Int. J. Dev. Biol. 53: 109-120 (2009)
doi: 10.1387/ijdb.082681ms

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Ceratitis capitata transformer-2 gene is required to establish and maintain the autoregulation of *Cctra*, the master gene for female sex determination

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ABSTRACT In *Drosophila melanogaster,* transformer-2 (TRA-2) which is a non-sex-specific auxiliary splicing factor, is required to promote female sexual differentiation by interaction with the female-specific TRA. The two proteins positively regulate the splicing of both *doublesex (dsx)* and *fruitless (fru)* pre-mRNAs, which in turn regulate phenotypic and behavioural sexual dimorphism. In the Mediterranean fruitfly *Ceratitis capitata*, the female-specific CcTRA is similarly required not only for *Ccdsx* splicing, but also to exert a novel autoregulatory function that consists of promoting female-specific splicing of *Cctra* pre-mRNA. This study reports the isolation and functional analysis of the *C. capitata* homologue of the *Drosophila transformer-2* gene *(Cctra-2)*. Transient RNAi against *Cctra-2* during embryonic development causes the full sex reversal of XX flies in adult fertile pseudo-males, as well as changes in the splicing pattern of *Cctra*, *Ccdsx* and *Ccfruitless (Ccfru)*. We propose that: 1) *Cctra-2*, as in *Drosophila*, is necessary for promoting *Ccdsx* and putative *Ccfru* pre-mRNA female-specific splicing and that 2) unlike in *Drosophila*, *Cctra-2* appears to be necessary for establishing female sex determination in early XX embryos and for maintaining the positive feedback regulation of *Cctra* during development.

KEY WORDS: *sex determination, Drosophila, Ceratitis, alternative splicing, autoregulation*

Introduction

The regulatory pathway controlling sex determination in *Drosophila melanogaster* is based on the sex-specific transcription and splicing of key regulatory genes such as *Sex-lethal* (*Sxl*), *transformer* (*tra*), *transformer-2* (*tra-2*), *doublesex* (*dsx*) and *fruitless* (*fru*) (Cline and Meyer, 1996). The XSEs (X-linked signalling elements) are the primary signals for sex determination (Erickson and Quintero, 2007), controlling the activity of downstream genes organized in a cascade of regulatory events (XSE> *Sxl*> *tra+tra-2*> *dsx/fru*). The choice between male and female development is made by the switch gene *Sxl* in response to this transient primary signal. The *Sxl* gene is only active in XX individuals, promoting femaleness through its downstream regulatory cascade, as well as ensuring female-specific maintenance of its own activation through a positive feedback loop. *Sxl* then promotes a genetic program of female sexual differentiation through the downstream *tra* gene. In XX individuals, the SXL protein promotes splicing of *tra* pre-mRNA so that full-length TRA is produced only in females (Inoue *et al.*, 1990, Sosnowski *et al.*, 1989, Valcarcel *et al.*, 1993). In males, the *Sxl* gene is "OFF", and *tra* male-specific splicing is governed by a default mechanism resulting in a small, non-functional TRA peptide (Boggs *et al.*, 1987, McKeown *et al.*, 1987). In XX individuals, TRA controls the downstream components of the cascade, *dsx* and *fru*. The sex-specific protein isoforms produced from *dsx* and *fru* are responsible for the development of sex-specific somatic tissues and behavioural traits (Burtis and Baker, 1989, Heinrichs *et al.*, 1998, Hoshijima *et*

Abbreviations used in this paper: dsRNA, double stranded RNA; ESE, exonic splicing enhancer; ISS, intronic splicing silencer; Medfly, Mediterranean Fly; PRE, purine-rich element; RNAi, RNA interference; RRM, RNA recognition motif; Tra, transformer.

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Accepted: 30 October 2008. *Published online:* 28 November 2008.

ISSN: Online 1696-3547, Print 0214-6282
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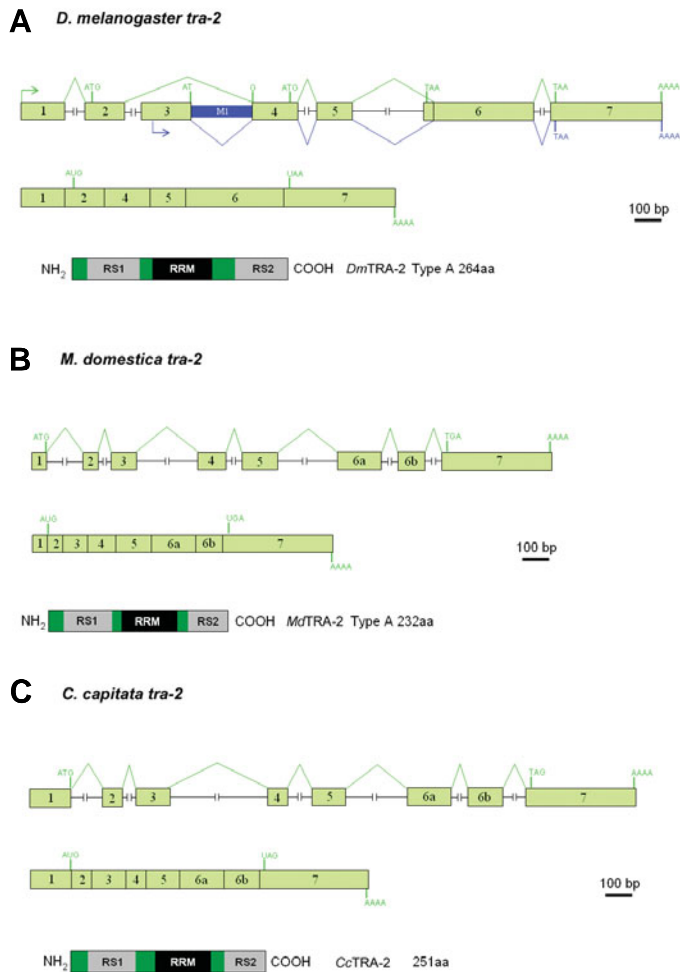

Fig. 1. Genomic organization of *Dmtra-2* (A), *Mdtra-2* (B) and *Cctra-2* (C). *The testis-specific transcription start site in* Dmtra-2 *is marked by a small blue arrow. M1 is an alternatively spliced intron in Dm. The structures of the major splice variants of* Dmtra-2, Mdtra-2 *and* Cctra-2 *transcripts and the corresponding proteins are reported (below). The RNA-recognition motif (RRM) is marked in black and the arginine-rich/serine-rich (RS) domains in grey.*

al., 1991, Ryner *et al.*, 1996). *dsx* and *fru* splicing regulation also require the product of the no-sex-specific *tra-2* gene as well as other general splicing factors (Amrein *et al.*, 1988, Burtis and Baker, 1989, Inoue *et al.*, 1992).

The *Drosophila tra-2* gene encodes three distinct protein isoforms (TRA-2^{264}, TRA-2^{226} and TRA-2^{179}), each containing an RNA-recognition motif (RRM) flanked by two arginine-rich/serine-rich regions (RS-domains) that mediate protein-protein interactions to facilitate the formation of both spliceosomal and regulatory splicing complexes. The three isoforms differ in their lengths, with TRA-2^{226} and TRA-2^{179} corresponding to two truncated versions of TRA-2^{264}, each lacking a different amino-terminal portion (Amrein *et al.*, 1994, Mattox *et al.*, 1996, Wu and Maniatis, 1993). The targets of *tra-2* regulation appear to be restricted by tissue type. In *Drosophila* XX larvae, the future females, TRA-2^{264} and TRA-2^{226}, redundantly together with TRA, bind to six nearly identical copies of an exonic splicing enhancer (ESE - called TRA/TRA-2 binding sites) and a single purine-rich element (PRE), building a splice-enhancing complex that promotes the use of a 3' splicing site upstream of the female-specific exon 4 of the *dsx* gene (Tian and Maniatis, 1993). This leads to the formation of an mRNA encoding the DSX^{F} protein that promotes female differentiation. In the nervous system, TRA-2 isoforms and TRA proteins direct female-specific utilization of an alternative 5' splice site in *fruitless* pre-mRNA resulting in sex-specific expression of Fruitless isoforms, which in turn promote the development of innate sex-specific behaviour (Ryner *et al.*, 1996). As in the case of *dsx*, this regulation depends on binding a specific set of TRA/TRA-2 binding sites in the *fru* pre-mRNA (Heinrichs *et al.*, 1998, Lam *et al.*, 2003). Both TRA-2^{226} and TRA-2^{179} are expressed in the male germ line, but only the former is necessary and sufficient for male fertility, acting independently of TRA and affecting sex-specific processing of pre-mRNA from *exuperantia* (*exu*), *alternative testis transcripts* (*att*), and *tra-2* itself (Hazelrigg and Tu, 1994, Madigan *et al.*, 1996, McGuffin *et al.*, 1998). TRA-2^{226} represses the removal of the M1 intron of *tra-2* pre-mRNA from mature mRNAs by binding to an intronic splicing silencer (*tra-2*-ISS), leading to the alternative TRA-2^{179} non-functional isoform (Chandler *et al.*, 2003, Mattox and Baker, 1991, Qi *et al.*, 2007). This autoregulatory mechanism affects a significant fraction of germ-line *tra-2* transcripts: about 50% retain the M1 intron in a manner dependent on functional TRA-2^{226} protein (Mattox *et al.*, 1996, McGuffin *et al.*, 1998).

The isolation of *Sxl*, *tra* and *dsx* homologues in the distantly related dipteran species *Ceratitis capitata* led to the discovery of the partial conservation of the *Drosophila* regulatory *tra>dsx* module despite 120 Myr of phylogenetic distance between the two species (Pane *et al.*, 2002, Saccone *et al.*, 2002; Saccone *et al.*, 1998). While *Ceratitis Sxl* is not regulated in a sex-specific manner, the *tra* and *dsx* homologues (*Cctra* and *Ccdsx*) produce sex-specific mRNA by alternative splicing, as in *Drosophila* (Pane *et al.*, 2002, Saccone *et al.*, 1998). Sequence analysis of the *C. capitata dsx* gene found the sequence conservation of two *Drosophila* regulatory elements: 1) a weak polypyrimidine tract at the 3' acceptor splice site before the female-specific exon and 2) four conserved putative TRA/TRA-2 binding sites in the female-specific 3' untranslated region of *Ccdsx* (Saccone *et al.*, 2008). These motifs support the notion that *Ceratitis dsx* female-specific splicing appears to be regulated by a conserved alternative splicing mechanism in which the male-specific mode is the default state, while the female-specific mode requires the positive action of the TRA/TRA-2 homologous splicing complex (Pane *et al.*, 2002). Moreover, a very interesting feature was found in *Cctra*: in contrast to the *Drosophila* homologue, the *Ceratitis tra* gene contains the very same TRA/TRA-2 binding sites within the male-regulated genomic region, suggesting an autoregulatory mechanism for splicing (Pane *et al.,* 2002). *Cctra* has a binary functional state (ON/OFF) which is controlled very early during embryogenesis, either directly or indirectly, by the primary signal, a Y-linked male determining factor M (Willhoeft and Franz, 1996). In XY embryos, M causes a default male-specific splicing of *Cctra* pre-mRNAs, which leads to the production of male-specific mRNAs containing very short, probably non-functional ORFs. In contrast, in XX embryos, *Cctra* splicing skips the male-specific exons, resulting in a female-specific transcript encoding full-length CcTRA protein. The complete sex reversion of XX adults, obtained with

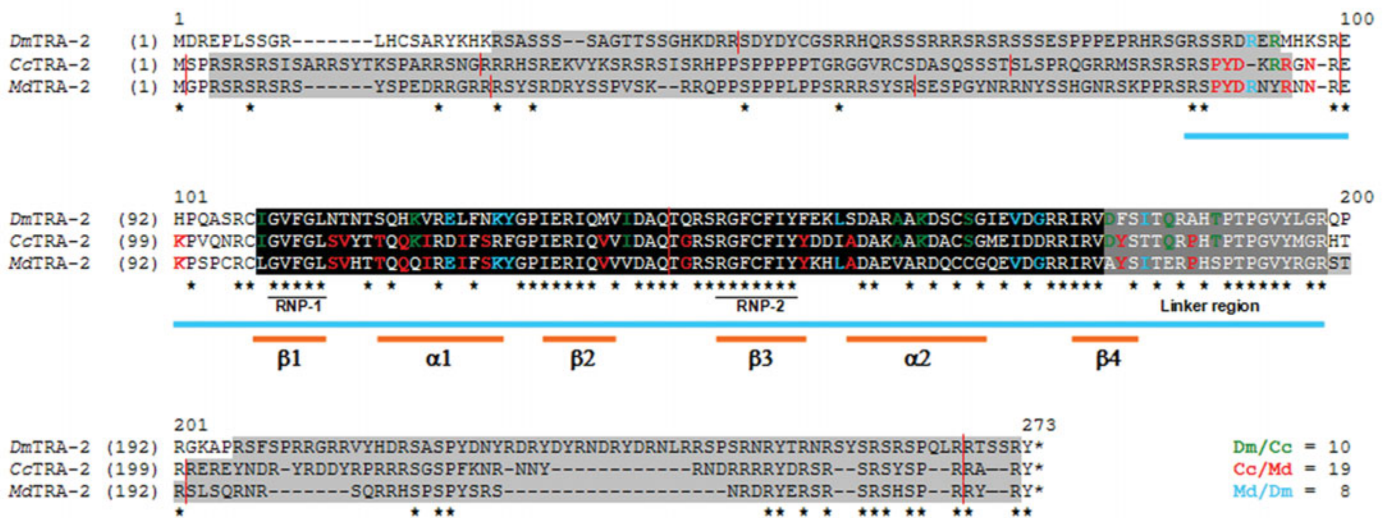

Fig. 2. Multiple sequence alignment of major TRA-2 protein variants in *D. melanogaster* (DmTRA-2), *Ceratitis capitata* (CcTRA-2) and *Musca domestica* (MdTRA-2). *Asterisks (*) indicate amino acid identity in all species. Intron positions are indicated by red vertical lines inside the amino acid sequences. The RNA recognition motif (RRM) is in black, RS domains are light grey, and the linker region is dark grey. RNP-1 and RNP-2 indicate the positions of two ribonucleoprotein identifier sequences, which are highly conserved between RRM proteins. Note the high number of identical amino acids within the RRM, whereas both RS domains are less conserved but are abundant in arginine and serine. Underlined in blue is the amino acid region subject to phylogenetic analysis between* Drosophila, Ceratitis *and* Musca *(see text). Underlined in orange are the amino acids in putative α-helix or β-sheet regions. Bold green letters indicate amino acid identity between* Drosophila *and* Ceratitis, *bold red letters indicate amino acid identity between* Ceratitis *and* Musca, *and bold blue letters indicate amino acid identity between* Drosophila *and* Musca.

transient RNAi against *Cctra* in *C. capitata* embryos, was surprisingly effective, supporting the notion of a peculiar *Cctra* autoregulation in maintaining its own female-specific activation (Pane *et al.*, 2002) by a positive feedback loop, which seems to be analogous to the *Drosophila Sxl* autoregulatory mechanism. *Cctra* acts as a key master switch and an epigenetic memory device for *Ceratitis* female sex determination, and indicates once again the existence of a functionally conserved *tra-2* homologue in *Ceratitis*, acting as a co-factor in maintaining *transformer* positive autoregulation.

This paper reports the cloning and functional analysis of the *C. capitata tra-2* homologue (*Cctra-2*). We present evidence supporting the model that, as expected, the *Cctra-2* gene is involved not only in the *Ccdsx* splicing but also in *Cctra* autoregulation. In addition, the sex-specific mRNA splicing of a putative *C. capitata fruitless* (*Ccfru*) homologue seems to depend on *Cctra* and *Cctra-2* expression. We propose that CcTRA and CcTRA-2 bind to the highly conserved TRA/TRA-2 elements to promote female-specific splicing of *Cctra*, *Ccdsx* and, presumably, *Ccfru* pre-mRNAs.

Results

Cloning and gene organization

To isolate *Ceratitis capitata tra-2* cDNA, PCR was performed with the degenerate primers MAR25, MAR5 and MAR17 (see Materials and Methods for details) designed from the sequence alignment of the *D. melanogaster*/human *tra-2* homologues, using adult *Ceratitis capitata Benakeion* wild-type cDNA as a template. One PCR product (240 bp) was obtained. The deduced amino acid sequence of the cDNA clone encodes for an 80 aa product with the highest identity to a putative TRA-2 from another Tephritidae (*Bactrocera oleae*, acc. numb. CAD67988), and identity to *Musca* domestica (Burghardt *et al.*, 2005) and *Drosophila melanogaster* TRA-2 homologues, as well as to TRA-2-related proteins from distantly related insect species, such as *Bombyx mori* and *Apis mellifera*. 5' and 3' RACE experiments on total RNA prepared from *Ceratitis* male and female adult flies allowed us to assemble a transcript 1.1 kb in length containing an ORF of 753 bp which encodes for a putative protein of 251 aa, with 70% identity (87/124) to *Musca* TRA-2 and 56% identity (94/166) to *Drosophila melanogaster* TRA-2 by Blast analysis. Genomic fragments covering the 3366 bp transcribed region were isolated by the Genomic Walker Kit and PCR from genomic templates. The genetic structure of the *C. capitata tra-2* gene was determined using cDNA and genomic clones. The *Cctra-2* transcript is composed of eight exons (Fig. 1). An alignment of genomic and cDNA sequences of *Cctra-2* with respect to *Dmtra-2* and *Mdtra-2* genes revealed that 3 of 7 introns are perfectly conserved at the corresponding positions. Exon 6 of *Dmtra-2* is split in *C. capitata* and *M. domestica* by an intron. We refer to these exons as 6a and 6b, following previous definitions (Burghardt *et al.*, 2005). The highest upstream putative translational start site in the assembled *Cctra-2* transcript is located at the very 3' end of exon 1, as in *Mdtra-2*.

Ceratitis *TRA-2 RRM is similar to* Musca *TRA-2 RRM*

D. melanogaster and *C. capitata* belong to the Acalyptratae group while *M. domestica* is more distantly related, classified in Calyptratae (Saccone *et al.*, 1998). A Clustal-W2 analysis (EMBL-EBi) of the entire *C. capitata*, *D. melanogaster* and *M. domestica* TRA-2 sequences revealed that CcTRA-2 is slightly closer to MdTRA-2 (guide tree, dnd file: Cctra2: 0.23248, Mdtra2: 0.22441, Dmtra2: 0.35317). Furthermore, the most conserved regions are five small segments in the RRM, which interestingly correspond

to the four beta sheets and one alpha helix of the putative secondary structure (Fig. 2). In addition, the following linker region is highly conserved, as previously observed in *Musca* and human TRA-2 proteins. The sequence conservation of a 112 amino acid segment including the RRM (72 aa) was further analyzed in *C. capitata*, *D. melanogaster* and *M. domestica* (Fig. 2. box underlined in blue). Again, a difference was observed between the established phylogenetic relationships of the three species and the "tree" obtained by Clustal-W2. Again, CcTRA-2 appeared to be more closely related to MdTRA-2 than to DmTRA-2. Within this protein domain, 37 positions out of 112 had a variable distribution of amino acid substitutions in the three species: 19 positions were conserved in *C. capitata* and *M. domestica*, while only ten were conserved in *C. capitata* and *D. melanogaster*, and only eight in *D. melanogaster* and–*M. domestica* (13 had no conservation). Similar results have been obtained using TRA-2 sequences missing the RRM domain. Similar Clustal-W analysis performed on another sex determination gene, *dsx*, showed, on the contrary, concordance with the established phylogenetic tree (Clustal-W2 guide tree, dnd file: CcdsxF: 0.06952, MddsxF: 0.27334, DmdsxF:0.18762).

Cctra-2 expression analysis

To analyze the developmental expression pattern of the *Cctra-2* gene, RT-PCR was performed on total RNA extracted from different stages and tissues, using a forward primer in exon 2 and a reverse primer in exon 6a. A unique amplification product of the expected size (0.5 Kb) was obtained in all samples (Fig. 3A). The presence of this fragment in samples derived from unfertilized eggs and ovaries (Fig. 3A) suggests that *Cctra-2* transcripts are maternally deposited in the eggs. Moreover, these results suggest that *Cctra-2* is expressed throughout development and that it produces a single transcript in both sexes, as in *M. domestica.* To detect minor differences in the size of putatively different *Cctra-2* transcripts in males and females, a further overlapping RT-PCR analysis was performed on RNA prepared from males, females, testis and ovaries using three different pairs of primers encompassing the complete CcTRA-2 ORF. No evidence of alternatively spliced products was found for *Cctra-2* (Fig. 3 B,C), and no evidence was found for the presence of an alternative testis-specific promoter, unlike in *Drosophila*. Thus, it appears that the regulation of transcription and splicing for *Cctra-2* is less complex than in *D. melanogaster*, and is more similar to *Mdtra-2*.

TABLE 1

***Cctra-2* RNAi EXPERIMENT**

Injected embryos	Larvae	Pupae	Adults	Males	Females	Intersexes
218	148	112	93	89	3	1
[Inj. dsRNA]	% Embryonal survival rate	% Larval survival rate	% Adults survival rate	% Males	% Females	% Intersexes
2,7 µM	67,8	51,3	42,6	95,7	3,2	1,1

Functional analysis

Cctra-2 dsRNA was synthesized using a 558 bp cDNA fragment derived from an adult female *Cctra-2* cDNA clone (see Materials and Methods). This fragment includes regions coding for the RS1 domain, the RRM domain and a part of the linker region. We injected 2.7 µM dsRNA into the posterior pole of preblastoderm stage embryos of wild type *Ceratitis capitata Benakeion* strain. Out of 218 injected embryos, 93 survived to adulthood (see Table 1). A strong sex ratio survival bias was observed in favour of males. Out of 93 flies, 89 showed an apparently normal male phenotype, three flies were females and one exhibited an intersexual phenotype, with typical female traits but male genitalia (Fig. 4). To assess the sexual karyotype of affected flies, PCR was performed on the genomic DNA of 20 randomly chosen phenotypic male flies and two of the three female flies using *C. capitata* Y-specific primers (Anleitner and Haymer, 1992). No products

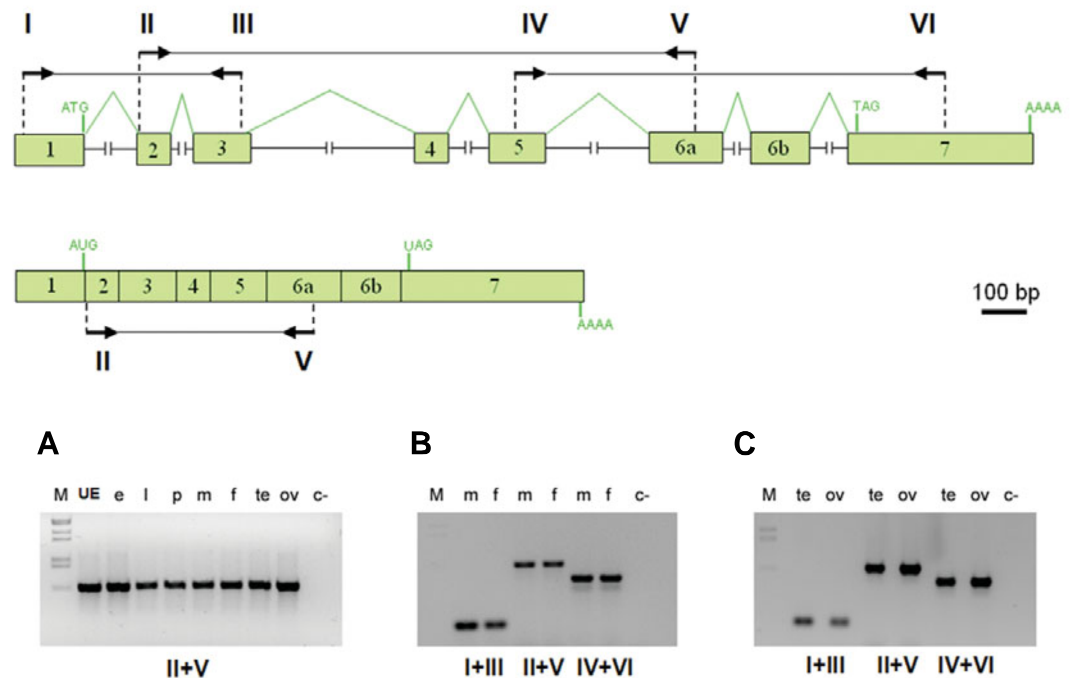

Fig. 3. Analysis of *Cctra-2* transcripts. *The molecular organization of the* Cctra-2 *gene and transcript is provided at the top, showing the locations of the primers indicated by short arrows and identified by Roman numerals.* **(A)** *Developmental RT-PCR amplification of* Cctra-2 *from unfertilized eggs (UE), 24 h old embryos (e), 3rd instar larvae (l), pupae (p), adult males (m), adult females (f), (te) testis and (ov) ovaries total mRNA samples. (M) Molecular weight marker. (c-) RT-PCR negative controls (reactions without template) are shown. A unique 0.5 kb product is present in all samples.* **(B,C)** *Overlapping RT-PCR analysis of RNA extracted from males (m), females (f), testis (te) and ovaries (ov) using 3 different pairs of primers that encompass the complete* Cctra-2 *ORF. See Materials and Methods for primer details.*

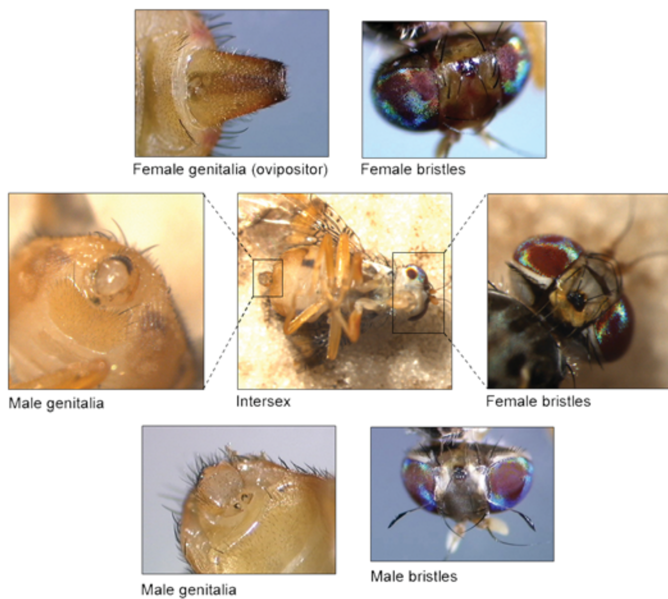

Fig. 4. Phenotypic analysis of the *Cctra-2* RNAi intersex individual. Ceratitis capitata *wild-type females exhibit bristles on the head and a prominent ovopositor. Wild-type males exhibit two additional spatulated bristles on the head and male genitalia. The unique intersex fly obtained by* Cctra-2 *dsRNA injection into the posterior pole of the embryo exhibits female-specific bristles on the head and male genitalia.*

were detected in single preparations of 11 out of 20 phenotypic males or in the two single preparations of females. This test revealed the absence of a Y chromosome, indicating that all these animals have a female XX karyotype (Fig. 5A). Moreover, apparently normal testes were observed, dissected from the same 20 males (Fig. 5E). Wild type ovaries were also observed in the two females (Fig. 5A, F1 and F2) that escaped the RNAi-induced masculinization (data not shown).

The masculinizing effect of *Cctra-2* dsRNA embryonic injection demonstrates a key role of this gene in female-specific development. On the other hand, no phenotypic abnormalities were observed in injected genotypically male flies. Thus, we can conclude that *Cctra-2*, like *Cctra*, is essential for female development of *Ceratitis capitata*.

Effect of embryonic Cctra-2*-specific RNAi on* Cctra-2*,* Cctra*,* Ccdsx *and* Ccfru *splicing patterns in adults*

In *D. melanogaster*, *tra-2* acts as an indispensable co-factor of *tra* in the female-specific splicing of *dsx* and *fru* pre-mRNA (Ryner *et al.*, 1996, Tian and Maniatis, 1993). In *C. capitata,* the injection of *Cctra*-dsRNA into XX embryos induces a permanent shift in the splicing pattern of *Cctra* and of *Ccdsx* from female to male mode. To test the effect of embryonic *Cctra-2*-specific RNAi on the splicing pattern of sex determining genes in *C. capitata* adults, an RT-PCR analysis was performed using gene-specific primers that allow for discrimination between sex-specific transcripts on RNA extracted from the unique intersex individual, four XX pseudo-males, and three XY adult males obtained by embryonic RNAi against *Cctra-2*. The molecular karyotype of flies (Fig. 6A-B) was assessed as described above (see Fig. 5). The *Cctra-2* gene in the XX intersexual fly and in the XX pseudo-males produced as expected a unique mRNA (Fig. 6C, lanes 1-5). The *Cctra* gene produced male-specific mRNAs in XX pseudo-male samples (Fig. 6D, lanes 2-5), as previously observed by Pane *et al.* (2002) with *Cctra*-specific RNAi. The intersex individual (lane 1) was a sexual mosaic, with both male and female *tra* splicing patterns. *Cctra-2*-specific RNAi had no effect on typical *Cctra-2* or *Cctra* splicing patterns in XY individuals (Fig. 6C and 6D, lanes 6-8). These data suggest that: (1) *Cctra-2* is required for *Cctra* female-specific splicing and (2) *Cctra-2* is not required for its own splicing. The *Cctra-2*-specific RNAi also caused a persistent change in *Ccdsx* regulation from a female-specific to a male-specific splicing mode in XX pseudo-male individuals (Fig. 6E, lanes 2-5), leading to male differentiation as previously observed following *Cctra*-specific RNAi (Pane *et al.*, 2002). Also in this RT-PCR experiment the intersex individual (lane 1) had both the male- and female-specific *dsx* splicing pattern. This data, together with the presence of TRA/TRA-2 binding sites in *Ccdsx* female-specific pre-mRNA, indicates that *Cctra-2* is also required for female splicing of *Ccdsx*.

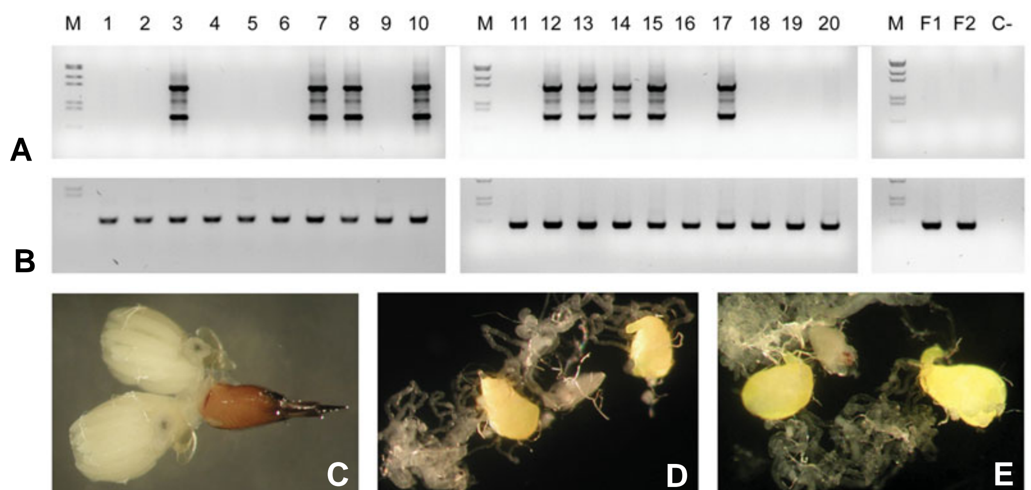

Fig. 5. Karyotypic analysis of RNAi treated individuals. (A) *PCR with Y-specific oligonucleotides on genomic DNA extracted from single flies developed from dsRNA-injected embryos. From lane 1 to 20, PCR on single male flies; lanes F1 and F2, PCR on two female "escapers" (see text); lanes C-, negative PCR control (PCR in the absence of template); lanes M, molecular weight marker. The PCR amplification patterns in lanes 3,7,8,10, 12-15 and 17 correspond to those of wild-type males, indicating that the analysed adults have an XY karyotype. By contrast, no signals are detected in lanes 1, 2, 4-6, 9, 11, 16, 18-20, indicating that these males lack a Y chromosome and are therefore XX sexually transformed males.* **(B)** *Positive PCR control with* RpP1 *specific primers showing that medfly genomic DNA is present in all samples.* **(C)** *Dissected wild-type ovaries from a non-injected female.* **(D)** *Dissected wild-type testis from a non-injected XY male.* **(E)** *Dissected testis from an injected XX pseudo-male.*

In a previous study (Davis *et al.*, 2000) a cDNA sequence was cloned corresponding to the putative *Ceratitis fruitless* (*Ccfru*) homologue (Accession N°AAF22477) coding for the BTB domain. Starting from this sequence, a 5' RACE experiment was performed on total RNA prepared from *Ceratitis* male and female adult flies. A male-specific *Ccfru* upstream cDNA sequence ($Ccfru^M$) was isolated which codes for a putative protein amino-terminal fragment, homologous to *A. gambiae* and *D. melanogaster* male-specific FRU^M isoforms (69% and 63% identity, respectively). RT-PCR analysis with $Ccfru^M$ specific primers of total RNA prepared from *Ceratitis* male and female adult flies showed an amplification fragment exclusively in the male sample. Hence, *Ccfru* is probably regulated by alternative splicing, as in *Drosophila*. Interestingly, RT-PCR analysis on the same RNA samples demonstrated that *Ccfru* male-specific mRNA was present in all XX pseudo-males and in the XX intersexual fly (Fig. 6F – lanes 1-5). These data suggest that (1) as in *Drosophila*, the sex-specific expression of *Ccfru* is under the control of *Cctra*/*Cctra-2*, and that (2) the change in *Ccfru* sex-specific regulation could cause a behavioural masculinization of XX interfered individuals.

Normal male sexual behaviour of XX males

C. capitata males release a long distance attractant pheromone, exposing a droplet of liquid with a balloon-like structure formed by a membranous portion of the rectal epithelium (Arita and Kaneshiro, 1986). Male courtship begins when a female approaches a male, who then displays the following behaviour patterns: (1) pheromone calling - a droplet of pheromone is exposed dorsally by calling males; (2) vibrating wings - the tip of the male abdomen is bent ventrally with the pheromone droplet present on the everted rectal membrane and the wings are moved up and down; (3) wing buzzing - during which the male wings are rhythmically moved forwards and backwards ("buzz wings") and the droplet of pheromone is usually reabsorbed; and (4) head rocking - rapid rotations of the male head ("head rock"). Successful courtship ends with copulation, during which the male mounts the female (5) (Briceno *et al.*, 1996). To test whether the XX pseudo-males have the same mating behaviour as XY wild-type males as suggested by the *Ccfru* RT-PCR analysis, and to test their fertility, 20 males were individually crossed with wild type virgin females (ratio 1 male: 3 females for each cage). The five typical male behaviours described above were visually analyzed for each of the 20 mating pools and for the 7 control pools. No obvious behavioural differences were noted in any of the 27 mating chambers (Fig. 7). Although we attempted to observe all interactions that occurred within all cages, we missed some head rocking and copulation events in some crosses. However, the presence of progeny in these cages led us to believe that these events occurred. Eighteen out of twenty matings were fertile. Eight out of twenty matings produced only female progeny,

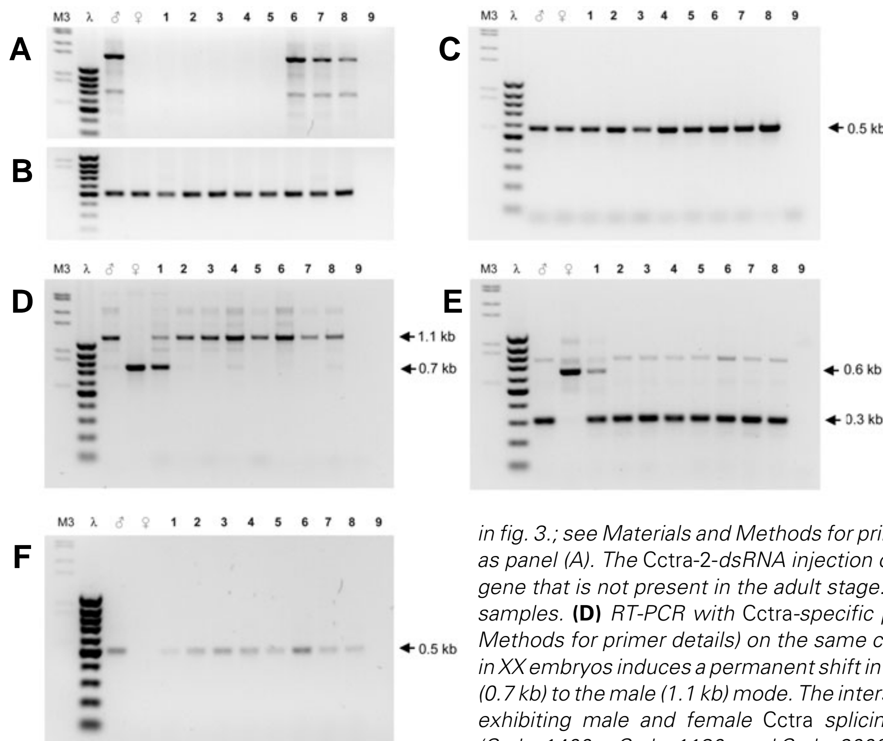

Fig. 6. *Cctra-2* is required for the auto-regulation of *Cctra* and for female splicing of *Ccdsx* and *Ccfru*. (A) *Genomic PCR with Y chromosome specific primers* Y-spec1 *and* Y-spec2 *on genomic DNA of XY wild type males (♂), XX wild type females (♀), the XX* Cctra-2-*dsRNA injected adult intersex individual (Lane 1), XX* Cctra-2-*dsRNA injected adult males (lanes 2-5) and XY* Cctra-2-*dsRNA injected adult males (lanes 6-8). Lane 9 is a negative PCR control. Two molecular weight markers are shown in lanes M3 and λ. Only genomic DNA extracted from Y chromosome-bearing flies have amplification signals.* **(B)** *Genomic positive control PCR with autosomal* RpP1 *gene-specific primers* RPP1+ *and* RPP1- *on the same genomic DNA samples used in (A).* **(C)** *RT-PCR with* Cctra-2-*specific primers (Cctra-2 II and Cctra-2 V shown in fig. 3.; see Materials and Methods for primer details) on cDNA samples from the same adult flies as panel (A). The* Cctra-2-*dsRNA injection caused a transient silencing of the endogenous* Cctra-2 *gene that is not present in the adult stage. A unique amplification signal of 0.6 kb is present in all samples.* **(D)** *RT-PCR with* Cctra-*specific primers (Cctra164+ and Cctra900–; see Materials and Methods for primer details) on the same cDNA samples used in (C). The* Cctra-2-*dsRNA injection in XX embryos induces a permanent shift in the splicing pattern of* Cctra *that turns it from the female (0.7 kb) to the male (1.1 kb) mode. The intersex individual (lane 1) is a sexual mosaic, simultaneously exhibiting male and female* Cctra *splicing patterns.* **(E)** *RT-PCR with* Ccdsx-*specific primers (Ccdsx1400+, Ccdsx1130– and Ccdsx2000–; see Materials and Methods for primer details) on the same cDNA samples used in (C). The 0.6 kb fragment corresponds to a region of* Ccdsx *major female-specific transcript, while the 0.3 kb fragment represents a region of* Ccdsx *major male-specific transcription. A consequence of* Cctra-2-*specific RNAi is a persistent change in* Ccdsx *regulation that turns from female-specific to male-specific splicing mode. Also in this RT-PCR experiment, the intersex individual (lane 1) shows both the male and female* Ccdsx *splicing patterns.* **(F)** *RT-PCR with* Ccfru *male-specific primers (CcfruM1+ and Ccfru2–; see Materials and Methods for primer details) on the same cDNA samples used in (C). In this RT-PCR experiment, the XX males (lanes 2-5) and the intersex individual (lane 1) show the male-specific* Ccfru *amplification signal observed in the XY males but not in XX females. This result suggests that the* Ccfru *gene is regulated by sex-specific alternative splicing and that* Cctra-2-*dsRNA injection in XX embryos could also induce a splicing switch between male transcripts and a putative female transcript.*

	Cross name	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	C1	C2	C3	C4	C5	C6	C7
Courtship behaviour	Pheromon Calling	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Vibrate wings	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Wing buzzing	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Head rocking	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+
	Copulation	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	-	+	-	+	-	+	-	+	+	+	+	-
Embryos deposition		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
Larvae		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
Pupae		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	-	+	+	+	+	+
Sex of progeny		♂♀	♀	♀	♀	♂♀	♀	♀	♂♀	♂♀	♀	♀	♂♀	♂♀	♂♀	♀	-	♂♀	♂♀	♂♀	-	♂♀	-	♂♀	♂♀	♂♀	♂♀	♂♀
Y-specific genomic molecular karyotyping																												
RpP1 genomic positive control																												

Fig. 7. Behavioural assays on *Cctra-2* dsRNA injected males. *Cages A1-A10 and B1-B10 contained one injected male and 3 virgin* Benakeion *wild type females, while C1-C7 correspond to control cages containing one* Benakeion *wild type male and 3 virgin* Benakeion *wild type females. The molecular karyotype of these flies was assessed as described in Fig. 5. "+", observed behavioural phenotype or life cycle stage; "-", no observed behavioural phenotype or life cycle stage.*

indicating that the XX males are fertile. The expected XX karyotype of the nine corresponding males was confirmed by genomic PCR. A Y-specific PCR band was missing in all these males, but not in the others (Fig. 7). Therefore, the XX pseudo-males produce fertile sperm carrying exclusively X chromosomes, and *Cctra-2* is required along with *Cctra* for female sex determination in both somatic and germ lines.

Discussion

Cctra-2 *is involved in sex determination in* Ceratitis capitata*, performing a novel function*

The *Cctra-2* gene is transcribed throughout development, and produces a single ORF-containing transcript in both sexes that encodes a putative 251 aa RNA binding protein, highly conserved compared to *M. domestica* and *D. melanogaster* TRA-2 proteins. Recently, following an EST analysis in *Ceratitis capitata, Cctra2* was also isolated and the present data corroborate the structural characterization and partial expression analysis reported therein (Gomulski et al., 2008). The function of *Cctra-2 in-vivo* was tested using RNA interference technique (RNAi). RNAi, is a transient and rapid phenomenon. For this reason, genes expressed in later development cannot easily be inactivated by embryonic RNAi (Kennerdell and Carthew, 1998, Misquitta and Paterson, 1999, Montgomery, 2004, Wianny and Zernicka-Goetz, 2000). For example, *M. domestica* embryonic RNAi against *Mddsx,* which is expressed throughout larval development and adulthood, had only mild effects on germ-line development and no effects on somatic tissues (Hediger *et al.*, 2004). In *Drosophila,* even a transgene-mediated RNAi against *tra-2* driven by a GAL4-dependent promoter caused only a partial masculinization (Fortier and Belote, 2000). The complete masculinization induced by a transient RNAi specific for *Cctra-2* is very striking and not easily explainable without the additional functions played by this gene in *C. capitata*. The apparently complete sexual reversion is paralleled by a permanent collapse during embryogenesis in *Cctra* positive autoregulation. Indeed, a stable change is observed in the splicing pattern of *Cctra* from female to male mode, in which mRNAs encode truncated and probably non-functional peptides. Furthermore, *Ccdsx* and the putative *Ccfru* gene shifted from the female to the male splicing mode. Knocking down *Cctra* caused a similar development of fertile XX pseudo-males and a permanent change in *Cctra* and *Ccdsx* splicing (Pane *et al.*, 2002). This similarity in RNAi efficiency and in phenotypic and molecular effects strongly supports a sex determination model in which *Cctra-2* is an auxiliary factor necessary in females for *Cctra* positive autoregulation (Fig. 8). In this model, *Cctra-2*, like *Cctra*, appears to be required for bypassing two male-specific exons in the *Cctra* pre-mRNA in XX individuals at embryonic stages and for the rest of their development. This early female-specific splicing leads to *Cctra* mRNAs encoding for the full length CcTRA protein, which is required, along with CcTRA-2, to maintain *Cctra* female-specific activation. CcTRA/CcTRA2 promotes female-specific splicing of the conserved downstream target genes *dsx* and *fru*, which in turn seem to control female differentiation and female sex behaviour.

Expression analysis shows that *Cctra-2* mRNA, like *Cctra*, is present in unfertilized eggs, suggesting a maternal genetic component. Note that unfertilized eggs also have maternal female-specific *Cctra* mRNAs (Pane *et al.*, 2002). The presence of both mRNAs suggests their embryonic involvement in the early female-specific activation of the *Cctra* positive feedback loop. The injection of *Cctra-2* dsRNA into embryos probably causes the degradation of maternally-inherited and possibly also zygotically-produced mRNAs. The transient disappearance of CcTRA-2 during these critical stages of development causes the failure of *Cctra* to initiate positive autoregulation in XX individuals.

A similar drastic sexual transformation induced by transient RNAi has also been observed for *Musca domestica tra-2* (*Mdtra-2*) (Burghardt *et al.*, 2005). The isolation and characterization of *Mdtra-2* led to the discovery that the gene has an essential function in the female development of the housefly, not only in regulating *Mddsx*, but also in maintaining the female state by autoregulation of the female determining factor F. Hence, *Mdtra-2* also has two genetically separable functions: (1) parallel to *F* as a co-factor for the regulation of downstream targets such as *Mddsx* and (2) upstream of *F* as a co-factor of the autocatalytic activity of *F* (Burghardt *et al.*, 2005). Activation of the *F* gene in the zygote depends on maternally provided activity (Dubendorfer and Hediger, 1998). Once *F* is activated, it remains active throughout development to induce female sexual differentiation; removal of its activity at later stages leads to male development (Hilfiker-Kleiner *et al.*, 1993). Based on these findings, it has been proposed that *F* relies on a feedback mechanism to maintain its female-promoting activity. The authors suggested that *Musca domestica F* could be the homologue of the *C. capitata trans-*

former autoregulatory gene, rather than the *D. melanogaster* counterpart, and that *Mdtra-2* is an essential auxiliary factor of *F* (Burghardt *et al.*, 2005, Dubendorfer *et al.*, 2002, Pane *et al.*, 2002).

Unlike CcTRA, CcTRA-2 has a high level of sequence conservation

CcTRA (400 aa) has very low levels of sequence and length conservation compared to DmTRA (200 aa), with only 23% identity in the protein regions that can be aligned. This is in contrast with the high sequence conservation of the corresponding TRA/TRA-2 binding sites identified not only in *Ccdsx,* but also newly identified in *Cctra* (Pane *et al.*, 2002). The expected physical interaction of the TRA/TRA-2 protein complex and the conserved TRA/TRA-2 RNA binding sites suggests a co-evolutionary dynamic of the two macromolecular structures. Considering the stronger RNA binding abilities of DmTRA-2 to the *cis* elements compared with DmTRA, and considering that TRA seems to lack a defined secondary structure, we expected to find higher sequence conservation in *tra-2* homologues across different dipteran species compared to *tra* homologues. The cloning of *Musca* and Ceratitis *tra-2* homologues supports this idea, since both encode RNA binding proteins that are highly conserved through 120-130 Myr of evolution. Interestingly, CcTRA-2 was found to be more similar to *Musca* TRA-2 than to *Drosophila* TRA-2, despite their respective phylogenetic distances. Hence, some evolutionary constraints could be more similar for *C. capitata* and *M. domestica* compared to *D. melanogaster.* Considering that MdTRA-2 seems to be involved, directly or indirectly, in the autoregulatory mechanism for maintaining active *F*, and considering that in *C. capitata* CcTRA-2 appears to be involved in the positive autoregulation of *Cctra* splicing, this peculiar similar novel function could help explain the slightly higher sequence similarity of Md/CcTRA-2. We propose that *Cctra-2* has functions more closely related to those of *Mdtra-2* than to those of *Dmtra-2* because of the epigenetic role played in maintaining female sex determination in *Ceratitis* and *Musca*. If the *M. domestica F* gene is structurally and functionally equivalent to the *C. capitata tra* gene, *Mdtra-2* should be considered functionally equivalent to *Cctra-2*, and then MdTRA-2 would be an auxiliary factor to the F product in the autoregulation of F, as CcTRA-2 is for *Cctra*.

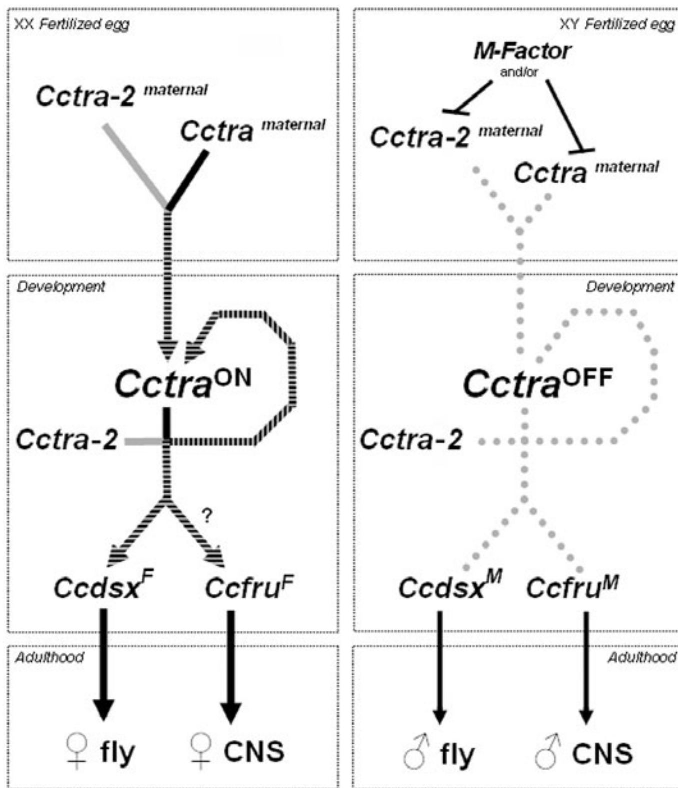

Fig. 8. Autoregulation model for sex determination in *Ceratitis capitata*. *In XX fertilized eggs, a maternal* Cctra *and* Cctra-2 *source (mRNA or protein) initiates positive feedback regulation via female-specific splicing of the zygotically transcribed* Cctra *pre-mRNA so that the new* Cctra *function can be activated.* Cctra*, together with the constitutive* Cctra-2 *gene, then controls the maintenance of* Cctra *autoregulation, the female-specific splicing of* Ccdsx *and, presumably, the female-specific splicing of* Ccfru *pre-mRNAs. Therefore, a CcDSX^F protein and presumably a CcFRU^F protein are produced, driving female somatic development and female sexual behaviour. In XY fertilized eggs,* Cctra *autoregulation is impaired by the male determining* M *factor. The* M *factor could prevent the action of maternal CcTRA and/or CcTRA-2 proteins, leading to male-specific* Cctra *mRNAs and hence to truncated non-functional CcTRA protein in XY embryos, and impairing the initiation of the autoregulatory loop. In the absence of CcTRA,* Ccdsx *and* Ccfru *are spliced in the male-specific manner by default, producing the CcDSX^M and CcFRU^M isoforms, which in turn induce male somatic development and male sexual behaviour.*

Cctra *and* Cctra-2*: a dual role in splicing regulation*

The conservation of TRA/TRA-2 binding sites in the *Ccdsx* female-specific exon and the male-specific region of *Cctra* strongly suggest a contribution of CcTRA and CcTRA-2 in the sex-specific splicing regulation of these genes. We propose that CcTRA-2 is able to directly bind, together with CcTRA: (1) the TRA/TRA-2 binding sites in *Ccdsx* pre-mRNA to promote female-specific splicing and the translation of the female-specific DSX^F isoform; (2) the TRA/TRA-2 binding sites of *Cctra* pre-mRNA to repress male-specific splicing and hence to promote translation of female-specific CcTRA, maintaining *transformer* positive autoregulation. Given their intrinsic ability to activate splicing through Exon Splicing Enhancer (ESE) elements, it is perhaps surprising that SR proteins and related factors like TRA and TRA-2 also repress splice site recognition in some pre-mRNAs. One striking example of this is the *Drosophila* TRA-2 protein. This protein is able to activate, along with TRA, the female-specific splicing of *dsx* and *fru* pre-mRNAs, to bind TRA/TRA-2 ESE sequences, and to repress the splicing of a specific intron (M1) in its own pre-mRNA in the male germ-line, binding a *tra-2*-ISS sequence (Intronic Splicing Silencer) (Qi *et al.*, 2007). The *Cctra-2* gene seems to lack this germ-line-specific autoregulatory mechanism, like *Musca domestica tra-2*. However, in *Ceratitis capitata,* the TRA and TRA-2 proteins exhibit a dual regulatory action for controlling the splicing of the sex determining genes *Ccdsx* and *Cctra*. In the case of *Ccdsx* splicing regulation, CcTRA and CcTRA-2 behave as splicing activators, promoting the inclusion of a female-specific exon into the mature mRNA. In the case of *Cctra* splicing regulation, the two proteins behave as splicing inhibitors, preventing the inclusion of a male-specific stop-containing region into the mature mRNA of females. In a recent comparative analysis of the *Cctra* gene among tephritids species, Ruiz *et al.* (2007) identified two conserved *tra-2*-ISS elements in the male-specific region of the *C.*

capitata and *Anastrepha tra* genes; these elements are absent in the *dsx* female-specific region in *C. capitata* and other tephritids species. Therefore, we propose that the binding of CcTRA-2 to these putative regulative sequences in *Cctra* pre-mRNA could be the discriminative event between the dual modes of action of CcTRA and CcTRA-2 as splicing regulators (Ruiz *et al.*, 2007).

Considering also that *Ccfru* sex-specific expression seems to be under the control of CcTRA/CcTRA-2 proteins, we anticipate that this gene conserves TRA/TRA-2 binding sites and sex-specific splicing regulation. It has recently been shown that *fru* sex-specific regulation is conserved within the context of 250 Myr of evolutionary divergence between *D. melanogaster* and *A. gambiae*. The male-specific mosquito FRU protein isoforms arise from conserved mechanisms of sex-specific activation and alternative exon splicing (Gailey *et al.*, 2006). The finding of a male-specific *Ccfru* mRNA detected only in XY males and in XX *Cctra-2*-RNAi treated pseudo-males, together with the normal male behaviours shown by these XX pseudo-males, strongly suggests that the *Ccfru* gene is involved in courtship regulation and that it produces sex-specific transcripts via alternative splicing under the control of the *Cctra* and *Cctra-2* genes, similar to *Drosophila*. Complete *Ccfru* characterization could help unravel the type of splicing regulation in Medfly. Further support for the notion that *Cctra-2* is involved in controlling these other sexual traits is found in a *Mdtra-2* study showing that it is also engaged in the control of sex-specific behaviours, fully implementing the female program of development (Burghardt *et al.*, 2005).

Cctra-2 auxiliary epigenetic function

The sex determination and sexual differentiation of *C. capitata* contrasts with the findings in *D. melanogaster* in at least three crucial aspects: the role of the *Sxl* gene, the nature of the primary signal and the apparent lack of fertility factors on the Y chromosome. The *Ceratitis Sxl* homologue has no sex-specific expression and its function remains to be defined (Saccone *et al.*, 1998), while the *Ceratitis* primary signal of sex determination is the still unknown dominant male determining factor mapped on the long arm of the Y chromosome (Willhoeft and Franz, 1996). Despite these differences between *Drosophila* and *Ceratitis*, the peculiar and novel function of *Cctra* and *Cctra-2* appears to be analogous to the *Drosophila Sxl* epigenetic autoregulatory function (Cline, 1984, Cline, 2005). We propose that *Cctra-2* plays an essential, auxiliary role in *Ceratitis transformer* female-specific autoregulation. Pane *et al.*, 2002, defined this autoregulation mechanism, which is able to switch OFF during the embryogenesis of XY individuals, as epigenetic because of its ability to maintain either the OFF or the ON state independently from the initial positive (maternal CcTRA) or negative (M factor) signal. Considering the novel functions of *Cctra* and *Cctra-2,* with respect to their *Drosophila* counterparts, we renamed *Cctra* and *Cctra-2* as *Cctra^{ep}* and *Cctra-2^{aux-ep}*, respectively, with ^{ep} meaning epigenetic and ^{aux} meaning auxiliary. This novel *Cctra/Cctra-2* epigenetic function could be a widely conserved mechanism, valid for all dipteran species which maintain a Ceratitis *transformer* homologue able to autoregulate, like *Bactrocera oleae* (Lagos *et al.*, 2007) and various *Anastrepha* species (Ruiz *et al.*, 2007). Hence, the novel results presented in this paper, the *Cctra-2* function for *Cctra* autoregulation and the *Cctra/Cctra-2* control of *Ccfru* splicing, allows us to update the sex determination cascade of *C. capitata* as follows: Y-linked M Factor> *Cctra^{ep}+Cctra-2^{aux-ep}* > *Ccdsx/Ccfru*.

As previously shown by Pane *et al.* (2002), *Ceratitis capitata* XX pseudo-males produced by RNAi against *Cctra-2* are fertile. Hence, the *Ceratitis* Y chromosome seems not to substantially contribute to male fertility, at least in cage experiments. Genetic and theoretical studies of Y chromosomes have led to the conclusion that they evolve to become functionally degenerate. However, it has been recently shown in *Drosophila melanogaster* that the Y chromosome not only contains male fertility genes, but it also regulates by unknown mechanisms hundreds of genes harboured on other chromosomes (Lemos *et al.*, 2008). Hence, the *Ceratitis* Y chromosome could be a sex chromosome with more degeneration, but it still could have male-specific functions only detectable by experiments approximating natural conditions, such as mating competition experiments (Rice and Friberg, 2008). The fitness advantage of a highly degenerate Y chromosome is illustrated in *Drosophila affinis,* in which the Y chromosome is not required for fertility, although males with no Y chromosome (XO) sire 25 to 38% fewer offspring when competing with XY males (Voelker and Kojiim, 1971). *Ceratitis capitata* is an important agricultural pest that can be controlled by the Sterile Insect Technique, which consists of mass rearing and then releasing sterilized males in large quantities (Robinson, 2002). The observed efficiency in producing male-only progeny by RNAi against *Cctra-2* suggests a potential biotechnological application for this gene, offering novel opportunities for future strategies aimed at developing transgenic strains. Therefore, studying the mating abilities of XX pseudo-males versus XY males in natural conditions will be of great interest from an evolutionary perspective and from a wider point of view.

Materials and Methods

Rearing of the C. capitata *strain*

The *C. capitata Benakeion* strain and the single injected adult flies were reared in standard laboratory conditions at 25°C, 70% relative humidity and 12:12 h light–dark regimen. *Benakeion* adult flies were fed yeast/sucrose powder (1:2). Eggs were collected in water dishes and transferred to larval food (30 g soft tissue paper, 30 g sugar, 30 g yeast extract, 10 ml cholesterol stock, 2 ml HCl stock, 8.5 ml benzoic stock, water 400 ml). Pupae were collected and stored in Petri dishes until eclosion.

Cloning and gene organization

The forward degenerate primers were designed from sequences located in the middle of the RRM; the reverse degenerate primer was designed from the extended homology region downstream of the RRM (linker region). The primers utilized were:

Forward

MAR25: 5'- TGY CTI GGN GTN TTY GGS YT R-3'

MAR5: 5'-MGN TCI CGI GGN TTY TGY TTY R-3'

Reverse

MAR17: 5'-GT RTG IGS ICG YTK NGT DAT NGA-3'

Ceratitis capitata Benakeion wild-type cDNA templates were prepared from adult males and females following standard procedures. A first round of PCR was performed with MAR25 and MAR17, followed by a second amplification with MAR5 (nested) and MAR17, following standard procedures. PCR conditions for the first amplification were denaturation at 95°C for 2 min, 35 cycles (denaturation at 95°C for 30 s, annealing at 42°C for 1 min and extension at 72°C for 1 min) and extension at 72°C for 5 min. The second amplification with nested primers was performed with the

following conditions: denaturation at 95°C for 2 min, 5 cycles (denaturation 95°C for 30 s, annealing at 42°C for 1 min and extension at 72°C for 1 min), then 25 cycles (denaturation 95°C for 30 s, annealing at 62°C for 1 min and extension at 72°C for 1 min) and a final extension at 72°C for 5 min. Amplified fragments were gel-eluted, subcloned and sequenced following standard procedures. 5' Race and 3' Race experiments were performed with the SMART RACE cDNA Amplification Kit (Clontech), following the manufacturer's instructions, which led to isolation of an additional 460 bp 5' sequence and a 600 bp 3' sequence. Genomic DNA templates were prepared from wild-type adult *Ceratitis capitata Benakeion* males and females. The Genome Walker kit (Clontech) was used to perform PCR on genomic DNA from *C. capitata* to obtain a 5' genomic clone of *Cctra-2*. Genomic PCRs were performed to determine the exon/intron junctions with primers designed from exonic regions conserved in *Musca domestica tra-2* (Burghardt *et al.*, 2005). The sequences of the genomic fragments generated were compared with the previously determined *Cctra-2* cDNA sequences. In this way, the exon/intron junctions were unambiguously identified.

5' Race primers:

Cctra-2 5'	5'-CCATTGCTGCGTCGAGCAGG-3'
Cctra-2 5' Nes	5'- TGCGGAAATGCTTCGGCTAC-3'
3' Race primers:	
Cctra-2 Sfa+	5'-GGATACAAGTAGTCATTGATGC-3'
Cctra-2 Sfa+ Nes	5'-ACTGGCCGATCTCGTGGCTT-3'
Genomic Walking primers:	
Cctra-2 M1a-	5'-CTTTGGCTGGCATCTGAACAG -3'
Cctra-2 M1a- Nes	5'-TGCGGAAATGCTTCGGCTAC-3'
Genomic PCR primers:	
Cctra-2 30+	5'-TCAATCAGCGGTAGTTTGTTGA-3'
Cctra-2 M1a-	5'-CTTTGGCTGGCATCTGAACAG-3'
Cctra-2 M1a+	5'-CTGTTCAGATGCCAGCCAAAG-3'
Cctra-2 ES4b-	5'-ATGGGAACGTGACATCCGACG-3'
Cctra-2 M1+	5'-GCGCAGACATACGCATATGTGA-3'
Cctra-2-	5'-ACGCCAGGTGTGGGAGTGT-3'
Cctra-2 300+	5'-GTACAAAACCGTTGTATAGGAG-3'
Cctra-2 900-	5'-AGTTCTAATAACGTGCACGC-3'

RT-PCR expression analysis

Total RNA was extracted, as described elsewhere (Andres and Thummel, 1994), from adult individuals and from unfertilized eggs, larvae, pupae, and dissected testis and ovaries. Oligo-dT-primed cDNA was prepared from DNAse I-treated total RNA of unfertilized eggs, larvae, pupae, male and female flies, testis and ovaries using the Advantage RT-for-PCR Kit (Clontech). RT-PCR expression analysis reported in Fig. 3A, B, and C was performed with the following primers: Cctra-2 I (5'-AAAGCTGGAATGAGTCCACGT-3') located in *Cctra-2* exon 1-exon2; Cctra-2 II (5'-GTAGCCGAAGCATTTCCGC-3') located in *Cctra-2* exon 2; Cctra-2 III (5'-CTTTGGCTGGCATCTGAACT-3') located in *Cctra-2* exon 3; Cctra-2 IV (5'-TACAAAACCGTTGTATAGGAG-3') located in *Cctra-2* exon 5; Cctra-2 V (5'-ACGCCAGGTGTGGGAGTG-3') located in *Cctra-2* exon 6a; Cctra-2 VI (5'-GGCGTGCACGTTATTAGAAC-3') located in *Cctra-2* exon 7.

Cycling conditions were denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, with a final 10 min extension at 72°C. The PCR products were gel-purified, cloned using the pGEM-T Easy Vector Kit (Promega) and sequenced with the Big Dye® Terminator v1.1 Sequencing Kit (Applied Biosystem).

RNAi

Cctra dsRNA was obtained and injected as described for *Drosophila* (Kennerdell and Carthew, 1998). A *Cctra-2* fragment from positions 17 to 575 was amplified with primers that introduced a T7 promoter sequence at each end. This template was used to produce dsRNA fragments by *in vitro* transcription with T7 RNA polymerase using the Megascript Kit (Ambion). The dsRNA was precipitated with ethanol and resuspended in injection buffer (Rubin and Spradling, 1982). Embryos were collected 1 hour AEL (after egg laying), hand dechorionated and microinjected with 2.7 μM dsRNA. Injected embryos were allowed to develop at room temperature.

Molecular karyotyping

Y-specific repetitive elements were amplified from genomic DNA extracted from 20 single treated phenotypically male adults and two enclosed treated females by PCR using Y-specific oligonucleotides, Y-spec1 (5'-TACGCTACGAATAACGAATTGG-3') and Y-spec2 (5'-GCGTTTAAATATACAAATGTGTG-3'). As a positive control, the *CcRpP1* specific primers RPP1+ (5'-TTGCGTTTACGTTGCTCTCG-3') and RPP1- (5'-AATCGAAGAGACCGAAACCC-3') were used on the same genomic DNA samples. Cycling conditions were denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 min, annealing at 59°C for Y-spec and 60°C for RPP1 for 1 minute and extension at 72°C for 2.5 minutes, with a final 10 minute extension at 72°C.

RT-PCR analysis of XX injected flies

RT-PCR experiments to analyze *Cctra*, *Cctra-2*, *Ccdsx* and *Ccfru* expression patterns in treated XX and XY males were performed by using the Advantage RT-for-PCR Kit (Clontech) with the following gene-specific primers:

Cctra-2 II (5'-GTAGCCGAAGCATTTCCGC-3')
located in *Cctra-2* exon 2;
Cctra-2 V (5'-ACGCCAGGTGTGGGAGTG-3')
located in *Cctra-2* exon 6a;
Cctra 164+ (5'-CAGTGGTTCGGTTCGGAAG-3')
located in *Cctra* exon 1;
Cctra 900- (5'-TCCATGATGTCGATATTGTCC-3')
located in *Cctra* exon 2;
CcdsxC 1400+ (5'-GGCATCAAGGCGTATAGAAGA-3')
located in *Ccdsx* common exon 3;
CcdsxM 1130– (5'-CTGGTGGTGACATCGTATCG-3')
located in *Ccdsx* male-specific exon 5;
CcdsxF 2000– (5'-ACGACGGCATGACCTTTAAC-3')
located in *Ccdsx* female-specific exon 4;
CcfruM1+ (5'-ATGTTGGCCATGTCACAAGG-3')
located in *Ccfru* male-specific exon;
Ccfru2- (5'-CGGTCTTGAGGAACATCGG-3')
located in *Ccfru* common exon, coding for the BTB domain.

Subcloning and sequencing of the candidate fragments were carried out as described above. Cycling conditions were denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 1 min, annealing for 1 min at 60°C and extension at 72°C for 2.5 minutes, with a final 10 min extension at 72°C.

Behavioural assays on injected males

Single crosses were performed in standard *Drosophila* vials, prepared with Instant *Drosophila* Medium (Sigma). Vials were closed with plastic nets and placed horizontally on small plastic boxes containing distilled water for embryo collection, in accordance with classic *Ceratitis* procedure. The flies were distributed in 27 cages, each containing 4 flies. Cages A1-A10 and B1-B10 contained one injected male and 3 virgin *Benakeion* females, while the control cages, C1-C7, contained one *Benakeion* male and 3 virgin *Benakeion* females. The 27 crossing cages were visually analyzed 3 times per hour, 6 hours a day, for 3 days. For each cage, we recorded whether the male was (1) "calling" (releasing pheromone), (2) vibrating his wings, (3) buzzing his wings when the female approached, (4) moving his head in a typical head rocking pattern, and (5) was successful in copulating with the female. For all cages, embryos were collected and reared until adulthood. At the end of the experiment, molecular karyotyping was performed on

genomic DNA extracted from each crossed male, as described above.

Sequence analysis

Protein and cDNA alignments were performed with MACAW software (NCBI, NIH, Bethesda, USA) with default settings and with DNA Fasta sequence comparison software.

GenBank Accession Numbers

Cctra-2 cDNA, EU999754; *Ccfru* male specific 5' cDNA end, EU999755.

Acknowledgements

We thank Leonard Rabinow and Lucas Sanchez for critical reading of the manuscript and for helpful suggestions. We thank also C. S. Levesque. This work was supported by grants from the Ministero della Ricerca Scientifica e Tecnologica (PRIN 2006, Italy) to L. C. Polito and from CDFA (USA) to P. Atkinson.

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