC-3'
CcdsxP+	5'-ATAGGCATCGTAGCTGTTCT-3'
CcdsxC 1400+	5'-GGCATCAAGGCGTATAGAAGA-3'
CcdsxM 1130–	5'-CTGGTGGTGACATCGTATCG-3'
CcdsxF 2000–	5'-ACGACGGCATGACCTTTAAC-3'
CCY+	5'-CACTGGAGTGGTTCCTGC-3'
CCY-	5'-ATTGCTCCCTACAATCTTCC-3'
DMF	5'-CCGCTATCCTTGGGAGCT-3'
DF	5'-TTGAGATTGGCTTGTATGCC-3'

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