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.Functional evolutionary studies of the sex-determining pathway of *Ceratitis capitata: MoY* ectopic expression and CRISPR/Cas9 targeting of *Cctra* and *Ccdsx* genes lead to sex reversals

Ph.D. course Coordinator: prof. Salvatore Cozzolino

Ph.D supervisor:
prof. Giuseppe Saccone

Ph.D. candidate:
Pasquale Primo

Ph.D.Co-supervisor:
prof. Ennio Giordano

Abstract

The *Ceratitis capitata*, also known as the Mediterranean fruit fly or Medfly, is one of the most dangerous agricultural pest insects considering its ability to parasite hundreds of fruits, nuts and crops. *Ceratitis capitata* and *Drosophila melanogaster* share a relatively recent common ancestry of about 120 million years ago. Based on this phylogenetic relationship, *Ceratitis* and *Drosophila* preserved segments of their sex respective determination pathways. In *Drosophila*, the sex determination pathway starts from a primary signal based on the number of X chromosomes. The presence of two chromosomes allows the functional and female-specific splicing of the master gene regulator of sex determination *Sex-lethal* (*Sxl*). *Sxl* is a splicing factor that drives the female-specific splicing of the downstream gene *transformer*. The *transformer* gene encodes for an SR-rich protein that regulates the female-specific splicing of the *doublesex* gene, a transcriptional factor involved in the transcription of the somatic female differentiation genes and the repression of the male-specific ones. Whilst, in the case of just one X chromosome, *Sxl* male-specific and *tra* non-sex-specific mRNAs contain truncated, non-functional, open reading frames, and *doublesex* produces by default a male-specific protein isoform promoting male somatic differentiation. In brief, the *Drosophila* sex determination pathway is based on the hierarchy *Sxl*>*tra*>*dsx*. Drosophila, also in Ceratitis female, a functional female-specific *tra* (*Cctra*), drives *doublesex* female-specific splicing. Differently from *Drosophila*, *Cctra* can autoregulate in XX embryos until adulthood, promoted by maternal mRNA, and maintained by a positive feedback loop its activation (Pane *et al*., 2002), with the help of the auxiliary factor *Cctra-2* (Salvemini et al., 2009). In males bearing the Y chromosome, *tra* mRNAs contain truncated open reading frames leading to non-functional peptides, and *doublesex* produces by default a male-specific isoform. However, a different primary signal is present in this species, based on a male-specific Y-linked dominant signal, M, repressing *Cctra* in XY, which in the master gene regulator is a *transformer* (*Cctra*). Recently, we have identified the primary Y-linked dominant signal necessary for male differentiation: *Maleness-on-the-Y* (*MoY*) (Meccariello et al., 2019). *MoY* encodes for a 70aa long protein, showing no apparent relationship with other *Tephritidae*. I have shown that injections of recombinant MOY in medfly XX embryos have partial masculinisation activity at the phenotypic and molecular level. We have also shown that transient RNAi targeting *MoY* confirms that *MoY* has a crucial role in maleness determination and that XY females are surprisingly fertile. During my PhD thesis, I have extended these studies and showed that embryos injections of *Bactrocera dorsalis* and *Bactrocera oleae* MOY recombinant proteins in XX-only embryos led to a molecular *Cctra* male-specific splicing but no to a significative phenotype alteration. Considering the efficacy of the CRISPR/Cas9 approach by Cas9 protein complexes embryos injections in the Medfly (Meccariello et al., 2017; idem, 2019), I have applied this technique targeted the *Cctransformer* and, more recently, the *Ccdoublesex* genes. Interestingly, I have found that *Cctra* Cas9 targeting led to a complete female to male sex reversal even in the absence of changes in the DNA

sequence due to an unexpected CRISPR/Cas9 interference (Primo *et al*., 2020). Furthermore, I have found that *Ccdsx* Cas9 targeting (common region present in both sex-specific isoforms) led either to masculinisation of XX individuals or feminisation of XY individuals rather than an expected intersexual phenotype. We have discovered that *Ccdsx* sex-specific isoforms seem to have regulatory feedbacks on the upstream *Cctra* gene, to either *Ccdsx*^{M} to stabilise male-specific *Cctra* splicing or *Ccdsx*F to stabilise *Cctra* female-specific splicing. We asked if these novel *dsx* feedback *tra* are evolutionarily conserved in *Drosophila melanogaster*, and we have found preliminary evidence supporting this model.
I have achieved the fully female-to-male reversion even without a high rate of indels introduction. In the end, I have targeted the *Ccdoublesex* gene showing that its disruption affects both *doublesex* splicing and *transformer* splicing, its upstream *regulator*.

Supervisor: Prof. Giuseppe Saccone

Co-Supervisor: prof. Ennio Giordano

1.Introduction

1.1 Insects

The *Arthropoda phylum* is the most abundant in terms of the number of species. The *Insecta* class represents a taxon of more than 1 million species and has colonised most of the land despite the various climatic differences. Nevertheless, the importance in studying insects relies not only on their interaction with the human being and ecosystems, but also on understanding the genetic pathway involved in various biological processes, following the establishment by T. H. Morgan of *Drosophila melanogaster* as a model organism for chromosome and genetic studies in the first 20 years of the 20th century and the developmental genetic studies of Ed Lewis during the 70s. It comes clear during the 90s how the knowledge of the pathways and the understanding of the biological basis of development and differentiation in insects can lead us to understand the molecular basis of human diseases and, more recently, to find new strategies for controlling pest insects. Among the insects, *Ceratitis capitata* is one of the most dangerous agricultural pest insects that can cause losses of billions of dollars in the fruit crop economy(Siebert and Cooper 1995).

1.2 Insect of interest: *Ceratitis capitata*

Ceratitis capitata (Wiedmann, 1824), also known as Medfly (Mediterranean fruit fly), is a pest insect belonging to the order of *Diptera*. It is a well-known insect because it can parasite hundreds of wild or cultivated food plants. *Ceratitis capitata* spread started from sub-Saharan Africa, where it originated (Gasperi *et al.* 1991), and it is now endemic to the whole Mediterranean area, South and Central America and Middle East Asia (Fig.1.1)(Malacrida *et al.* 1992). This insect harmfulness relies on its reproduction. After the mating, the female lays its eggs beneath the epicarp fruit, using its extendable ovipositor. The Medfly's life cycle lasts 21 days considering optimal temperature and humidity conditions. The eggs are curved, smooth and white, with an anterior micropyle. After 2-4 days, eggs develop in larvae. The larvae are about 7mm-9mm and cylindrically elongated, their anterior end is recurved ventrally, and the larval development is composed of 3 substages called "instar". The last "instar" is known as "jumpy" because of its capability to jump. Then the larva develops in pupae, which is a cylindrical and brown shape. During this stage, metamorphosis occurs. The pupae enclose in adults showing a marked sexual dimorphism. The male has a pair of bristles with enlarged spatulated tips next to the eyes. It also has bristles on the anterior legs and blue reflections in the eyes; furthermore, the males are smaller than females and have a male reproductive apparatus. The female has green reflections in the eyes, and they can extend the can ovipositor up to 1.2 mm (Fig.1.2). After enclosion, the adults require about 2-3 days to be mature for mating (Thomas *et al*. 2001). After that, male courtship can start. The courtship is composed of three phases: continuous vibrating wings, when

the male meets the female and his abdomen is bent ventrally with a pheromone drop, then the wings vibrate stronger in rhythm and back and forward. Then, in the end, a rapid rotation of the head(Briceño, Ramos, and Eberhard 1996). After the mating, the female can lay hundreds of eggs in its lifetime (about 300). To lay, the female extends the ovipositor beneath the fruit's exocarp. The eggs are fed by the vegetative part of the fruits causing their spoil. Thus, considering the numbers of eggs per female per lifetime, the *Ceratitis capitata* population can increase exponentially in optimal temperature and humidity conditions. In *Drosophila*, egg development has a peculiar and fascinating division. Since the flies lay the eggs, they follow many divisions without cytokinesis, resulting in a stage called syncytial blastoderm: different *nuclei* for just one membrane. (Fig.1.3)
To eradicate, or at least suppress, the Medfly presence in some areas, scientists used chemical and biological-based strategies. During the 90s in the USA, the Sterile Insect Technique (SIT) successfully eradicated *Ceratitis capitata*. This technique aims at a massive rearing of the insect in bio-factories, then separation of males from females, terilizedon of males using a mutagenic rays (γ or χ) and in-field release of sterile males. Released sterile males will compete with the wild-type males in mating females (Morrison et al. 2010). The sterile males are not able to produce vital progeny (Knipling 1955). To this aim, for *Ceratitis capitata* fighting, a *Genetic Sexing Strain* (GSS) has been developed. This strain easily distinguishes females from males based on pupal colour, thanks to the *white* pupae (*wp*) gene and the temperature-sensitive lethal (*tsl*) gene, leading females to death if exposed at 42ºC during early embryonic stages. In detail, this strain has a 5 to Y chromosome translocation of tsl^{+} and wp^{+} *wild-type* alleles. Thus, males are heterozygous ($XY^{tsl+/wp+}/AA^{tsl-/wp-}$) whilst female has just two non functional copies of these genes ($XX/A^{tsl-/wp-}A^{tsl-/wp-}$)(Fig.1.4) . *Wild-type* alleles confer to the male individual a brown pupal colour and a temperature resistance if exposed to a higher temperature. On the contrary, females bearing two non-functional alleles have a white pupal colour and die if exposed at 42ºC *heat shock* (Robinson 2002). Once sorted, males can be terilized and released in-field. This method results in a fast, cost-effective, time-saving and eco-friendly strategy for insect pest-fighting. Hence, the knowledge in sex determination genes can arise in new strategies for insect pest control.

1.3 Sex determination in *Drosophila melanogaster*

Most of the insect species have a marked sexual dimorphism. Sexual dimorphism depends on the early embryonic molecular pathway of genes involved in the sex determination mechanism. In *Drosophila melanogaster*, the model organism, the number of X chromosomes dictates the sexual differentiation pathway and the cell's phenotype (H. Salz and Erickson 2010). Females bear two X chromosomes. Thus they have a double copy of X-linked signaling elements (XSEs), while males have just one X chromosome. The genes that compose the primary signal can be clustered in 3 groups: numerators, denominators and maternal. All of them encode transcriptional factors. The numerator genes, so-called because their action is

dependent on the number of X chromosomes, include *sisterless-A (sisA)* and *sisterless-B (sisB)* genes that encode the transcriptional factors SisA and SisB, respectively. The SisA elements are a family of basic leucine zipper factors (bZIP) that bind *Sex-lethal* (*Sxl)* early promoter along with not-yet-known protein. The SisB factor has a basic helix-loop-helix domain (bHLH) that interacts with the maternally provided *daughterless (da)* to regulate the early female-specific promoter *Sxl* transcription (Cline 1993; Erickson and Cline 1998). Moreover, SisA and SisB have protein-protein interaction domain. Their interactors are the maternal *daughterless* (*Da*) and *groucho* (*gro*) and the denominator gene *deadpan* (*Dpn*). In males, having one copy of the X chromosome, DPN and DA bind SisA and SisB. This interaction results in an inactive multicomplex. For this reason, the *Sex-lethal* transcription is not activated by the early promoter. In females, expressing a double quantity of SisA and SisB with the respect of males, the inhibitors fail to counteract them, letting SisA and SisB able to regulate *Sex-lethal* early transcription (H. Salz and Erickson 2010). *Sex-lethal* (*Sxl*) is the master gene regulator for female sex differentiation in drosophilids, and it is involved in three different processes: somatic and germline development, sexual development, and dosage compensation. *Sxl* genomic locus has two promoters: a later promoter, active in both females and males, and an early promoter only active in females as mentioned above (H. K. Salz et al. 1989). The transcript arising from the later promoter includes early stop codon, resulting in a truncated, thus non-functional, SXL protein. The transcript produced by the early promoter, *Sxlpe (Sex-lethal early promoter)* transcript, is translated into a functional protein. The functional SXL is a splicing regulatory element able to sex-specific direct its own splicing of the pre-mRNA transcribed starting from the *late* promoter in a female-specific manner. In males, the absence of a functional SXL protein results in a default *Sex-lethal* splicing, including the third exon bearing a STOP codon. In females, the presence of a functional SXL protein (arising from the early promoter thanks to the presence of a double amount of SisA and SisB) drives the splicing of the pre-mRNA from the late promoter in a female-specific manner via *exon-skipping*, then the third exon can be removed from the mature mRNA and its translation results in a full-length functional SXL protein. Once the autoregulative epigenetic loop is established, the SXL functional protein can drive its splicing and direct the splicing of the downstream gene *transformer* (*tra*) in a female-specific manner. The female-specific spliced *tra* mRNA encodes a functional splicing factor protein, called TRA, that interacts with TRA-2 an autosomal non-sex-specific splicing factor able to regulate the downstream gene *doublesex* (*dsx*) sex-specific splicing in a female-specific way (Belote *et al*. 1989). The presence of consensus regulation sequences in the *transformer* transcript recognised by SXL dictates the regulation of *transformer* by *Sex-lethal*. The *tra* pre-mRNA is present in both females and males, but it is processed via *3' alternative* splicing just in females. Precisely, the alternative splicing results in the inclusion (in males) or the exclusion (in females) of a STOP codon in the open reading frame (ORF). A STOP codon in the coding sequence results in a truncated, thus non-functional TRA protein in males. The presence of a functional SXL protein allows the *transformer* pre-mRNA to be spliced in a full length,

thus functional protein (Boggs *et al*. 1987). The competition between the U2AF splicing factor and SXL for the same binding sites regulates this molecular mechanism (Valcárcel *et al*. 1993). *Doublesex* is a transcriptional factor that drives both the expression and the suppression of genes encoding sex-specific traits. In brief, the *Drosophila melanogaster* sex determination pathway is hierarchy regulated by sex-specific splicing of the genes involved, in particular, *Sxl>tra>dsx*. Moreover, as in females, sex-specific splicing is needed to translate a functional product. Whilst, in males, only default splicing is needed. The term “default splicing” process is meant to be a splicing process in which no other splicing factors than the spliceosome are required (Fig 1.5).

1.4 Sex determination in *Ceratitis capitata*

The molecular sex determination pathway seems to be conserved among the insect *taxa* (Bopp, Saccone, and Beye 2014). In particular, the genes at the bottom of the regulatory cascade are well conserved compared to those at the top(Graham, Penn, and Schedl 2003). In *Ceratitis capitata*, and other species belonging to the *Tephritidae* family, the primary sex determination signal is the presence on the Y chromosome of a dominant maleness gene, so-called *Maleness-on-the-Y* (*MoY*). According to which *MoY* can express its function, the molecular mechanism is still unknown, while the downstream genes involved in the pathway have already been genetically and functionally characterised. The *Ceratitis capitata Sxl-lethal* orthologue seems to be not involved in sex determination and has no sex-specific splicing neither (Saccone *et al*. 1998). On the other hand, the *transformer* orthologue represents the master gene for female regulation in Medfly. The *Ceratitis capitata transformer* gene (*Cctra*) locus is about 3kb long and organised in 5 exons, two of them only present in the male-specific isoform. The zygotic *Cctra* (z*Cctra*) pre-mRNA is spliced by the maternal provided CcTRA that, along with CcTRA-2, splices its own zygotic pre-mRNA in a female-specific manner and produces $CcTRA^{F}$ via “exon-skipping” by removing the second and the third male-specific exons. As *Sex-lethal* acts in *Drosophila* by establishing an autoregulatory epigenetic loop, so *Cctra* acts in *Ceratitis* (Salvemini *et al*. 2003). Precisely, the female-specific *Cctra* isoform encodes a 429 aa SR-rich protein belonging to the class of splicing factors. In brief, *Cctra* was maternally provided as mRNA in the eggs, and the mRNA is then translated and drives the splicing of the newly zygotically transcribed *Cctra* pre-mRNA. This autoregulatory system can be maintained thanks to 5 repetitive elements 13 bp long on the pre-mRNA called TRA/TRA-2 binding sites. On the other hand, in males, the *Cctra* pre-mRNA is spliced by default, including two male-specific exons in two different male-specific isoforms, both carrying premature stop codon(Pane *et al*. 2002)(Fig.4). The TRA/TRA-2 binding sites are also present on *Ceratitis capitata doublesex* gene (*Ccdsx*), indicating a regulatory action of *Cctra* on *Ccdsx*(G Saccone, Salvemini, and Polito 2011, 200). *Ccdsx* genomic locus has four exons. The first and second exons are present in both females and males, the third

is a female-specific exon, which carries TRA/TRA-2 bindings sites, and the fourth is a male-specific exon. Differently from *Cctra*, *Ccdsx* is spliced via “alternative 3’-acceptor”. This 3’ alternative acceptor is the reason why male- and female-specific transcripts just differ in their 3’ region. The alternative splicing is co-adiuvated by TRA/TRA-2 complex enhancing the female-specific *Ccdsx* splicing, preserving cis-regulatory element, in both *Drosophila* and *Ceratitis*, suggest a positive selection in both species and the sex-specific and functional conservation (G. Saccone 1997). The sexual fate is established in the early embryonic development, 5-6 hours after egg laying and in *Ceratitis,* the presence of a Y chromosome is crucial for male fate. Functional studies based on RNAi silencing *Cctra* and *Cctra-2* have demonstrated that the inhibition of the autoregulatory loop at early embryonic stages results in a complete female-to-male reversion despite the XX karyotype(Salvemini *et al*. 2003; Pane *et al*. 2002)(see Fig. 1.5).

1.5 Sex determination and dosage compensation: two sides of the same coin?

In many organisms, the presence of two heterochromatic chromosomes represents the best way to achieve sexual somatic, germline and neuronal differentiation. In both *Drosophila* and mammals, the male or female cell fate is established by the presence of one (in males) or two (in females) X chromosomes. As this sexing mechanism arose during the evolution, different strategies aiming to counterbalance the genetic products of the different X chromosome numbers that were employed. In mammals, the compensation is given by the inactivation, in females, of one of the two X chromosomes, thanks to the presence of a long non-coding RNA *Xist* (Ballabio and Willard 1992). Whilst in *Drosophila*, the compensation is based on the X chromosome hyperactivation in males. In this insect, a group of proteins and RNAs are implicated in modelling the chromosome structure to allow the X hyperactivation. The RNAs are indicated as *roX* (RNAs on the X). While the group of the proteins involved in this mechanism are called the Male-Specific Lethal protein (*msl*), so-called because their disruption induces male-lethality, these five proteins are respectively encoded by five genes clustered on the X chromosomes: *maleless* (*mls*), *male-specific lethal 1*, *male-specific lethal 2*, *male-specific lethal 3*(*msl-1, msl-2, msl-3*) and *male-absent on the first* (*mof*) (Morales *et al*. 2004; Kadlec *et al*. 2011). All these components are necessary for X hyperactivation, considering their pivotal role in the acetylation of the Lysine-16 on the Histone 4 (H4K16Ac) thanks to an acetylation domain present in the MOF protein (Hallacli and Akhtar 2009). This acetylation process removes the positive lysine charge, resulting in a less attractive histone tail for the DNA. This charge loss gives the DNA a flexible structure, thus easily accessible to transcriptional factors and RNA polymerase (Gelbart *et al*. 2009). The MSL-1, MSL-2, MSL-3, and MLS proteins seem to have a crucial role in letting the MOF protein target the X chromosome. The *msl-2* splicing is regulated by *Sex-lethal*, which represents the link between sex determination and dosage

compensation. The *msl-2* pre-mRNA sequence reveals different *Sxl* putative binding sites, which consist of Poly-U sequences. These poly-U sequences are present in both 5' and 3' UTR transcript regions. The removal of an intron in the 5'UTR region is the main characteristic of the most *msl-2* male-specific mRNAs. Whilst, in females, the intron is retained. *Sxl* has a pivotal role in regulating this splicing event by preventing the intron splicing at 5' termini (Kelley *et al*. 1997). *Sex-lethal* is employed in two different molecular pathway that is, somehow, connected.

1.5 The insects' primary signals: identification and evolution

The *transformer* and *doublesex* genes are functionally conserved in lots of insect orders: *Lepidoptera*, *Hymenoptera* and *Diptera*(Bopp, Saccone, and Beye 2014). Nevertheless, the primary signals triggering the sex-specific splicing of downstream genes seem to be variable in their identity and function. The presence of a male determining factor is a common theme in at least most vector disease and agricultural pest insects such as *Anopheles gambiae*, *Aedes aegypti* and *Ceratitis capitata*. A male determining factor should have three main characteristics: present only in males, located in a single copy of the genome and expressed in the very early stages during embryogenesis. The isolation and characterisation of the M-factor have been recently improved thanks to next-generation sequencing (NGS) techniques and Chromosome Quotient (CQ) approach. The NGSs allow access to both males and females' genomes and transcriptomes rapidly during developmental stages. M-factors are usually located in a high-repetitive region such as a Y chromosome, hard to sequence even using NGS. So to exceed the limits of repetitive elements, a new tool has been developed: the Chromosome Quotient (CQ). The Chromosome Quotient is a bioinformatic tool based on the alignment of the transcriptome, from embryos at early stages, to the genome (Hall *et al*. 2013). This tool application results in identifying genes sex-biased expressed genes, *i.e.* expressed in one sex rather than in the other. Thus, chromosome quotients have led to the isolation of male-biased genes expressed during early embryonic stages, hence potential candidates to be the male-specific factors. Subsequently to the bioinformatic analysis, a molecular and functional approach is needed to confirm the *in silico* data. In *Aedes aegypti*, the yellow fever primary vector, this workflow led to the isolation of 24 putative M-factor sequences. Among these contigs, one results to be an orthologue of *tra-2* able to instruct the male-specific splicing pathway when present and called *Nix*. This gene encodes a 288 amino acids long protein with two RNA binding motifs, and it is present only in the male genome and found to be expressed 3-4h after egg laying. Its disruption reverts males into females, whilst its ectopic expression in females results in a female-to-male reversion. In conclusion, *Nix* was necessary and sufficient for maleness sexual fate. It is also conserved in other *Aedes* genera and was also found in *Aedes albopictus* (Hall *et al*. 2015). According to the same workflow, the *Anopheles gambiae* maleness factor has been identified. It has no similarity with *Nix*, has a three exons organisation, and, in

addition to *Nix*, is involved in the male-determining pathway and the dosage compensation. The isolated gene was then able to instruct a male-determination program and called *Yob*. Unusually, an M-factor is also involved in a pathway different from sex determination. However, in *Anopheles stephensi,* the maleness factor *Guy1* seems to be involved in dosage compensation, suggesting a common ancestor of these genes was involved in such molecular processes (Criscione et al. 2013). In *Apis mellifera*, the sex determination signal is based on an aplo-diplo mechanism of *csd* (*complementary sex determination*) gene, *i.e.* the presence of two different *csd* alleles the same organism acts as a female initiation program on a *transformer* orthologue: *fem* (*feminizer*). On the other hand, the presence of one allele or two homoalleles results in a male-specific development pathway(Beye *et al*. 2003). In *Bombyx mori*, sex determination is based on a heteromorphic chromosome, but males are homomorphic (ZZ) while females carry two different chromosomes (ZW), in which the primary signal is a small PIWI-interacting RNA(Kiuchi et al. 2014). *Musca domestica* represents a particular primary signal variability example, in which, depending on the population considered, the primary signal can be Y-linked, autosomal, environmental or also X-linked(Tomita and Wada 1989). Recently, the *Musca domestica* male determining factor, named *MdMd*, has been isolated thanks to the approach explained ahead, resulting in a paralog of *Mdncm*, a splicing factor(Xu *et al*. 2017, 2017).

1.6 The *Maleness-on-the-Y* (*MoY*) gene in *Ceratitis capitata*

The *Tephritidae* family comprises more than 5000 species, and tens of them are incredibly harmful to hundreds of agricultural plants. The knowledge arising from sex determination studies poses the basis for developing newer and more accurate strategies for dangerous species fighting and control. *Tephritidae* have two heteromorphic sexual chromosomes, *i.e.* XY individuals are males and XX are females. Hence, male traits are determined by the presence of a maleness gene on the Y chromosome. Because of repetitive sequences, the sequencing and characterisation of the Y related genes were challenging. In 1996, the region coding for the maleness factor in *Ceratitis capitata* was mapped on the pericentromeric region on the Y long arm(Willhoeft and Franz 1996). However, the molecular identification of the genomic region coding for the maleness gene awaited new sequencing new approaches and sequencing technologies. Following the NGS and CQ approach, the putative M factor was isolated in 2017 (Meccariello 2017). One of them, the *non-coding-1*, resulted in the *Maleness-on-the-Y* gene. In the beginning, the idea was that the gene could act as a silencer RNA and, for this is the reason it was called "*non-coding-1*", and the first studies were carried out using RNAi and ectopic expression. Silencing in XY embryos of the *nc-1* gene resulted in a complete male-to-female reversion, and, on the contrary, its ectopic expression in XX resulted in a female-to-male reversion. Considering the crucial role in maleness pathway initiation and its presence on the Y chromosome, this gene was called *Maleness-on-the-Y* (*MoY*). *Ceratitis* and *Bactrocera* shared a common ancestor 80

million years ago. Despite the relatively recent divergence, the *MoY* locus is not conserved in terms of the nucleotide sequence. However, the identification of a putative 70 amino acids long coding sequence, which appeared to be conserved among Tephritidae, has led to the hypothesis that *MoY* could act as protein. The putative protein was indeed partially conserved in *Bactrocera* species (30% similarity), allowing the identification of the *MoY* locus also in this *Tephritidae* taxon. Based on these findings, the transient silencing of the same locus by embryonic RNAi was performed in *Bactrocera dorsalis* and *Bactrocera oleae*, confirming that *MoY* locus is conserved the *Tephritidae* family (Meccariello *et al.*, 2019). Along with functional analysis carried on *Bactrocera dorsalis* and *Bactrocera oleae*, other bioinformatic investigations were performed to search the *MoY* locus in other *Bactrocera* species in both their transcriptomes and genomes. *MoY* orthologs in the genomes and/or transcriptomes were isolated in eight other *Tephritidae* species. And in four of them, the position of this locus on the Y chromosome has been demonstrated (Fig. 1.8). *MoY* molecular pathway is still unknown, hypothesis arising from its amino acids constitutions (basic and positively charged) let speculate about its implication as a nucleic acid interacting protein. It might interact with the maternal provided *Cctra* by blocking its translation or disrupting the mRNA in very early embryonic stages or a TRA/TRA-2 complex inhibitor and, hence, by blocking the initiation of the establishment of the epigenetic autoregulatory loop (Meccariello *et al*. 2019).

1.7 The *Ceratitis capitata transformer* gene

The *Drosophila melanogaster* transformer gene was first described in 1945. This mutation ultimately reverts genetically XX, which should develop in females in a fully reverted male. Even though the individuals were XX and their sex determination genes have wild-type alleles, these flies became male because of the mutation of a locus on the 3rd chromosome, later called a *transformer*, because of its capability of transforming female to male. Furthermore, the gene *lethal(3)73Ah* shares its 3'UTR with the *transformer* gene, transcribed on the complementary strand (Irminger-Finger and Nöthiger 1995). Based on this knowledge, the *Ceratitis capitata transformer* gene was isolated and characterised by synteny, *i.e.* starting from a 400bp *Drosophila melanogaster lethal(3)73Ah* probe and hybridisation on *Ceratitis capitata* cDNA were performed. The *Ceratitis capitata lethal(3)73Ah* resulted well conserved. Then, to isolate the *Cctra* gene, a chromosome walking downstream the new isolated *lethal(3)73Ah* was performed, 4 kb downstream, a putative partially conserved *transformer* ORF has been characterised. Even though the *Ceratitis capitata transformer* nucleotide sequence was partially conserved, the gene resulted in an ortholog of *Dmtra* and expressing a pivotal role in sex determination. As *Sex-lethal* in *Drosophila*, *transformer* in *Ceratitis capitata* establish an epigenetic autoregulatory loop by maintaining sexual memory in somatic cells (Pane *et al*. 2002). *Cctra* has five exons. Two of them (2nd and third) are male-specific and spliced via *exon-skipping* by the TRA/TRA-2 complex. Thus, in males, two *Cctra*

isoforms are present and encoding for two truncated TRA(OFF) proteins, M1(59 amino acids) and M2 (99 amino acids), that seem not to have any implications in sex determination. the other hand, in females, the *Cctra* female-specific transcript encodes a full-length functional CcTRA(ON) protein belonging to the SR-rich protein, usually involved in splicing processes (see Fig.1.6). The CcTRA interacts with CcTRA-2 protein, a non-sex-specific protein but crucial to co-adiuvante the female-specific splicing of both *Cctra* itself and *Ccdsx* pre-mRNAs(Salvemini *et al*. 2003). The CcTRA/CcTRA-2 complex binds the *Cctra* pre-mRNA thanks to the presence of 13 bp five times repeated sequence called TRA/TRA-2 binding sites. The splicing occurs in the first hours after oviposition (5-6h)(Gabrieli *et al*. 2010), and the ON/OFF switch is based on the presence of the Y chromosome and thus on the *MoY* action in sex determination. Functional studies based on RNAi targeting the maternal *Cctra* have demonstrated that the collapse of the epigenetic autoregulatory loop at the early development stage results in a complete female-to-male reversion. A transgenic line expressing *Cctra* RNAi (paper in submission) controlled by an ovary-specific promoter can lead to XX flies with male traits and fertile, concluding that the Y is not necessary for fertility. The cross of XX males and XX females results in all XX females progeny, useful for male-determining studies (Fig.1.9).

1.8 The *Ceratitis capitata doublesex* gene

The *doublesex* gene is the sexual differentiation pathway, the last effector. In *Drosophila,* the hierarchy controlling males and females traits involve the two isoforms DSX^F and DSX^M , at its bottom. As in most insects, also in *Ceratitis capitata,* the doublesex gene has a male and a female-specific transcript differ just for their dimerisation domain (N-terminal), whilst they share a common DNA binding domain at their C-terminal end. The *doublesex* pre-mRNA splicing is controlled by the TRA/TRA-2 complex (Fig.1.7). The female and the male-specific *doublesex* isoform encoded for transcriptional factor regulating the transcription of different targets (Burtis *et al*. 1991). It seems that *doublesex* is involved in processes determining the secondary sexual traits. The DSX proteins have many interactors. The interaction with the homeotic *Abd-B* led to the expression of one germinal trait instead of the other, depending on the sexual development program. The interaction with the *bric-a-brac* gene led this complex to control the fly pigmentation (darker in males). In *Ceratitis capitata,* the *doublesex* ortholog is composed of 4 exons. The pre-mRNA is spliced via a 3'-alternative acceptor leading to two proteins different for their 3' end. The N-terminal encodes 397 amino acids whilst the sex-specific C-terminal of either 152 (DSX^M) or 30 (DSX^F) amino acids. The *Ccdsx* splicing is driven by the presence of TRA/TRA-2 binding site in female-specific exon only, thus, in the female, the splicing complex enhances the recruitment of female-splicing acceptor rather than the male splicing-acceptor, whilst in males, the splicing acceptor is coded by default. Unlike the *transformer*, *doublesex* is well conserved among all the insect *taxa*, and its male-specific ectopic expression in *Drosophila melanogaster* results in partial masculinisation of XX individuals (Saccone *et al*. 2002).

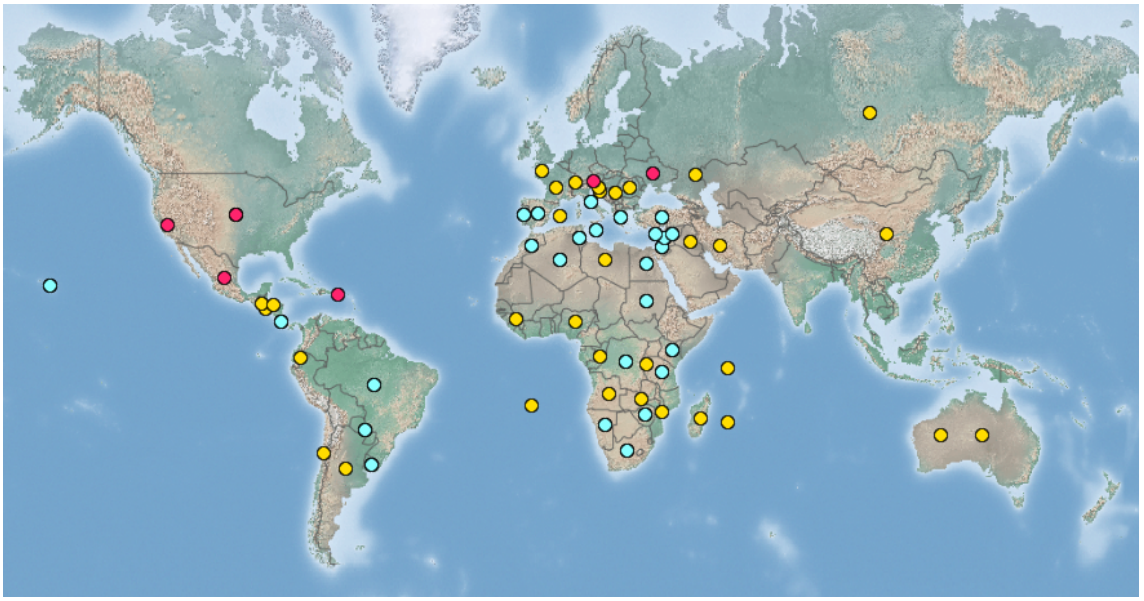

Figure 1.1- Medfly distribution throughout the globe, blue spots highlight the areas where *Ceratitis capitata* is widespread, the yellow one highlights areas where it is localised and the red one the areas where it has been eradicated. (https://www.cabi.org/isc/datasheet/12367#toDistributionMaps)

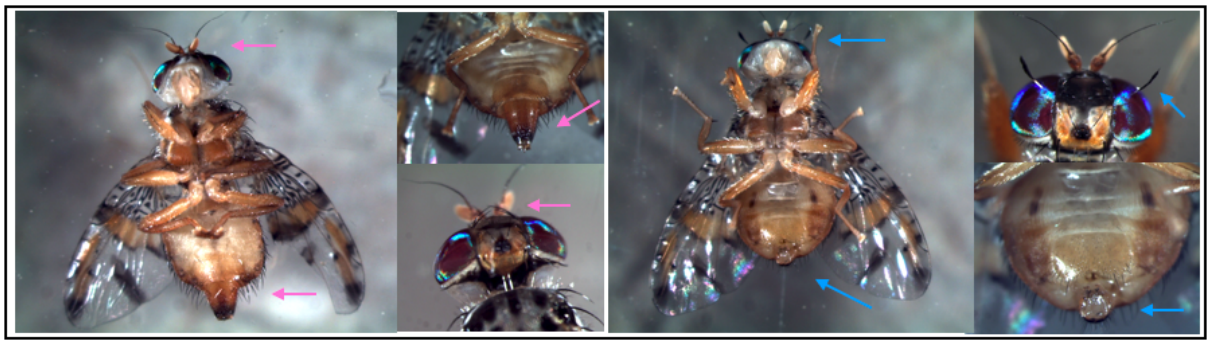

Figure 1.2- *Ceratitis capitata* sexual dimorphism. On the left, the female, on the right the male. The pink arrows highlight the female-specific sex traits, whilst the light blue arrows indicate the male-specific ones.

Figure 1.3 - *Drosophila melanogaster* development (Developmental biology - Scott F. Gilbert, 8th Edition, Sinauer Associates, Inc., Publishers Sunderland, Massachusetts USA, pag. 254)

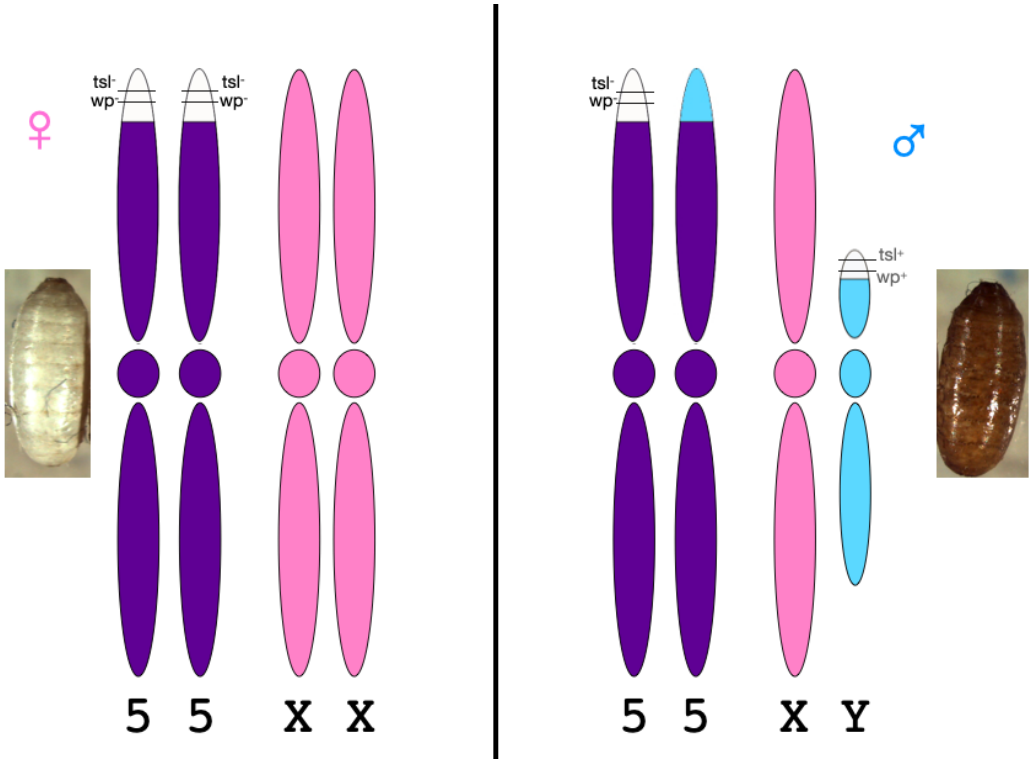

Figure 1.4 - Graphical representation of *wp/tsl* strain. In grey, the two X chromosomes, in black the Y chromosome and white the two autosomes 5. On the left, the female bears the two non-functional alleles for *wp* and *tsl*. Whilst, on the right, the male carries the two wild-type alleles, translocated from chromosome 5.

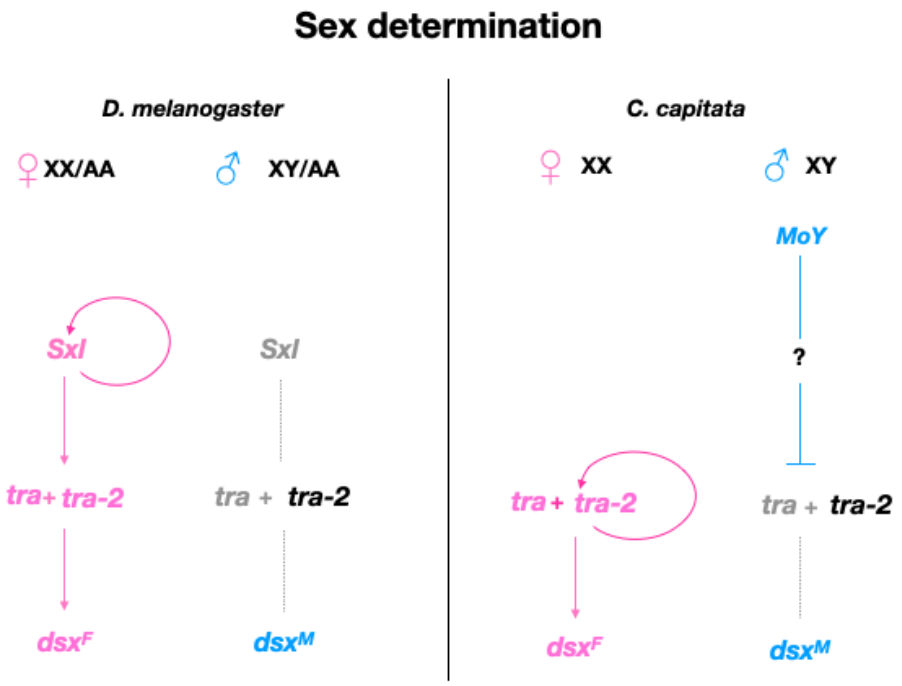

Figure 1.5- A comparison between *Drosophila melanogaster* and *Ceratitis capitata* sex determination pathway. In *Dm* on the left, *Sex-lethal* acts in XX in a positive-autoregulatory manner to establish the female sex-specific splicing of downstream genes and itself, whilst in XY individuals, the non-functional SXL protein leads to a male-specific development. In *Ceratitis capitata* on the right, the autoregulatory loop is established by the *Cctra* gene, acting like *Sxl* in *Drosophila*, by maintaining the feedback loop.

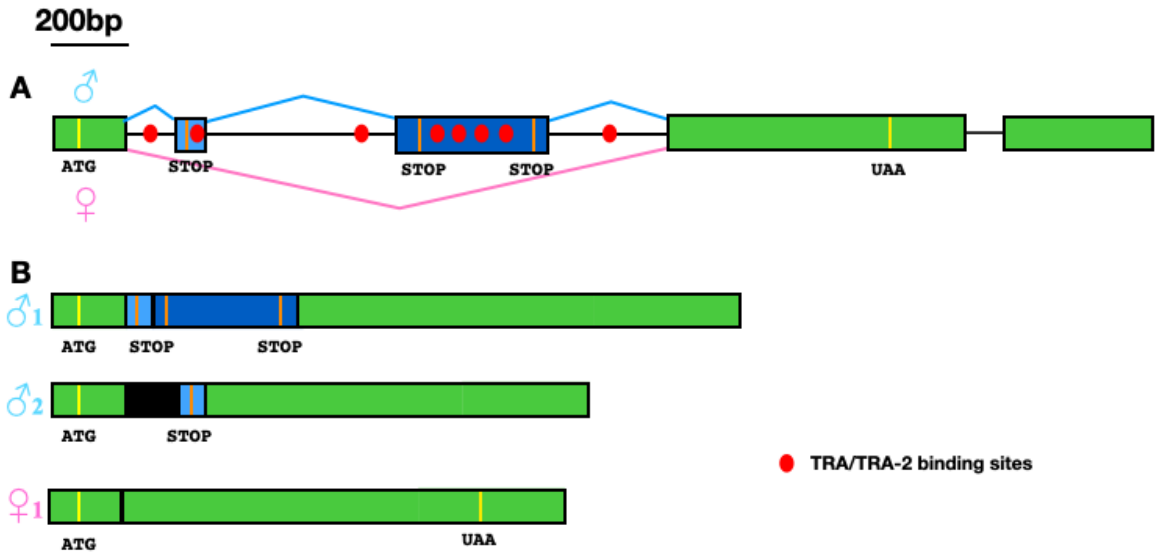

Figure 1.6- A) Representation of the *Ceratitis capitata transformer* genomic locus and its splicing regulation, in green are the exons present in both male and female transcript, whilst in blue and dark blue the ones present in the males-specific transcript. Red dots indicate the TRA/TRA-2 binding sites necessary to promote the

female-specific splicing. Using a yellow line, start and stop translation sites are marked. **B)** *Cctra* sex-specific splicing isoform. In males, the inclusion of male-specific exons in the transcript led to a truncated *Cctra* protein.

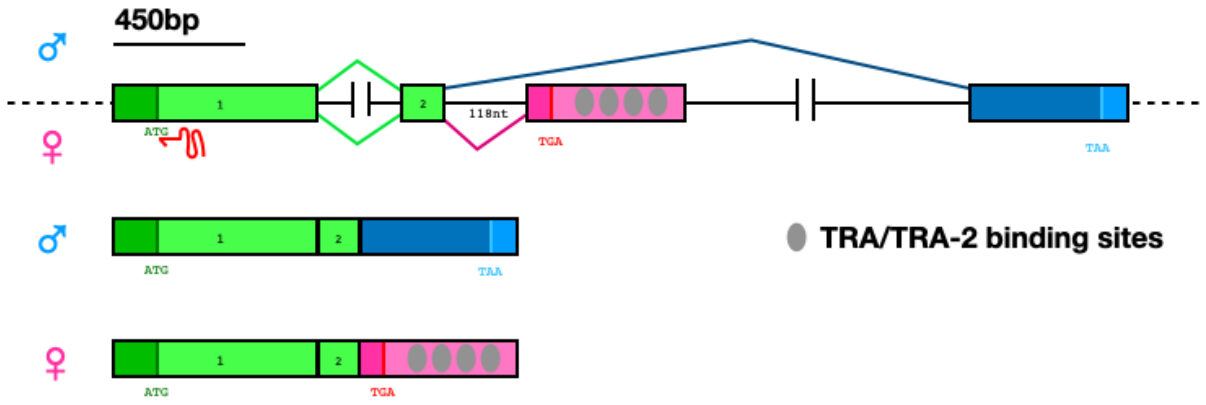

Figure 1.7- A) Representation of the *Ceratitis capitata doublesex* genomic locus and its sex specific splicing regulation. In green, the exons present in both females and males transcripts, whilst in pink, the female-specific exon and blu the male-specific exon. It has been highlighted in grey the TRA/TRA-2 binding sites. On the bottom the two isoforms of the gene.

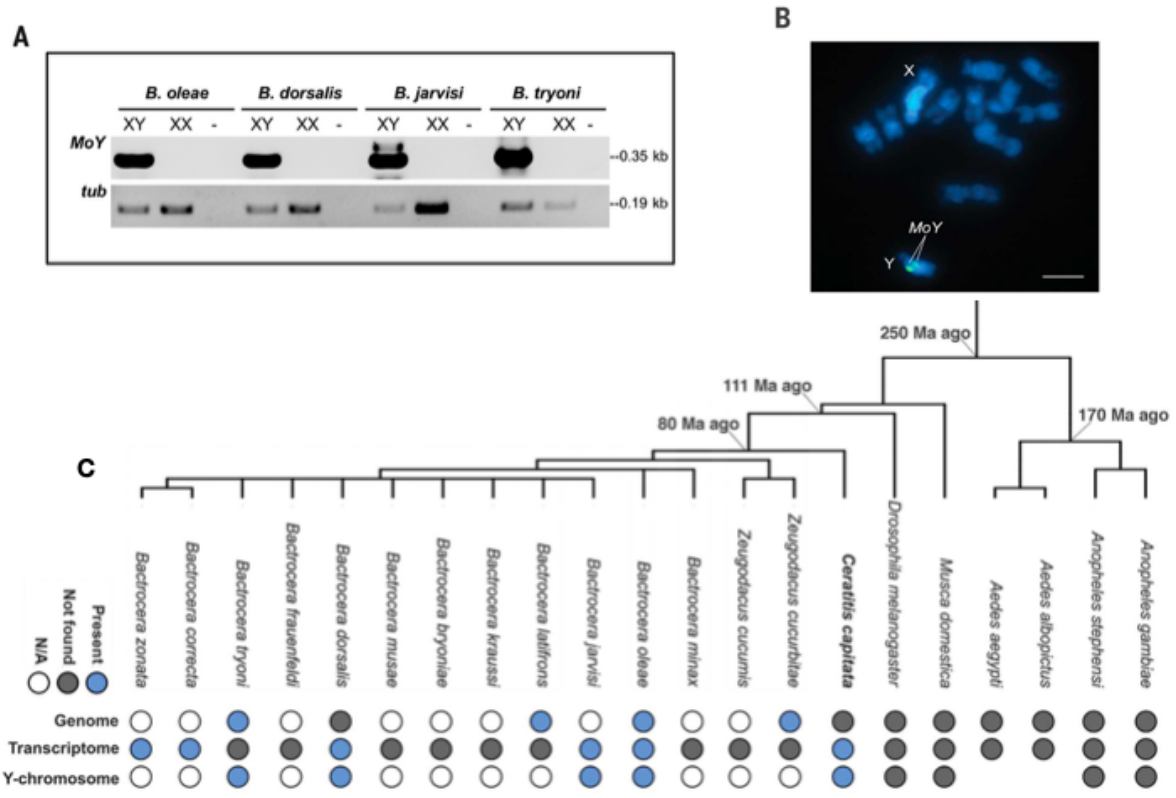

Figure 1.8- A) PCR on genomic DNA demonstrating *MoY* conservation in *Bactrocera dorsalis*, *oleae*, *jarvisi* and *tryoni* **B)** *In situ* hybridisation mapping the *Ceratitis capitata MoY* on the Y chromosome **C)** Evolutionary analysis for *MoY* conservation in *Tephritidae* (Meccariello *et al*. 2019).

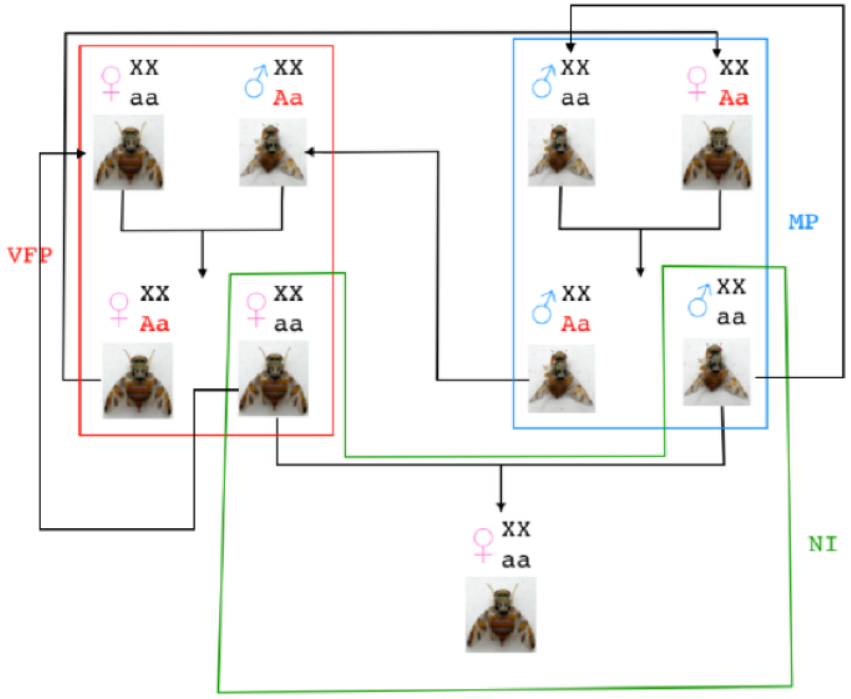

Figure 1.9 - Transgenic line expressing *Cctra*-RNAi controlled by an ovary-specific promoter. The transgenic construct expresses the double-stranded RNA targeting the *Cctra* mRNA. The mother deposits the dsRNA in the eggs, thus flies with a transgenic mother will develop in males despite the presence or absence of the Y chromosome, allowing us to obtain the XX males. In detail, a transgenic female arises from the crosses of XX transgenic male with wild-type female (highlighted in red, Virgin Female Producer (VFP) strain). Transgenic females can be crossed with a non-transgenic XX male from a transgenic mother (highlighted in blue, Male Producer (MP) strain). XX males and XX females can be crossed to obtain only XX females (highlighted in green, Non-interfered (NI) strain).

2. Results

2.1 *Ceratitis capitata Maleness-on-the-Y* gene acts as a protein

In 2019, Meccariello et al. identified the *Maleness-on-the-Y* (*MoY*) gene *in Ceratitis capi*tata, the gene responsible for the maleness development program. Thus, *MoY* is an intronless, Y-linked gene expressed during the early embryonic development stages to instruct the *transformer* male-specific splicing and result in male sexual development. Meccariello et al. have shown that the genomic region containing *MoY*, a 5kb extended region, can fully revert XX females into XX males. Moreover, its silencing results in a complete feminisation of XY embryos into fertile adult XY females. *MoY* has a unique ~600bp long transcript, in which a 70 aa long putative ORF is present. It has no similarity to other known protein, and it is able to promote the male-specific splicing, directly or not, of genes involved in the sex determination pathway. *MoY* conservation studies in *Tephritidae* have identified its orthologues in *Bactrocera dorsalis* and *Bactrocera oleae* , showing 21% identity and 44% similarity to Medfly MOY, Meccariello et al., 2019). Functional studies based on embryonic RNAi and CRISPR/Cas9 showed its conservation as primary signalling for male sex determination also in these species. (Meccariello et al., 2019).

My contributions to this work are:

1) AM, PP and GS selected *MoY* as a first *M* candidate from the list of putative male-specific transcripts by MDR and the list of novel putative male-specific transcripts by ZT and BH.
2) PP, AG, MAG, FF, DI and MMP maintained the strains, performed crosses and DNA/RNA molecular analyses.
3) PP performed MOY protein embryos injections.

See Attachment :

MoY orchestrates male sex determination in major agricultural fruit fly pests

In conclusion, I have shown that the orthologues of MOY in four other Tephritidae species are Y-linked. I have confirmed that XY females can be fertile and that the recombinant Medfly MOY protein can partially revert Medfly XX females to XX intersexes toward masculinity.
Despite the low sequence similarity to Medfly MOY, I planned to investigate during my PhD, if the BdMOY and BoMOY might be able to perform their masculinisation function when injected in *Ceratitis capitata* XX embryos.

2.2 *Bactrocera oleae* and *Bactrocera dorsalis* MOY ortholog proteins can very partially revert *Ceratitis capitata transformer* splicing from female to male in XX individuals

Recombinant BdMOY and BoMOY proteins have been developed and provided by L., Vitagliano, A., Ruggiero (IBB-CNR, Naples; unpub, res.; Giovanni Barra, master thesis) can molecularly masculinise the *Cctra* splicing in XX-only *Ceratitis capitata* embryos despite the low sequence similarity in terms of amino acids sequence. The highly conserved short regions could play a significant role in this potential inter-specific functional conservation.

XX-only progeny arises from a cross among XX females, and XX fertile male obtained by embryonic RNA interference targeting the *Cctra* gene (Pane et al., 2002; Meccariello et al., 2019, see Fig. 1.9). In this unisexual progeny, a female-to-male complete molecular and phenotypic reversion, even if rare, would be more easily detectable rather than in a bisexual one. Injections either with BoMOY or BdMOY proteins (~70ngr/µL) were performed in XX embryos 1h after egg laying (AEL) and dechorionated one by one manually. After embryogenesis, I detached 50 embryos from the glass slides, pulled them together and extracted RNA. I performed RT-PCR analyses and gel-electrophoresis to monitor if the female-specific *Cctra* splicing expected for XX-only embryos was shifted toward a male-specific pattern (Fig. 2.2.1). In adults a ~600 bp long male-specific and a ~200 bp female-specific *Cctra* cDNA fragments are detected (Pane et al., 2002). The ~200 bp band is detected as a weak band very rarely also in male samples as in this case. I detected by RT-PCR in XX/XY embryos and in injected XX embryos lanes, *Cctra* splicing intermediates and possibly those of the male-specific length. In contrast, XX embryos untreated (Fig. 2.2.1) as well XX embryos injected with injection buffer, BdMOY buffer, BoMOY buffer and BSA (See also Meccariello et al., 2019; see also Fig. 2.2.5 and Fig. 2.2.6) showed only a female-specific band.

In both *Bo*MOY and *Bd*MOY injected XX pools (BoMOY and BoMOY injection were carried in duplicates, see Fig. 2.2.1 and Fig. 2.2.5), I detected very week bands of cDNA amplicons corresponding to male-specific *Cctra* transcripts in addition to the female-specific ones. Moreover, other amplicons of intermediate lengths corresponding to the major female-specific and male-specific ones are detected in XX/XY, not injected embryos as in the XX injected embryos. These amplicons may be explained because the *Cctra* splicing could pass through different intermediate stages. Moreover, injected XX embryos have a *Cctra* splicing pattern similar to the untreated XX/XY embryos pool. In XX non-injected embryos, just the female-specific *Cctra* isoform is detected as expected. To confirm that the XX embryos pools were -free of any XY embryos, I have carried out an RT-PCR reaction to detect RNAs derived from a Y-specific gene (*MoY*) and hence to monitor the potential presence of the Y chromosome indirectly. Gel electrophoresis showed that *MoY* RNA is detected only in the reference (positive control) cDNAs of XX/XY embryos and XY adult flies, but not in XX-only embryos and XX adult flies (Fig. 2.2.1).

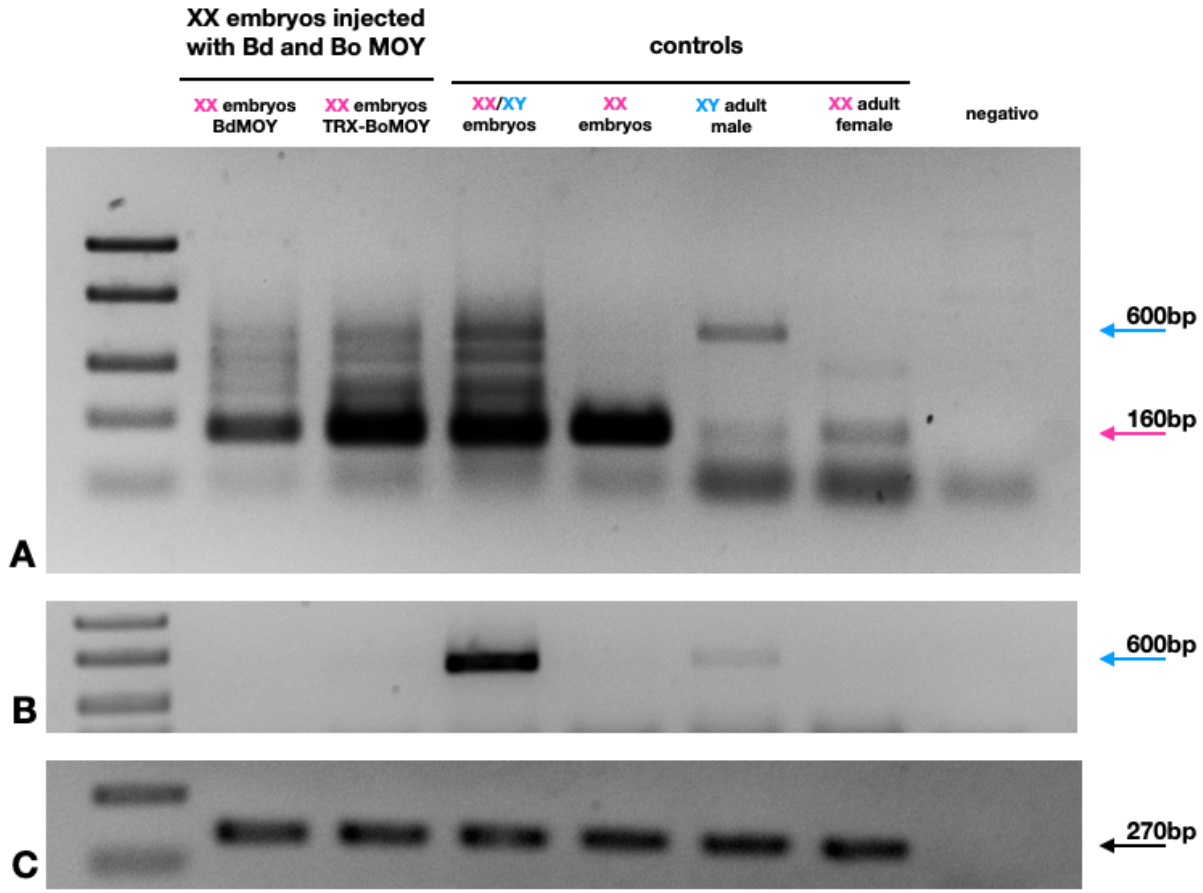

Fig. 2.2.1 - RT-PCR on BoMOY and BdMOY in XX injected embryos. A) *Cctra* splicing pattern analysis. B) *MoY* detection. C) *Ccsupeoxide-dismutase* (*housekeeping*). The first lane, XX embryos injected using the BoMOY recombinant protein, the second lane is the BdMOY recombinant protein, the third lane non-injected mixed XX/XY embryos, the fourth lane XX-only non injected embryos, the fifth lane the male adult and the sixth lane XX female adult.

We know from Meccariello et al. (2019) that *MoY* is expressed mainly during embryogenesis and very low at adult stages. From this result, it seems that the orthologous proteins of MOY in *Bactrocera dorsalis* and *Bactrocera oleae* as recombinant proteins can promote only very weakly male-specific *Cctra* splicing. I performed injections of the recombinant BdMOY and BoMOY protein in XX/XY mixed embryos and XX-only embryos to verify also if the partial molecular masculinisation of *Cctra* splicing results into a phenotypic anomaly at adult stages (Tab. 1). However, I did not observe the expected intersexual adult phenotype in the developed flies in neither of the sets of injections (Tab.1).

# Set	Injection Mix	Embryos karyotype	n. Injected embryos	Larvae	Pupae	Females /tot	Males/ tot	Intersexes/ tot	%survival
#1	BdMOY	XX/XY	180	48	21	14	5	0	26,7
#2	BdMOY	XX/XY	81	24	17	6	9	0	29,6
#3	BdMOY	XX	180	32	3	2	0	0	17,8
#4	BoMOY	XX/XY	150	92	59	37	22	0	61,3
#5	BoMOY	XX	150	67	39	35	0	0	44,7
#6	BoMOY	XX	150	54	37	32	0	0	36,0
#7	MOY (syn)	XX	180	31	14	12	0	1	17,2
#8	Buffer	XX/XY	150	63	59	28	31	0	42,0

Tab.1 - BdMOY and BoMOY injection in XX-only (#3,#5,#6) and XX/XY (#1,#2#4). (#7) Synthetic MOY injection in XX-only embryos).

A synthetic MOY protein has been developed and provided by Ruvo Menotti (IBB_CNR, Naples) to try to solve problems to solubility and concentration that also Dr. L.Vitagliano, Dr. A. Ruggiero, prof. E.Pizzo and Dr. A.Bosso (our collaborators for protein recombinant expression and purification) experienced. Injection of synthetic MOY in *Ceratitis capitata* embryos has led to a not completely developed fly (not hatched) showing an intersexual phenotype out of 13 adult flies, confirming the data of Meccariello et al., 2019.

The partially developed and reverted fly has no male-specific antenna but a male-specific reproductive apparatus. To investigate the partial masculinisation of the XX individual, and to confirm its intersexuality, RT-PCR on *Cctra* was performed.

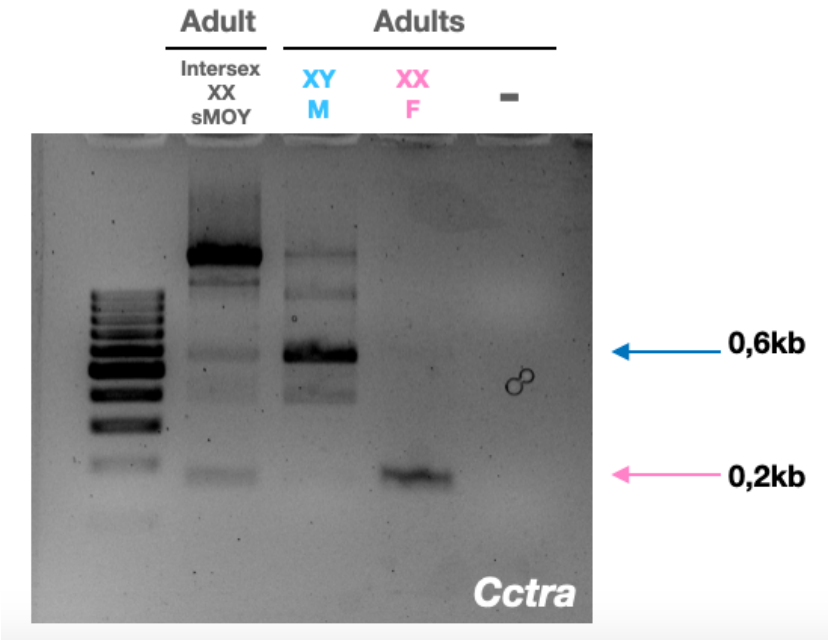

Fig. 2.2.4 - RT-PCR of *Cctra* RNAs on the single XX intersex obtained after synthetic MOY injection. The first lane intersex, the second lane is an adult male, the third is a female adult, and the fourth lane is a non-template negative control.

Hence, RT-PCR analysis has demonstrated both males-specific and female-specific amplicons in the same fly, suggesting that this is a gynandromorphic fly at the molecular level as expected. Molecular analysis has revealed an intersexual *Cctra* splicing pattern, presenting both female-specific and male-specific amplicon.

I also investigated if the *Bactrocera oleae* MOY can masculinize the *Cctra* splicing pattern, if injected in *Ceratitis capitata* XX embryos and monitored a few hours later. The recombinant BoMOY protein (~70ngr/µL) was injected in XX/XY and XX-only embryos at 0.-1h. 50 XX-only embryos were injected using the BdMOY, BoMOY, synthetic MOY recombinant proteins and injection buffer (Fig. 2.2.5).

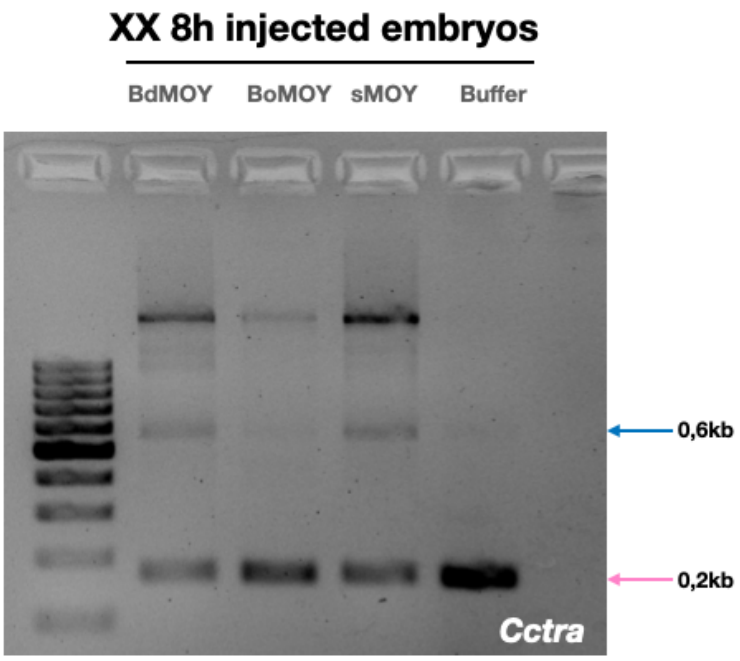

Fig. 2.2.5 - RT-PCR on XX-only 1h AEL embryos injected respectively with BdMOY, BoMOY, sMOY (synthetic MOY) and buffer as a control and processed 8h AEL.

In the control, XX embryos injected with the injection buffer showed only the female-specific *Cctra* RNAs, as expected in case of no effect. On the contrary, BdMOY and sMOY shifted part of the splicing pattern of *Cctra* toward the male-specific isoform significantly and BoMOY only mildly. I have detected only in protein injected embryos, a longer cDNA product (> 900bp) likely corresponding to an unspliced *Cctra* product, previously described also in XX adult flies but not XY (Pane *et al.,* 2002).

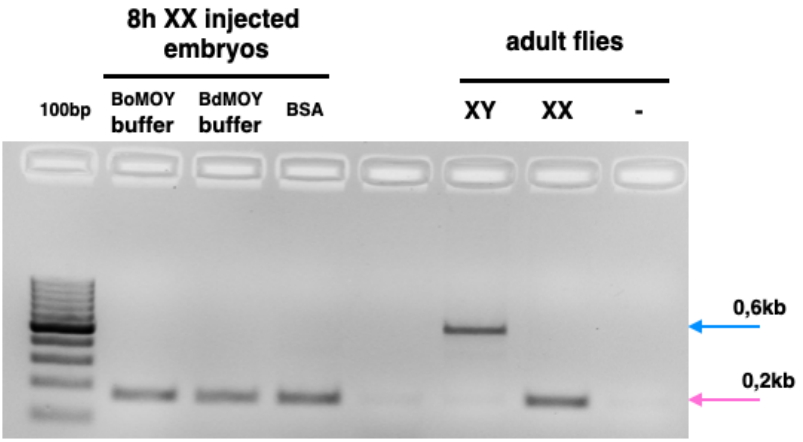

Fig. 2.2.6 – Control experiments: RT-PCR analysis the *Cctra* splicing pattern on XX-only embryos injected 1h AEL and processed 8h AEL. In the first lane 50 XX-only embryos were injected using the BoMOY buffer, in the second lane 50-XX only embryos were injected using the BdMOY buffer, the third lane 50-XX only embryos were injected using the BSA.

Moreover, I performed control experiments using the protein resuspension buffer only and a BSA protein and I confirm that the molecular *Cctra* shift from female-to-male can only be ascribed to the specific injection of BoMOY or BdMOY(Fig.2.2.6). Indeed, no male-specific *Cctra* cDNA fragment was amplified. .

2.3 *Ceratitis capitata transformer* CRISPR/Cas9 mediated targeting

The *transformer* is a bistable master gene involved in sex determination regulating its splicing and epigenetic autoregulative loop (Pane *et al.,* 2002). Pane et al. (2002) observed a collapse of the autoregulatory loop once embryonic RNAi targeting the *transformer* gene was performed. This collapse resulted in a full XX reverted male showing male-specific *Cctra* RNAs. When *Cctra* is switched off in XX, the *tra* pre-mRNA is spliced in two male-specific transcripts, both encodings truncated TRA proteins (The M1 transcript encodes for a 49 aa long protein, whilst the transcript M2 encodes for a 99aa long protein) likely non-functional. Our study aimed to induce, using the CRISPR/Cas9 system, frame-shift loss of function mutations in the *transformer* in the first coding exon. The ORF disruption would also affect the male-specific CcTRA short polypeptides. Hence we could verify functionally if these putative non functional truncated CcTRA proteins are dispensable in phenotypic XY males (as well as in expected reverted XX) for male differentiation.
Therefore, XX female-only embryos were injected using a CRISPR/Cas9 protein pre-assembled with a sgRNA targeting the *Cctra* first common exon (Meccariello et al., 2017). As biallelic mutations induced by Cas9 can be observed often at low frequency in somatic and germline cells, we planned a complex scheme of injections of embryos and crosses of the injected G_0 adult flies to produce a G_1 progeny having at least a few flies with a heteroallelic combination of induced *Cctra* mutations. However, surprisingly we observed already at G_0 full reverted XX males. Furthermore, more surprisingly, DNA and RNA sequencing of DNA/cDNA clones, from the adult XX reverted males and XX females, hatched from injected embryos revealed a very low frequency of mutations induced at the *Cctra* locus by Cas9. I have also observed in XX adult males a complete shift in the *Cctra* splicing pattern from female-specific to male-specific. We speculated and offered data in support this idea, that the masculinization of XX individuals was apparently due to an unexpected CRISPR interference. Injections of a plasmid expressing a dead Cas9 (dCas9, able to bind but not to cut) and sgRNA binding to *Cctra*, led to masculinization of XX, as predicted.
Cctra positive autoregulation in XX embryos seems to be very sensitive to transient molecular disturbances even at the transcriptional levels, leading to a shift to the male-specific splicing pattern which is maintained during all development.

See Attachment:

Targeting the autosomal *Ceratitis capitata transformer* gene using Cas9 or dCas9 to masculinize XX individuals without inducing mutations

2.4 *Ceratitis capitata* and *Drosophila melanogaster doublesex* Cas9 targeting

In the sex determination pathway of *Drosophila melanogaster, doublesex* is the downstream gene acting in its two sex-specific as transcriptional factors. The male-specific isoform activates the genes involved in the male developmental process and suppresses the ones involved in female differentiation. On the contrary, the female-specific isoform activates the genes involved in female developmental programming and represses the ones involved in the male differentiation program. (Nagoshi *et al*. 1988). As in *Drosophila* and *Ceratitis,* the *Ccdsx* female-specific splicing factors are controlled by CcTRA and CcTRA-2 splicing factors (Pane et al., 2002; Saccone et al., 2008). The *Ccdoublesex* genomic region on the 3rd chromosome is composed of 4 exons, with two of them present in both female and male isoforms, whilst the third is female-specific, and the fourth is male-specific, thanks to the 3' alternative acceptor (Fig. 2.4.1)

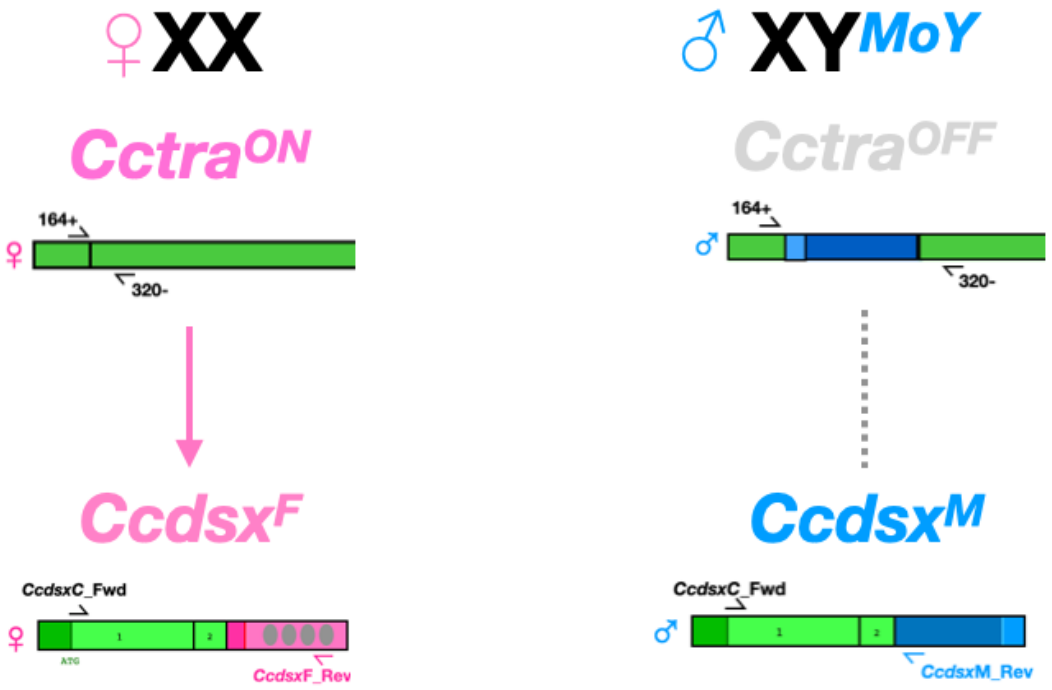

Fig. 2.4.1 - Representation of *Cctransformer* and *Ccdoublesex* and their interaction, highlighting the primer used for the RT-PCR. In females, the *CctraON* isoform results from the splicing out of the male-specific exon and thus in a shorter transcript, using the primers 164+ and 320- both the male-specific (higher) and the female-specific (lower) can be detected on gel electrophoresis. For *Ccdsx*, one common forward primer and two different reverse primers can be used to detect both female and male-specific isoforms in the same sample. Highlighted in grey the TRA/TRA-2 binding sites only present in female-specific exon.

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We aimed to target by CRISPR/Cas9, the first common *Ccdsx* exon, downstream and proximal to the ATG translation starting site. Based on the *D. melanogaster* intersexual phenotypes observed in mutant *dsx* loss of function strains, we expected to observe in the Medfly, in case of evolutionary functional conservation, similar phenotypes as a consequence of *Ccdsx* gene disruption (following the biallelic loss of function mutations induced by CRISPR/Cas9). The *Ccdsx* gene disruption would be provoked by indels in the dimerisation domain (DM). The dimerisation domain is common, so both male and females should be affected by its *loss of function*.
A first CRISPR/Cas9 experiment was carried out on injecting XX generated by the cross fertile XX males (Pane et al., 2002), resulting from an *RNAi-Cctra* at early development stages, and XX *wild-type* females (see Fig. 1.9). After injections of the Cas9 protein complex in embryos, some cells and their descendants will carry no mutations, some other cells and descendants will carry monoallelic mutations, and finally, some others cells and descendants will carry biallelic mutations (Primo *et al*., 2020). For example, targeting of the autosomal *Ccwhite* gene by embryos injection of Cas9 purified protein and *in vitro* synthesised RNA led to biallelic mutations in somatic cells and patches of white eye cells within red ones (Meccariello *et al*. 2017). We expected to observe an intersexual phenotype in the G_0 injected progeny if biallelic mutations were present in the adult fly cells composing those body regions showing sexual dimorphic traits in the head (male-specific spatulated bristles) and the reproductive apparatus.
I also planned to test a different arrangement of the previous Cas9 technique applied in the Medfly to substitute the purified Cas9 protein with a plasmid encoding a Cas9. I have used a plasmid carrying a Cas9 synthetic gene that encodes Cas9 based on *Spodoptera frugiperda* codon usage and controlled by the *Baculovirus* IE1 promoter very active in insect cells (Mabashi-Asazuma and Jarvis 2017). I injected 189 embryos (see Tab.2) with CRISPR/Cas9 complexes targeting the first common *Ccdsx* exon. This led to the development of 5 flies, 3 females and 2 intersexes (Fig 2.4.2), while no effect was observed in XX embryos injected with the buffer .

# Set	Injection Mix	karyotype	Injected embryos	Larvae	Pupae	Adults	# females	#males	#intersexses	% survival
1	Cas9 sg*Ccdsx*	XX	189	12	7	5	3	0	2	6,3
2	Buffer	XX	132	54	46	42	39	0	0	40,9

Table 2 - Injection table in XX-only embryos of CRISPR/Cas9 targeting the *Ccdsx* gene and buffer injection set.

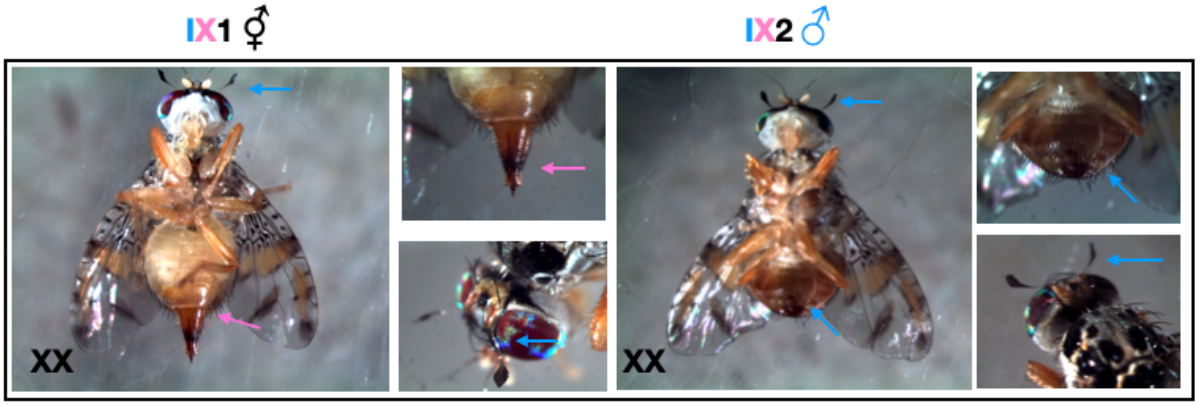

Figure 2.4.2 - Two intersexes developed from CRISPR/Cas9 embryos injection targeting *Ccdsx*. **On the left,** the first intersex shows male trait on the head and female ovipositor at the end of the abdomen. **On the right,** the second intersex shows a more substantial masculinisation effect by presenting male-specific bristles on the head and a not fully formed male-specific apparatus.

Of the two intersexes observed, one XX fly presents male bristles and a male-like apparatus. The male-like apparatus is not fully formed, indicating an almost complete masculinisation. Even though the experiment aimed to introduce insertions or deletion on the genomic target site, I have extracted RNA because I had still to develop a method to extract from the same sample both DNA and RNA. As one of the two intersexes showed an almost complete masculinisation, which was unexpected based on the *Drosophila dsx* loss of function phenotype, I planned to investigate if a change in the splicing pattern of *Ccdsx* and *Cctra*, although also unexpected, was underlying this drastic developmental change.
After RNA extraction, cDNA synthesis and PCR were performed (Fig. 2.4.3).

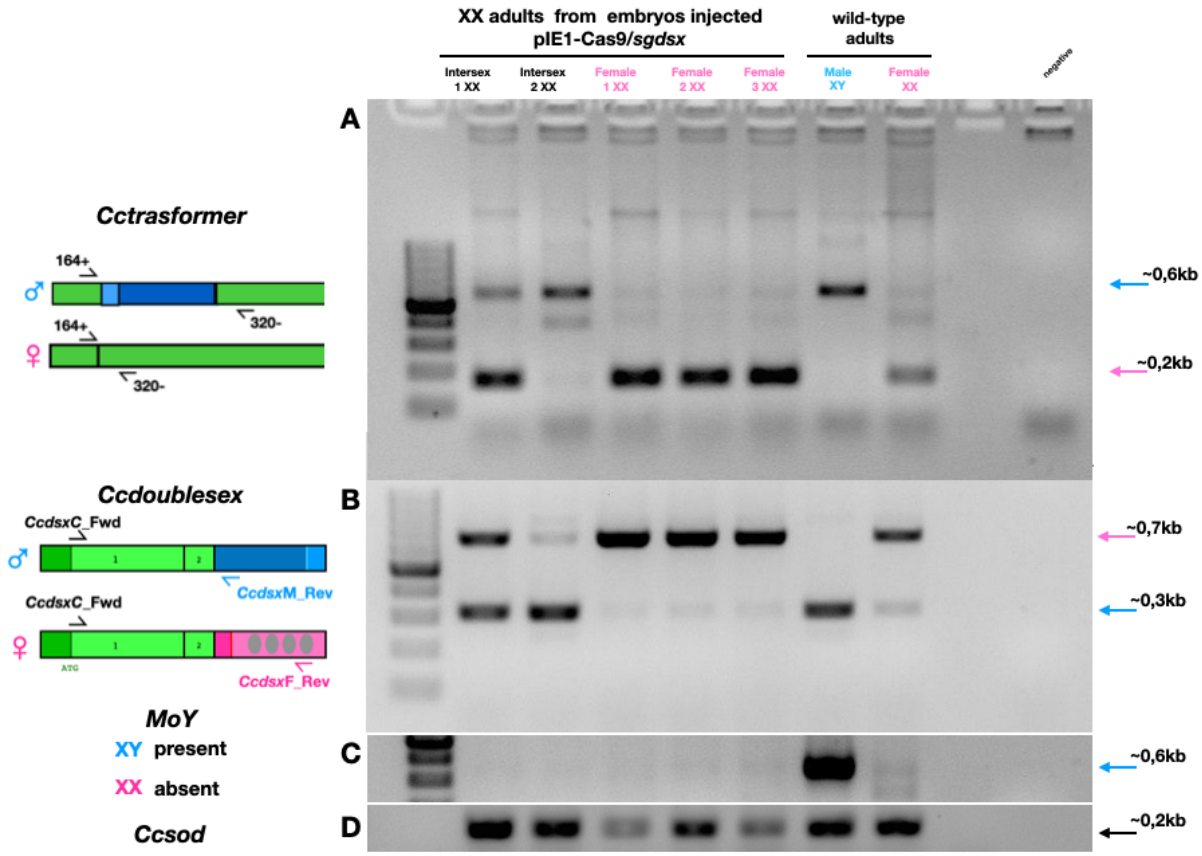

Figure 2.4.3 - RT-PCR on five XX flies from injected XX-only embryos (tab. 2): 1st lane the intersex 1, 2nd lane the intersex 2, third,-fifth lanes XX females, 6th and 7th lanes respectively a *wild-type* male, and female and the 9th lane a no-RNA-template reaction. On the left a graphic representation of *Cctra* and *Ccdsx* sex-specific isoforms and the primers used for their detection. **A)** *Cctransformer*, in the first and second lane, the two XX intersexes show the additional presence of a male-specific *Cctra* transcript, the three XX females show a regular female-specific *Cctra* splicing pattern, as expected based on the normal phenotype; **B)** *Ccdoublesex*, multiplex-RT-PCR (three primers used to amplify both female-specific and male-specific isoforms), show that IX1 and IX2 express both female- and male-specific isoforms, with the last one being more abundant in IX. The three XX females show the expected female-specific Ccdsx isoform, **C)** *YM1* RT-PCR confirms the absence of the Y chromosome in the five analysed flies and in the XX female its presence in the XY male control. **D)** RT-PCR on the housekeeping gene *Ccsuperoxide-dismutase* as a positive control On the right a graphical representation of sex-specific splicing of analysed transcripts.

At first, *housekeeping* gene and *Y-linked* marker analysis ensure cDNA quality, genomic DNA contamination absence and molecular karyotyping. Then, *Cctra* is composed of five exons, the first common exon, the second and the third male-specific exons and the fourth and the fifth common exon. This results in mature mRNA differences in length in male (longer) and female (shorter). Then *Ccdsx* and

Cctra splicing analysis were then carried out by RT-PCR. *Ccdsx* differs in its female and male isoform for its 3' ends, hence to detect both male and female isoform, a multiplex PCR was carried using a forward primer on the first exon (common) and two different reverse primers, one male-specific and one female-specific to detect, in the same PCR reaction, both transcript isoforms.

In XX-only embryos, normally the *Cctra* splicing is ON, and its regulated gene, *Ccdsx* is expressed in a female-specific splicing pattern. The *Ccdsx* gene disruption of the common region should not have altered the *Ccdsx* splicing considering that *Ccdsx* is not a splicing factor and it is not known to be involved in its autoregulation. Its sequence disruption in XX-only flies should have resulted in a female-specific *Ccdsx* isoform bearing indels on the targeted site. However, as shown in fig. 2.4.3, RT-PCR analysis showed that in both intersexes, not only the female-specific isoforms of *Ccdsx* and *Cctra* but also their male-specific ones can be detected. It was unexpected to find a change in the splicing pattern of *Ccdsx* following CRISPR/Cas9. One possibility could be that Cas9 binding to *Ccdsx* locus can interfere with its splicing as it is coupled with transcription. Furthermore it was very unexpected that *Ccdsx* Cas9 targeting also affected *Cctra* splicing, the upstream regulator of *Ccdsx* female-specific splicing. Moreover, mutations induced on the first exon should also be present in the cDNA sequence.
Moreover, out of 7 sequences from the first XX intersex, just one was bearing indels. In the almost fully reverted XX males out of 5 sequences, no one clone showed indels on the targeted site. These results indicate that the *Ccdsx* CRISPR/Cas9 targeting masculinizes XX individuals and affects both *Cctra* and *Ccdsx* splicing, even having a low rate in inducing indels on the target site and hence likely functioning as CRISPR interference.
The *Ccdsx* targeting has led to an almost complete XX female-to-XX male reversion and a *Cctra* splicing pattern shift toward a male-specific one. Thus, a *Ccdsx* targeting was also required to evaluate the effect of *Ccdsx* targeting in XY individuals. I have performed either the CRISPR/Cas9 complex injections or co-injection of Cas9-plasmid and sgRNA to target *Ccdsx* in XX/XY embryos. I repeated these two experiments two times to have them in duplicates (Tab.3).
Two independent experiments have been injected two times in two different ways in XX/XY: as a pre-assembled ribonucleic complex and as plasmid encoding Cas9 protein and *in vitro* synthesised sgRNA (see Tab.3).
The *Ccdsx* target is in a non-sex specific gene region, thus affects both female (XX) and males (XY). Considering results from injection in only XX strain (XX females reverted in intersexes), in this injection set we expected to obtain not only XX intersex and again XX males, but also XY intersexes as *CcdsxM* is expected to be required for male differentiation as in *D. melanogaster*.

# of set	Injection Mix	karyotype	Injected embryos	Larvae	Pupae	Adults	# females	#males	#intersexes	% survival
1	Cas9 sg*Ccdsx*	XX/XY	194	32	13	10	8	2	0	16,5
2	pIE1_Cas9 sg*Ccdsx*	XX/XY	250	83	34	33	14	19	0	33,2
3	Cas9 sg*Ccdsx*	XX/XY	182	69	28	17	4	13	0	37,9
4	pIE1_Cas9 sg*Ccdsx*	XX/XY	111	31	12	9	2	7	0	27,9
5	Cas9	XX/XY	200	101	70	69	-	-	-	50,5
6	sgRNA	XX/XY	150	76	73	82	-	-	-	50,7
7	Buffer	XX/XY	200	105	114	106	-	-	-	52,5
8	N/A	XX/XY	200	122	118	148	-	-	-	61,0

Table 3 - Injection set of Cas9+sg*Ccdsx* (row 1,3) and pCas9+sg*Ccdsx* (row 2,4). Lane #5 injection set performed using the Cas9 protein, lane #6 the injection set using the sgRNA, lane #7 the injection set using the injection buffer and the lane #8 the set not injected but only hand dechorionated. For the controls, the number of females and males was not reported.

All the injection sets performed did not lead to obtaining intersexes. The number of surviving individuals was not sufficient to statistically evaluated the presence of sex ratio distortion. I have extracted DNA and RNA from individual fly to investigate 1) molecularly the karyotype of the flies, 2) the splicing pattern of *Ccdsx* and *Cctra*, 3) clone and sequence the Cas9 targeted *Ccdxsx* region. The first injection set (row #1, table 3) led to 8 females and 2 males.

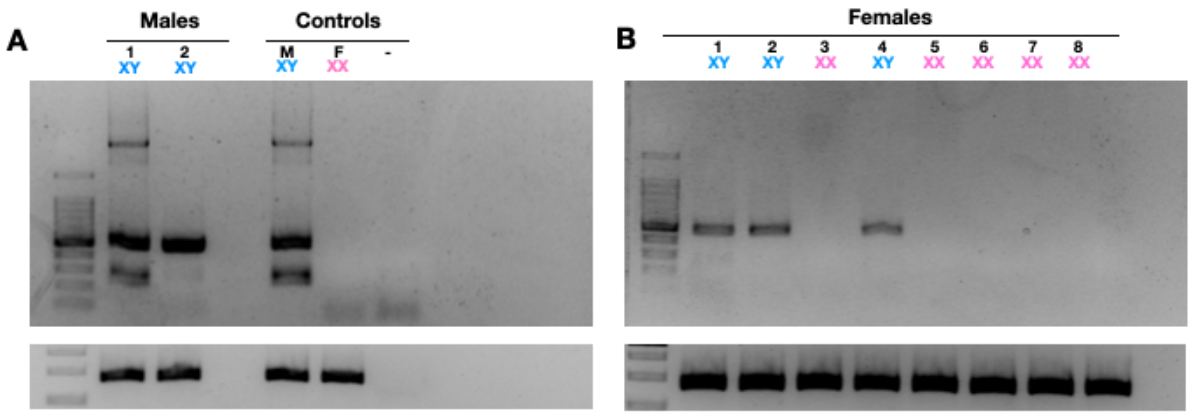

Figure 2.4.6 - Molecular karyotyping using and a housekeeping gene on the #1 injection set individuals **A)** the 2 males, in **B)** the 8 females.

The molecular analyses by PCR of this showed that the two males were normal XY, but unexpectedly, 3 out of 8 females were XY (Fig. 2.4.6, B), suggesting a complete feminization of XY individuals.

The second injection set (row#2, table 3) has led to 14 females and 19 males.

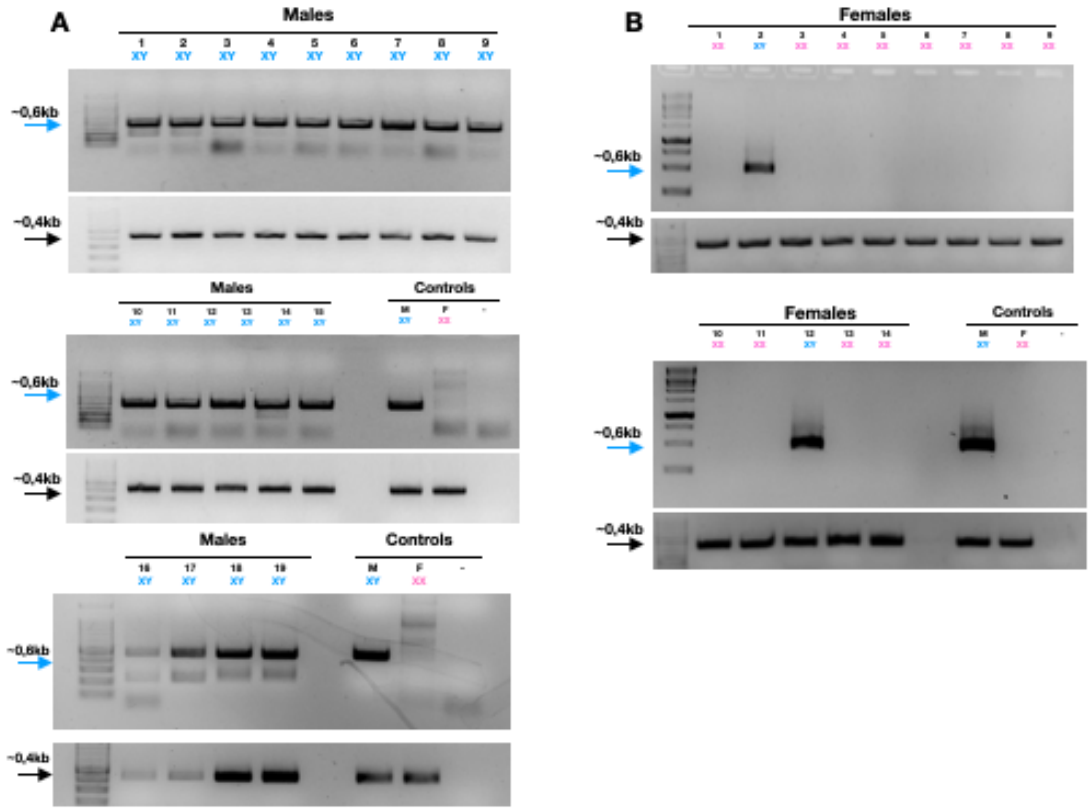

Figure 2.4.7 - Molecular karyotyping using *MoY* (YM1; 0.6Kb)) and a housekeeping gene (0.4 Kb) on the #2 injection set individuals in **A)** the 19 males from the XX/XY embryos injection using the sg*Ccdsx*, in **B)** the 14 females obtained from the XX/XY embryos injection using Cas9/sg*Ccdsx*. Unexpectedly, two out of 14 females were XY.

The PCR on the second injection set has revealed the presence of two reverted XY females, so in this case, a fully male-to-female reversion was achieved. The third injection set (row#3, table 3) resulted in 13 males and 4 females.

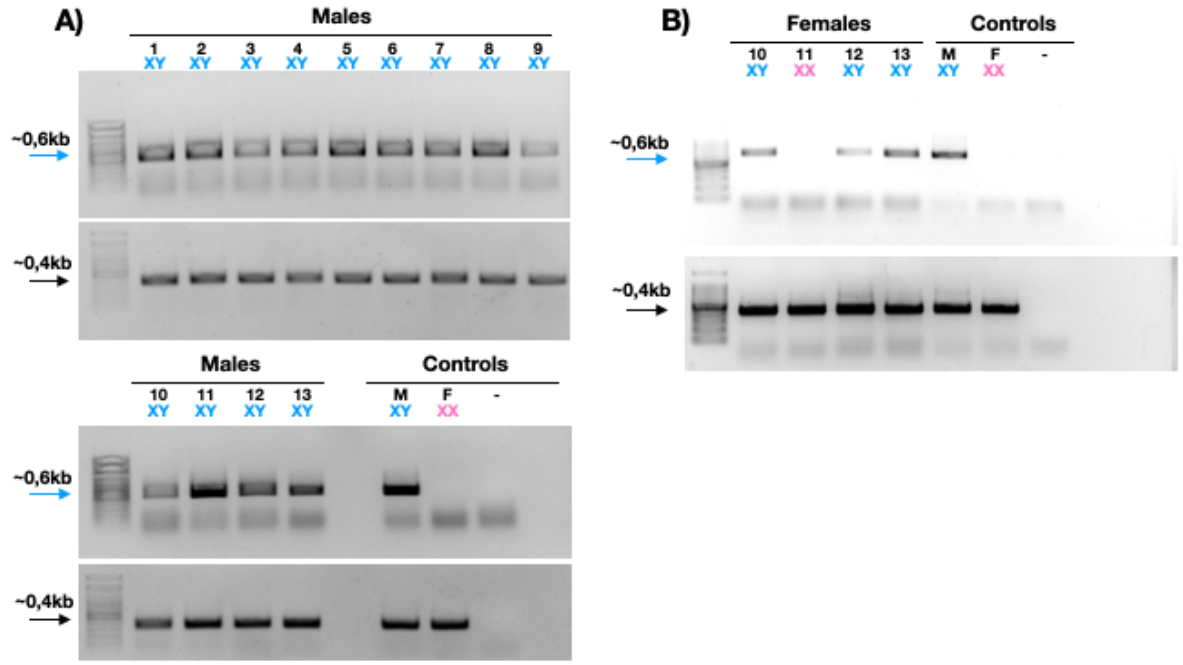

Figure 2.4.8 - Molecular karyotyping using *MoY* (YM1) and a housekeeping gene on the third injection set individuals (row#3, table 3) in **A)** the 13 males, in **B)** the 4 females.

The fourth injection set (row#4, table 3) resulted in 7 males and 2 females. The fourth injection set has led to the detection of 3 out of 4 reverted XY females.

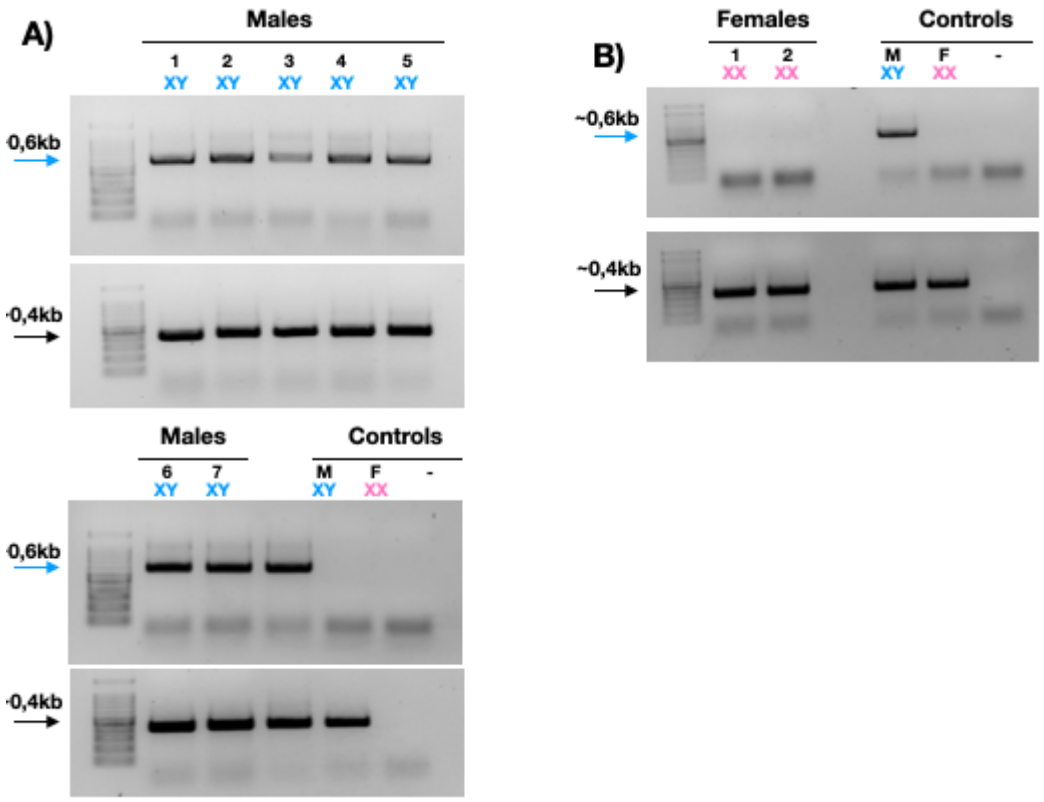

Figure 2.4.9 - Molecular karyotyping using and a housekeeping gene on the third injection set individuals in **A)** the 7 males, in **B)** the 2 females.

In brief, in three out of four embryonic injection sets (Tab. 3), using either a plasmid encoding the Cas9 protein or a purified Cas9 protein to target *Ccdsx*, I observed some XY fully reverted females. It is not clear why we failed to observe again XX partially masculinized individuals in the 4 sets of XX/XY injected embryos experiments as in the previous set of injections into XX only embryos. We are presently repeating the Cas9-sgRNA-*Ccdsx* targeting in XX only embryos and confirmining the previous data with more replicates. We expected that the XY reverted females have extensive biallelic mutations in the *Ccdsx* gene. Furthermore, considering the data collected from the 4 sets of injections in XX/XY embryos we speculated that in contrast to *D. melanogaster* the *Ccdsx* gene in *C. capitata* has a major function in XY individuals rather than in both sexes.
Preliminary data from cloning and sequencing of the targeted region, revealed only low frequency of Cas9-induced mutations (data not shown, as in progress), suggesting that 1) the chosen *Ccdsx* sgRNA is not efficient, 2) it is likely sufficient a CRISPR/Cas9 interference to affect *Ccdsx* function.
Thus, the 7 females from injection set #1, #2 and #3 were processed. RNA was extracted along with the DNA samples using the TRIzol™. Different RT-PCRs were performed for the detection of *Cctra* splicing and *Ccdsx* splicing. For *Cctra* splicing, we used two primers which annealed on the first common and the second common exon. The application of this primer pair led to the detection of both females and

males *Cctra* splicing. In males, two longer amplicons were present. In females, one small amplicon and an unspliced amplicon can be detected. For the *Ccdsx* splicing, I used a multiplex RT-PCR using one forward primer and two reverse primers. The forward primer anneals on the common *doublesex* region are present in both males and females, while the two reverse primers are for the male-specific portion and one for the female-specific portion. I have also amplified the *MoY* region to confirm that *MoY* is either weakly or not expressed in XY females.

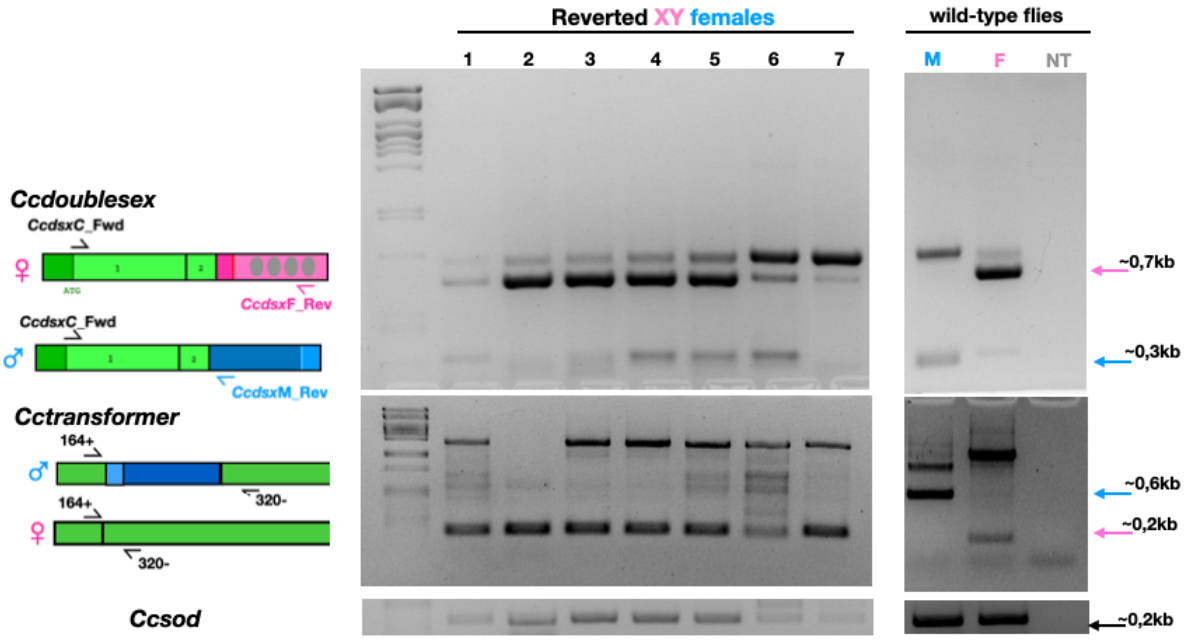

Figure 2.4.10 - RT-PCR on RNA samples of 7 XY reverted *Ccdsx is* targeting females. In A) RT-PCR for *Cctra* splicing, in B) RT-PCR for *Ccdsx* splicing, in C) RT-PCR on *MoY* gene and in D) RT-PCR on *CcSOD*, a housekeeping gene.

RT-PCR reactions of *Ccdsx* have shown that in wild type males and females either a female-specific expected 0.6 Kb long cDNA fragments in XX and a male-specific expected 0.2 Kb one in XY are present. The 0.9 Kb long band in XY could be likely due to intermediate splicing or an uncharacterized isoform of *Ccdsx*.
In all the seven reverted XY females, the female-specific 0.2 Kb long band is present, with only four XY females showing a weak male-specific *Ccdsx* band and three no one. These data indicate that the reverted XY individuals are feminized at least partially also at the molecular level of *Ccdsx*.
RT-PCR reactions of *Cctra* have shown that in wild type males and females either a female-specific expected 0.2 Kb long cDNA fragments in XX and a male-specific expected 0.6 Kb one in XY are present. Longer cDNA bands could be likely due to intermediate splicing, unspliced products or an uncharacterized isoform of *Cctra*. Again, all seven XY females showed a mostly female-specific *Cctra* splicing pattern with only a very weak male-specific *Cctra* band in a few individuals.

An unexpected high mortality rate was observed when the CRISPR/Cas9 complex targeting the *Ceratitis capitata doublesex* was injected (Table 5).

As a control to investigate if there is a genetic variability of *Ccdsx* and *Cctra* splicing patterns in the flies we reared even in the absence of treatments, I performed RT-PCR of *Ccdsx* and *Cctra* on 17 XY males and 16 XY females, hatched from the sample strain used for the experiments and the same petri dish of pupae. The XX and XY flies were reach sexual maturity 2 days and left to mate for 1 day before process them to extract RNA and DNA.

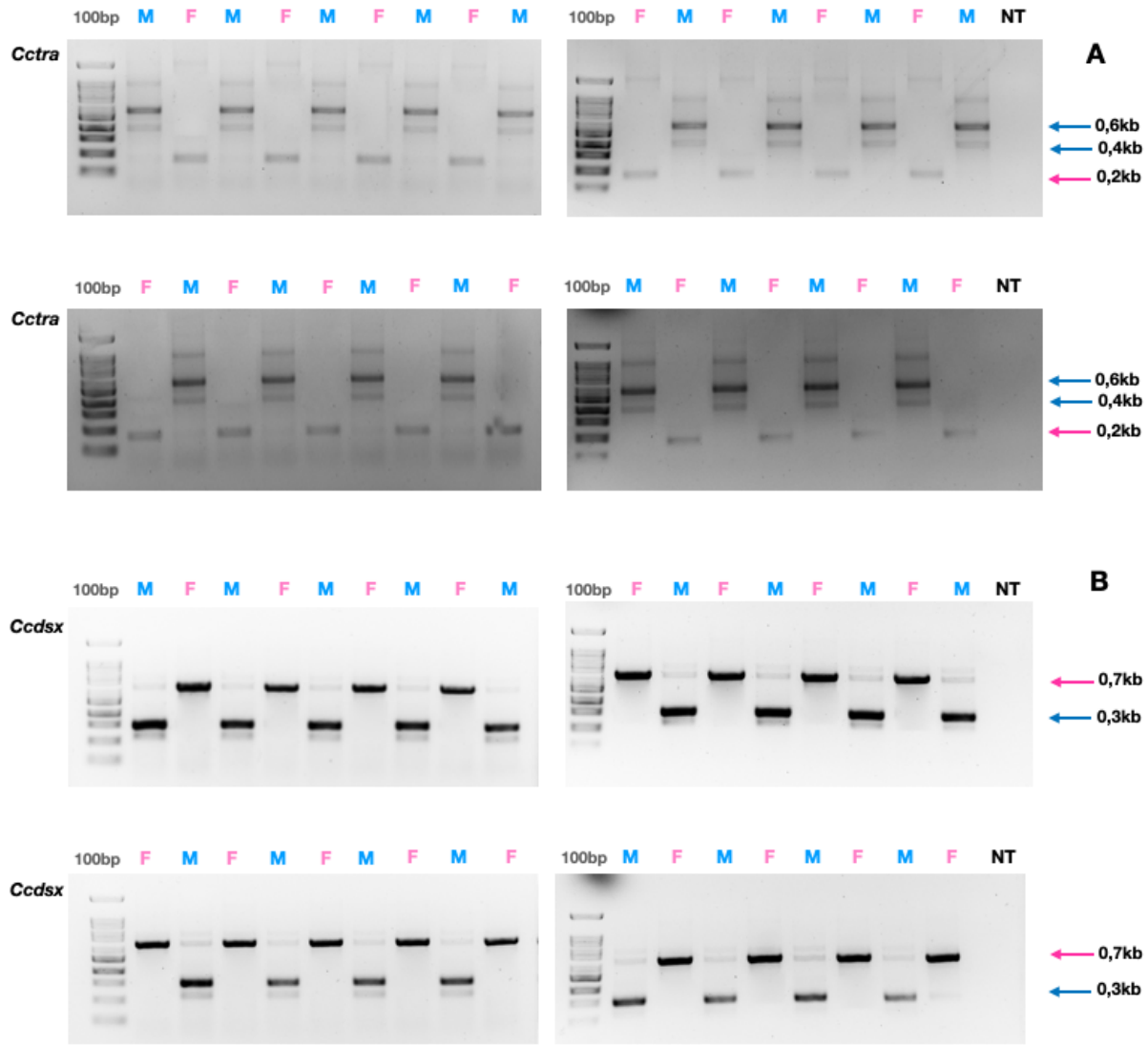

Figure 2.4.15 - RT-PCR analysis on wild-type XY males and wild-type XX females, likely mated. In A) and ijn B) RT-PCR of respectively *Cctra* and *Ccdsx* on individual adult flies. In the 17 XY males only male-specific and in the 16 XX females only female-specific splicing patterns are detected for each gene.

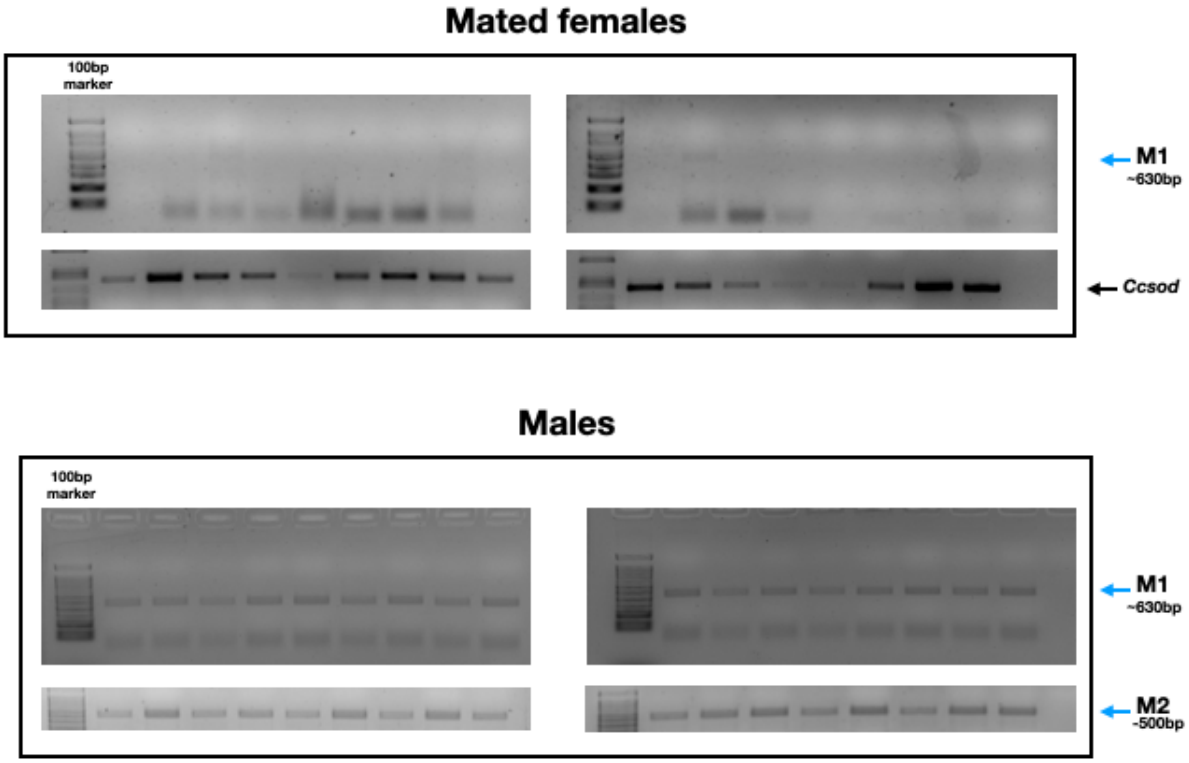

Figure 2.4.16 - On the top, molecular karyotyping of 17 mated females, in which both an autosomal and a Y-linked marker are shown. On the bottom, molecular karyotyping of 17 males, using two different Y-linked markers.

I also planned to control if in the detection of XY females in the previous experiments, was due to misinterpretation of the data. I investigated if the Y-derived amplicon detected from these adult reverted XY females might be the result on the contrary of XX females having in their spermatheca, sperms bearing the Y chromosome. Thus, gDNA analysis of *Y-marked* amplification on mated XX females was necessary to clarify this point. The presence of the Y-linked amplicon was detected only in XY males but not in XX females, left in the petri dish to mate (**Fig. 2.4.16)**.

We concluded that the Cas9-sgRNA targeting of *Ccdsx* affected consistently XY individuals phenotype leading to sexual reversion into XY females, feminized *Ccdsx* and *Cctra* splicing pattern. This effect is likely due to CRISPR interference rather that gene disruption. Furthermore the change in the splicing pattern of *Cctra*, upstream regulator of *Ccdsx* suggest that this second gene is required during embryogenesis in XY for male-specific splicing of *Cctra* and masculinization induced by *MoY* from the Y chromosome. *MoY* is transiently expressed during embryogenesis and its masculinization activity can be overcome by the Cas9 transient interference targeting *Ccdsx*, which likely is necessary together with *MoY* to block the feminization activity of the maternal *Cctra* input used to determine female sex in XX embryos (Pane et al., 2002; Meccariello et al., 2019). The use of dead Cas9 to target *Ccdsx* is underway to confirm this model

Cas9 targeting of dsx in D. melanogaster

We have asked if also in *D. melanogaster*, a CRISPR/Cas9 mediated targeting of *dsx* orthologue would lead to similar drastic sexual reversions, instead of, or together with, intersexual phenotypes. I injected the Cas9 purified protein pre-assembled with an *in vitro* synthesised sgRNA twice, and one more injection set was performed a plasmid coding for a functional Cas9 protein along with the same as previous sgRNA (see Tab.6).

# set	Injection Mix	Karyotype	Injected embryos	Adults	Females	Males	Intersex	%survival
1	Cas9 protein/ sg*Dmdsx*	XX/XY	160	39	26	13	/	24,4
2	pIE1_Cas9/ sg*Dmdsx*	XX/XY	80	18	15	6	/	22,5
3	Cas9 protein/ sg*Dmdsx*	XX/XY	80	12	4	7	1	15,0
4	Buffer	XX/XY	120	53	28	25	/	44,2
5	Cas9	XX/XY	150	61	28	31	/	40,7
6	sgRNA	XX/XY	110	49	26	23	/	44,5

Table 3 - Injection sets of the pre-assembled complex (row 1 and 3), plasmid encoding the Cas9 protein and sgRNA (row 2), the injection buffer (row 4), the Cas9 only (row 5) and the sgRNA (row 6) and the plasmid encoding a functional Cas9 protein and the in vitro synthesis sg*dsx*.

The expected phenotype for a *Drosophila dsx* targeting was an intersex individual. In this experiment, just one intersex individual hatched in the second set, whilst no intersexes individual hatched from the first and third set, but a solid female bias can be detected. So the double amount of females seen in the first two sets of injections, can be justified in a male-to-female reversion due to a *doublesex* disruption. To this aim, both DNA and RNA were extracted from a single adult, and then PCR for a *housekeeping* gene and a Y-linked molecular marker (Dimitri et al. 2009) were performed.

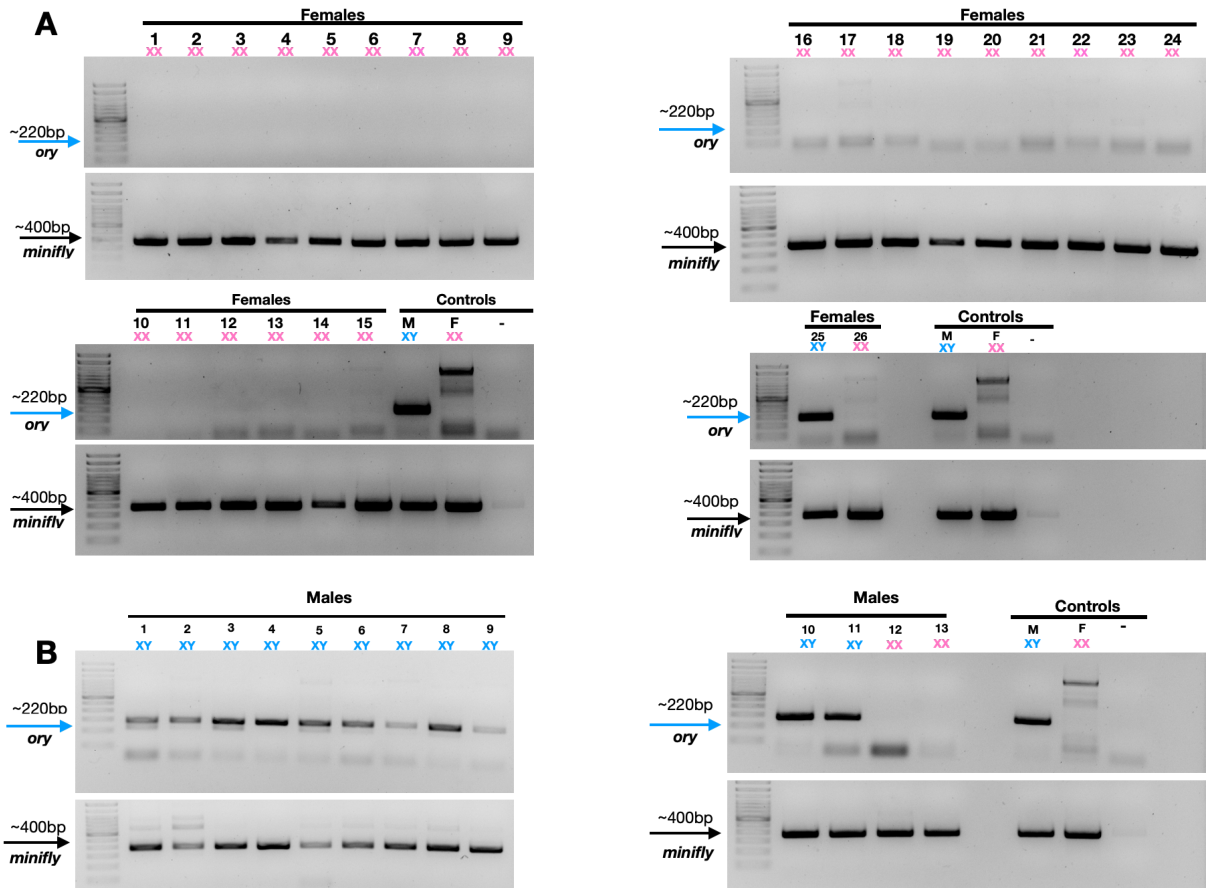

Figure 2.4.11 - PCR on genomic DNA of the 26 females (A) and from 13 males (B) from the first set (Cas9 purified protein + sg*dsx*). In the upper gel, the molecular Y-linked marker, whilst in the lower gel, the *housekeeping* autosomal minifly gene.

Out of 26 females, one resulted to be positive for the Y-linked marker and hence a XY reverted individual (n. 25; Fig. **Fig. 2.4.11**). Out of 13 males, 2 resulted in an XX reverted males.

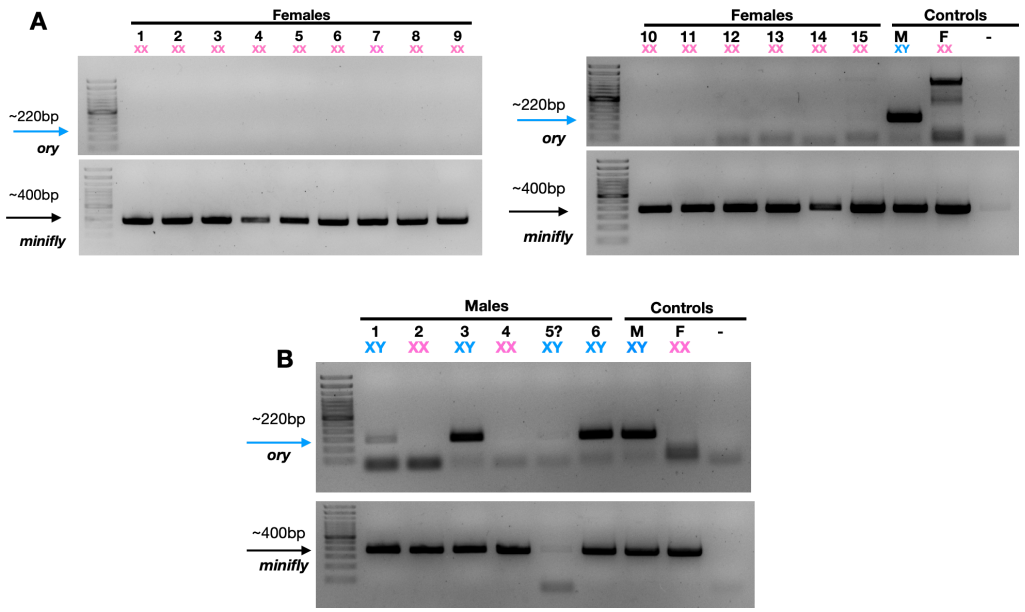

Figure 2.4.12 - PCR on genomic DNA on *ory* (the molecular Y linked marker, indicated using a blue arrow) and *minifly* (an autosomal housekeeping gene, indicated using a black arrow) of the 15 females (**A**) and 6 males (**B**) from the second set (pIE1_Cas9 + sg*dsx*).

In the second set of injections, 2 out of 6 males resulted XX reverted males. The male n. 5 presents a question mark because the *minifly* gene has a mild signal as the Y-linked marker. For this reason, I deduce that the mild amplification is due to a not proper conservation of the sample. On the other hand male number 2 and male nuber 4 have no *ory* amplification, indicating their XX karyotype (n.4 and n.2, Fig. 2.4.12, B).

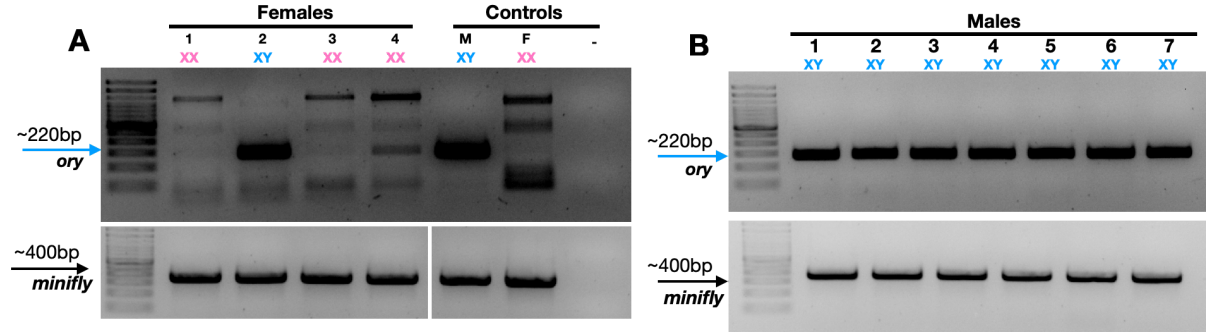

Figure 2.4.13 - PCR on genomic DNA of the 4 females (**A**) and 7 males (**B**) from the third set (pIE1_Cas9 + sg*dsx*). In the upper gel, the molecular Y-linked marker, whilst in the lower gel, the *housekeeping minifly* gene.

In the third injection set, 2 out of 4 females has Y-band.
Also, the intersex was karyotyped and resulted to be an XY intersex.

Preliminary data on cloning and sequencing of the *dsx* Cas9 targeted region, revealed that low mutation rate is observed and that biallelic extensive mutations are not the reason of the sexual reversion. Instead, CRISPR/cas9 transient interference is likely the mechanism of action again as we speculated in *C. capitata* experiments.

Similarly to my *C. capitata Ccdsx* study, I performed also RNA analyses of *dsx*, *tra* but also of *Sxl* and *msl-2* genes, respectively the master gene for sex determination, active in XX females and a key gene for dosage compensation, active in XY males. *Sxl* is responsible in XX females to directly control the female-specific splicing of *tra*, activating it, but also of *msl-2*, repressing it. In *D. melanogaster*, in contrast to *C. capitata*, tra gene has a non-sex-specific splicing and a female-specific splicing (**Fig. 2.4.14**: 0.4 Kb and 0.2 Kb). For RNA analysis, RT-PCR reactions were performed. Since both *Sxl* and *tra* have an exon-skipping alternative splicing mechanism, the RT-PCRs were performed using two primes, both annealings on exons flanking the male-specific exon, expecting one long amplicon for the males and one smaller amplicon for females. For RT-PCR on *dsx*, a multiplex RT-PCR was performed to amplify the male and the female-specific isoform as previously done for *Ceratitis*. Moreover, considering we observed an higher number of XX individuals than XY in two sets, an hypothesis of a male-biased lethality linked potentially to dosage compensation needs to be considered. For this reason, an RT-PCR on *msl-2* can clarify if the reverted individuals have a functional or non-functional *msl-2* isoform.

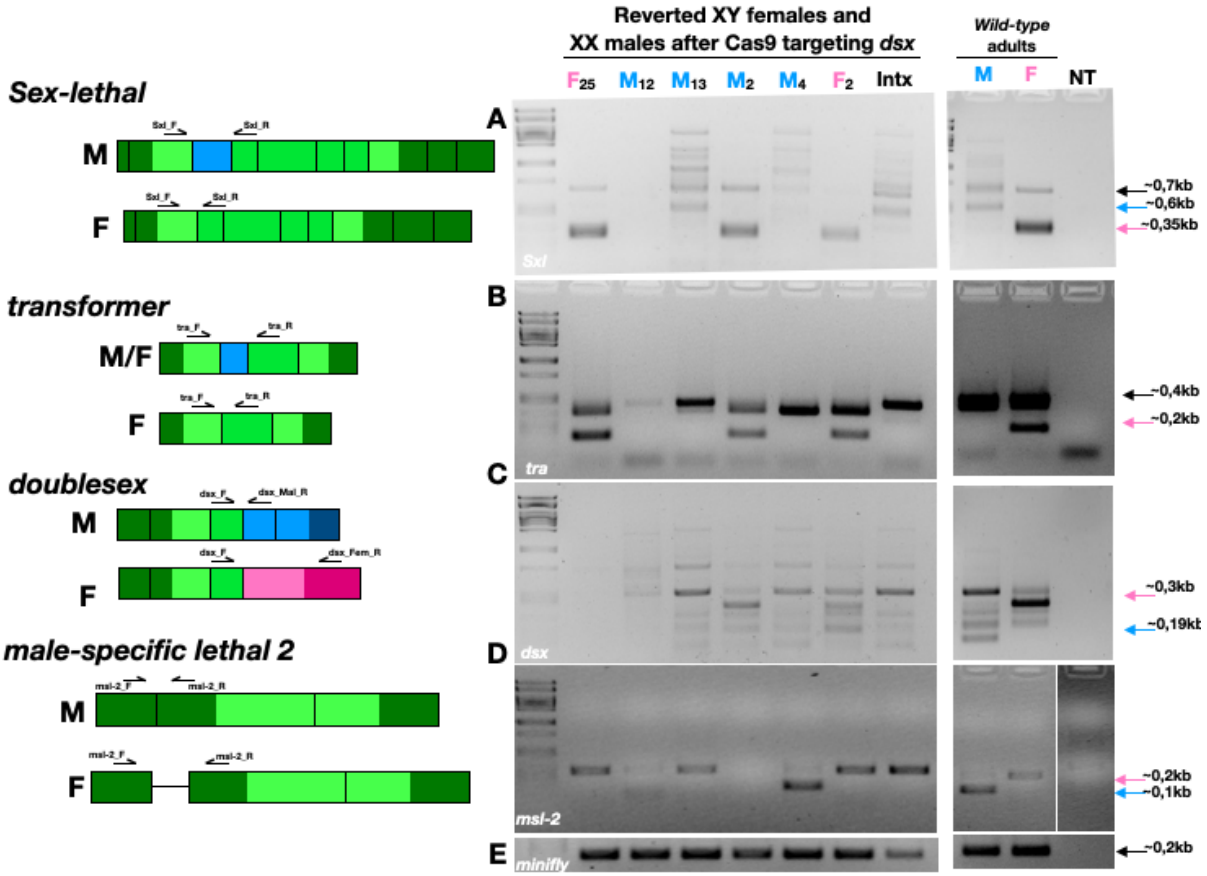

Figure 2.4.14 - RT-PCR on reverted *Drosophila* adults targeted in dsx second common exon using CRISPR/Cas9. in **A**) RT-PCR on *Sxl* gene, in **B**) RT-PCR on *dsx* gene, in **C**) RT-PCR on *msl-2* gene, in **D**) RT-PCR on *tra* gene and in **E**) RT-PCR on *minifly*, a housekeeping gene.

As we have seen previously, the CRISPR/Cas9 targeting the second common *dsx* region resulted in both a complete female-to-male or male-to-female reversion. I performed RT-PCR of 4 genes on one intersex, two reverted XY females and three reverted XX males (**Fig. 2.4.14**). The XY females n. 25 and n. 2 show female-specific *tra* and Sxl splicing patterns but a male-specific *msl-2* splicing pattern (so active *msl-2* and dosage compensation ON, compatible with XY alive individual).
The PCR reaction failed to amplify the *dsx* transcript in the female XY n. 25 but intersexual *dsx* splicing pattern was detected in the second XY female n.2. The female phenotype of XY individuals is consistent with the splicing pattern shift in *tra* and *Sxl* splicing, unexpectedly observed also in *D. melanogaster* as in *C. capitata*. This data indicates that *dsx* is required in XY individuals to maintain *tra* non sex-specific splicing and *Sxl* male-specific (non functional splicing) and hence masculinization.
Three out of four XX males (M12, M13 and M4; in **Fig. 2.4.14**) showed a male-specific pattern of *Sxl*, *tra* and *dsx,* coherent with their sexual reversion. However, again the msl-2 gene showed a pattern not always coherent with the vitality of the flies. For example male XX n. 4 showed male-specific hence functional *msl-2*, which should lead to hypertrascription of the two XX and lethality, while males XX n. 12 and 13 show a female-specific *msl-2* splicing pattern indicating no dosage compensation, which is compatible with XX individuals alive. A more complex study is required to confirm and extend these data toward understanding these unexpected phenotypes and splicing patterns related to *msl-2* and dosage compensation.
The data indicate that a transient Cas9 targeting of *dsx* in *Drosophila* embryos affected also the XX individuals leading to their masculinization at the phenotypic and molecular levels. We propose that *dsx* is required in XX to maintain not only tra female-specific splicing but also and mainly *Sxl* female-specific splicing by a mechanism which has to be still clarified.
Compared to *C. capitata* where we have found *Ccdsx* (*CcdsxM* isoform likely) involved in XY in the control of male-specific *Cctra* splicing possibly together with *MoY*, in *D. melanogaster dsx* seems to be involved in both sexes in sending feedback signals to maintain the sex-specific splicing not only of *tra* but also of *Sxl*.

2.5 *Bactrocera oleae* autosomal *white-eye* gene targeting

In 2017, Meccariello et al., have demonstrated that the efficiency of gene targeting can reach up to 99% on both alleles in germ-line while targeting the autosomal orthologue of the white-eye gene in *Ceratitis capitata*. Thus, the CRISPR/Cas9 targeting of the *white-eye* gene was also performed in *Bactrocera oleae*, another Tephritidae close to Ceratitis. My contributions to this work were the establishment of rearing and injection methods for *Bactrocera oleae* in our lab.

See Attachment:

Targeted somatic mutagenesis through CRISPR/Cas9 ribonucleoprotein complexes in the olive fruit fly, *Bactrocera oleae*

3. Discussion

Experimental programs in *Ceratitis capitata* have been carried out since the ‘80s. Despite the vast numbers of publications and findings in this insect, some molecular pathways are still unknown. Clarifying its sex determination pathway represents one of the most challenging aims because of the complexity of gene interactions. Above it, the functional, genetic and transgenic studies aim to identify crucial sex determination genes and molecular or phenotypic marker. In 2017, the CRISPR/Cas9 technique was successfully applied to target the autosomal orthologue of the *white-eye* gene in *Ceratitis capitata* resulting in somatic mosaicism of the eyes. Furthermore, the CRISPR^{ant} were crossed with *we*$^{-}$/*we*$^{-}$ flies, resulting in about 99% mutational rate in the germline. Demonstrating that both copies of the autosomal *white-eye* gene were targeted in the germline (Meccariello *et al*., 2017). In 2017, the CRISPR/Cas9 technique based on purified Cas9 protein complexed with the sgRNA was established, in Ceratitis. Furthermore, the know-how was transferred to the target of the *white-eye* gene in *Bactrocera oleae*, the olive fly, phylogenetically close to *Ceratitis*. The CRISPR/CAs9 targeting the white-eye gene in *Bactrocera oleae* resulted, in some cases, in a complete eye pigmentation loss (Meccariello *et al*. 2020). *Ceratitis capitata* resulted in a cost-effective, easy reading and suitable for transgenic and gene-editing experiments and these reasons might be interesting considering Ceratitis capitata a model organism for the *Tephritidae* family. Nevertheless, the *Maleness-on-the-Y* gene has been identified and resulted in an intronless single-copy y-linked gene encoding for a 70aa basic and positively charged peptide, but its interactors and molecular mechanism are still unknown. RNAi and CRISPR/Cas9 induced disruption has been used to show that *MoY* genetic locus is necessary for male sex determination. The bioinformatic analysis revealed no similarity with known protein or domain but orthologues found in the

Bactrocera genera. Also, in *Bactrocera*, the *MoY* orthologues are Y-linked and *Bactrocera dorsalis* and *Bactrocera oleae* RNAi target the *MoY* gene during the early embryonic stage,, resulted in feminisation of the XY individuals. Injections of DNA fragment of MoY demonstrated that it is sufficient for male sex determination. Furthermore, MOY recombinant protein has been demonstrated to be capable of partial female-to-male reversion but failed to revert female-to-male completely. Different possible scenarios can explain this phenomenon: MOY protein concentration (70ngr/μL) was not enough, in terms of quantity, to complete revert females in males; *in vivo* MOY protein might carry post-translational modifications not added during the *in vitro* synthesis; *in vivo* MOY protein might act as an oligomeric complex whilst *in vitro* synthesised MOY is monomeric; MOY protein may interact with other different products in the 5kb region capable of completing reversion females to males. Nevertheless, the BdMOY and BoMOY orthologues were cloned and recombinantly expressed in *the E.coli* strain. Once injected the BdMOY, but not BoMOY, has a mild effect in reverting the molecular *Cctra* splicing from female-to-male. New questions are arising from the sex development pathway.
Thus we aimed to disrupt both *Cctra* and *Ccdsx* using the CRISPR/Cas9 strategy. Both purified His-tagged Cas9 protein or a plasmid encoding for Cas9 protein under the control of a constitutive promoter (pIE1_Cas9) (Mabashi-Asazuma and Jarvis 2017) and a single-guide RNA synthesised *in vitro* are used.
The targeting of *Cctra* revealed an unexpected CRISPR interference with reversion of XX individuals into males in the absence of mutations (Primo al al. 2020).
Based on our knowledge, in *Ceratitis capitata,* the hierarchy of genes involved in sex determination is as follow: in XY *MoY*>*CctraOFF*>*CcdsxM* and in XX *CctraF* maternal>*CctraON*>*CcdsxF* The *Ceratitis capitata* sex determination is based on the ON/OFF switch of genes involved in this molecular pathway. In females, the *Cctra* isoform is “ON" because of its capability to encode a functional CcTRA protein, whilst, in males, the *Cctra* isoforms are “OFF” because they both encode truncated proteins that seem to be not functional (M1 and M2). On the contrary, both *Ccdsx* isoforms encode a functional protein but different for their C-terminal, thus regulating different target genes. *Cctra* is a splicing factor, acting as a splicing silencer when it binds its pre-mRNA, or splicing enhancer, when it interacts with the *Ccdsx* pre-mRNA (Pane et al., 2002; Saccone et al., 2008). In detail, the functional CcTRA protein binds its own pre-mRNA and, by blocking the male-specific splicing sites, splicing it in a female-specific manner. On the other hand, when CcTRA binds the *Ccdsx* pre-mRNA, it enhances the inclusion of the female-specific exon in the mature transcript. Thus, *Cctra* is the master gene regulator for female sexual differentiation, triggered by a maternal *Cctra*. The *Ccdsx* is a transcriptional factor able to activate or block the transcription of its target genes. The female-specific *doublesex* isoform blocks the transcription of genes involved in male sex determination and activates the ones involved in female sex determination. On the contrary, the male-specific *doublesex* isoform activates the genes involved in the male determination pathway and blocks the ones involved in the female differentiation program. After *Ccdsx* targeting there was an unexpected shift (from female-to-male) of both *doublesex* and

transformer. The splicing aberration was unexpected, considering that the targeted gene is a transcriptional factor. It seems that *doublesex* does not just activate or inactivate the splicing of downstream genes, but it might also regulate, probably, indirectly the splicing of upstream genes. Moreover, when the CRISPR/Cas9 complex targets the *doublesex* gene injected in *Ceratitis capita* a high mortality rate can be observed. This suggests a possible involvement of the *doublesex* gene in some vital function. Even thought, the lethality observed in *Ceratitis capitata* seems to be sex-biased (the low number of sample didn't allow a proper statistical test), I speculate that doublesex can be involved in the dosage compensation process, not yet demonstrated in *Ceratitis* capitata. This speculation is supported by a rather astonishing result from the analysis of the *msl-2* splicing in XX males and XY females in *Drosophila melanogaster* flies injected using the CRISPR/Cas9 complex targeting the *doublesex* gene in the same region chosen for *Ceratitis capitata*. In *Drosophila* CRISPR^{ant} flies, some XX males have female-specific *msl-2* splicing that should not be compatible with life, whilst some of the XY females have male-specific *msl-2* splicing, also, in this case, should be not compatible with life. Further experiments will aim to further confirm this new model in which *doublesex* has a crucial role in the regulation of gene upstream. We planned to inject in sexed Ceratitis capitata strain to ensure the proper karyotyping not only molecularly but also phenotypically, considering that a *Ceratitis capitata* strain allows to identify XY individuals and XX individuals based on pupal color (XY individuals have a brown pupae, whilst XX individuals have a white pupae). We planned to target a second sequence in the same *dsx* region to double check that the targeting of this region is employed also in splicing regulation. Moreover, since we speculate that the *CcdsxM* and *CcdsxF* have a crucial role in feedback respectively the maleness and femaleness pathway, we planned to target the male-specific isoform in XX/XY embryos and in XX-only embryos. For *Drosophila*, the *doublesex* targeting should be carried in only females progeny strain. Presently, we are using the *Drosophila white-eye* gene to sex females and males. Crossing white eyed female (X^{we-}/X^{we-}) and red eyed males (X^{we+}/Y) the progeny will result in a red eyed females and white eyed males. This cross will led the overcome of the molecular PCR karyotyping avoiding potential mis-interpretation of the data because of only PCR-based analysis. Moreover, only females progeny strains are present for *Drosophila*, so we plan also to use this strain to replicates the experiment performed in XX-only embryos in *Ceratitis capitata*. And as for *Ceratitis*, we planned to target the same region using a different target sequence and to target the male-specific and the female-specific *dsx* isoform.

4. Conclusion

Ceratitis capitata represents one of the most suitable insects for evolutionary and functional studies nowadays. This insect can be reared and injected easily. Moreover, the established protocol and the gene-editing techniques lay the foundations for establishing this insect as a model organism for the significant

agricultural pest insects. Moreover, since the *Ceratitis capitata* sex determination has been well studied during the years, the knowledge for a better understanding of this pathway and the development of new strategies fighting the agricultural pest insects can be found in this insect, a fertile ground for improvement. In 1988, the sex determination pathway was clarified, establishing a model in which master genes instruct the slave because of their epistatic interactions. The top of the cascade, the master genes, seems to not be well conserved in the insect taxon. On the other end, the “slave genes”, the ones at the bottom of the cascade, such as *doublesex*, seem to be well conserved in most insects, and P. Graham explained this concept in 2003. Future studies should aim to clarify the implication of the *doublesex* gene also in the dosage compensation process. This recent finding may enlighten the road for a novel concept in which the sex determination pathway should be considered not only as an epistatic interaction between the genes involved, but should also considering the influence of the “slave” (*dsx* and *Ccdsx*) upon “masters”(*Sxl* and Cctra), in other words, the sex determination seems not to be a dictatorship, but a democracy.

5. Materials and Methods

5.1 *Ceratitis capitata* strains

The strains used in this study are as follows: 1) Benakeion (developed by P. A. Mourikis, *Benakeion* Institute of Phytopathology, Athens, Greece) 2) Transgenic flies carrying a dsRNA transmitted maternally. The flies were reared under laboratory standards conditions, *i.e.* 50% humidity, 25ºC and 12h light-dark cycle. Adults were fed using a 1:2 sucrose/yeast powder compound, whilst larval food was made by 30 gr paper, 400 mL dH_2O, 2 mL HCl (C_i=2%), 10 mL Cholesterol (C_i= 2,5%), 7,5 mL Benzoic acid (C_i= 4% pH=2.8), 30 gr yeast powder (Piccioni lab) and 30 gr sucrose. Then pupae were collected in petri dishes until enclosure.

5.2 Embryos collection and microinjection

The injection slide is prepared as follows: liquid glue (prepared dissolving double tape Scotch® in heptane) and a copper yarn. Embryos were collected in 100 mL trays, 0-45 min after egg laying (AEL). Once collected, embryos were hand dechorionated using surgery and aligned on an injection slide. Once aligned, they are dried in a petri dish containing $CaCl_2$ crystals for 2-3 min. Then they are covered using a tiny Halocarbon 700 by Sigma Aldrich layer. The needle is prepared by

pulling a boride silicate capillary using an NP-7 Narishige puller. The needle is then set on a nitrogen pump.

5.3 sgRNA synthesis and injection mix

The single guide RNA is in vitro transcribed using Ambion® MegaScript according to manufacturer instructions. As a template, we use DNA fragment from a PCR reaction performed using two overlapping primers, reported as follow:
Primer forward:
5'_GAAATTAATACGACTCACTATAGG**[CRISPR20nt]**gttttagagctagaaatagc_3'

sg*Ccdsx*: TCAAAGGCCGACGCATGTGG
sg*Dmdsx*: TCAAAGAACGACGTCTGTGG

Reverse forward:
5'_AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATT TTAACTTgctatttctagctctaaaac_3'

Once transcribed, sgRNA quantitative and qualitative analysis was performed using Nanodrop from Applied Biosystem ® and gel electrophoresis on a 2X MOPS gel. A 200 ngr/µL sg*dsx*, 1,8µgr/µL Cas9 encoding plasmid (kindly provided by dr. D. Jarvis) and 300mM KCl was then loaded in the ready-to-inject needle. The plasmid was purified using basic lysis, then phenol/chloroform purification.

5.3 DNA and RNA extraction

DNA has been extracted by Holmes-Bonner protocol (1988) or by TRIzol® RNA was extracted from the sample using TRIzol® according to manufacturer instruction and retro-transcribed using an M-MLV based protocol and Oligo-dT primers from Promega®. For 8h AEL embryos RNA extraction, the embryos were removed from the injection slide using Heptane (Sigma-Aldrich). Once detached, the embryos were homogenised using a mortal and RNA extraction was performed according to the manufacturer's instruction.

5.4 cDNA synthesis and PCR analysis

RNA quality was ensured on electrophoresis 1% agarose gel. cDNA was then synthesized using Lunascript (NEB®), an oligo-dT reverse transcriptase based protocol. RT-PCR for indels detection (*Ccdsx*CommFor/*Ccdsx*CommR), *Cctra* splicing(164+/320-), *Ccdsx* splicing (*Ccdsx*CommFor /*Ccdsx*MaleR+*Ccdsx*FemaleR), *MoY* (MoY_F/MoY_R, named YM1) (MoY_F2/MoY_R3, named YM1) for Y detection and *Ccsod* (SOD+/SOD-) were performed (for primer list see the Primo et al., 2021 and Meccariello et al., 2019).

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SHORT COMMUNICATION

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Targeted somatic mutagenesis through CRISPR/Cas9 ribonucleoprotein complexes in the olive fruit fly, *Bactrocera oleae*

Angela Meccariello[1,2] | **Konstantina T. Tsoumani**[3] | **Andrea Gravina**[1] | **Pasquale Primo**[1] | **Martina Buonanno**[4] | **Kostas D. Mathiopoulos**[3] | **Giuseppe Saccone**[1]

[1]Department of Biology, University of Naples "Federico II", Naples, Italy

[2]Department of Life Sciences, Imperial College London, London, UK

[3]Department of Biochemistry and Biotechnology, University of Thessaly, Larissa, Greece

[4]Institute of Biostructures and Bioimaging (IBB), CNR, Naples, Italy

Correspondence
Konstantina T. Tsoumani, Department of Biochemistry and Biotechnology, University of Thessaly, 41500 Larissa, Greece.
Email: kotsouma@bio.uth.gr

Giuseppe Saccone, Department of Biology, University of Naples "Federico II", 80126 Naples, Italy.
Email: giuseppe.saccone@unina.it

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Abstract

The olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae), is the most destructive insect pest of olive cultivation, causing significant economic and production losses. Here, we present the establishment of the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 methodology for gene disruption in this species. We performed targeted mutagenesis of the autosomal gene *white* (*Bo-we*), by injecting into early embryos in vitro preassembled and solubilized Cas9 ribonucleoprotein complexes loaded with two gene-specific single-guide RNAs. Gene disruption of *Bo-we* led to somatic mosaicism of the adult eye color. Large eye patches or even an entire eye lost the iridescent reddish color, indicating the successful biallelic mutagenesis in somatic cells. Cas9 induced either indels in each of the two simultaneously targeted *Bo-we* sites or a large deletion of the intervening region. This study demonstrates the first efficient implementation of the CRISPR/Cas9 technology in the olive fly, providing new opportunities towards the development of novel genetic tools for its control.

KEYWORDS

CRISPR/Cas9 ribonucleoprotein complexes, genome engineering, insect pest control, Tephritidae, *white* gene

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1 | INTRODUCTION

The olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae), is a monophagous species that infests olive fruits, causing serious damages in olive production, and resulting in significant economic losses (Daane & Johnson, 2010). The major research focus of such insect pests is directed towards the development of novel strategies for their effective control (Ant et al., 2012). Although the olive fly research has entered the realm of genomics (Pavlidi et al., 2017; Sagri et al., 2017, 2014) reliable tools for reverse genetics, functional analyses, and applied research are still under development, hampering the progress towards the establishment of genetic control technologies. The availability of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology and its ability to induce gene-specific mutations is expected to revolutionize the genome engineering toolbox also in the olive fly. Indeed, the CRISPR/Cas9 system has been successfully used for gene knockout in several insect pests (Taning, Van Eynde, Yu, Ma, & Smagghe, 2017). An ideal genetic target to test CRISPR/Cas9 mutagenesis in *Drosophila melanogaster* was the *white* gene (Bassett, Tibbit, Ponting, & Liu, 2013) because mutations on this X-linked gene that controls the red eye color are easily screened by visual observation in males (Green, 2010). The *white* gene was previously found structurally and functionally conserved in *Ceratitis capitata* as an autosomal locus (Loukeris, Livadaras, Arcà, Zabalou, & Savakis, 1995; Zwiebel et al., 1995) and was successfully targeted by CRISPR/Cas9 in this (Meccariello et al., 2017) and other Tephritidae species (see references in Table 1).

The main initiative of this study was to transfer the CRISPR/Cas9 editing technology into the olive fruit fly. To this end, we identified by sequence similarity the genomic locus of the eye pigmentation gene *white* (*Bo-we*) in *B. oleae*, which showed high homology with its orthologue in *C. capitata*. Unlike in *Drosophila* and as in *Ceratitis capitata*, *Bo-we* is located in an autosomal region (Drosopoulou, Nakou, & Mavragani-Tsipidou, 2014). Therefore, we expected to observe phenotypic effects on the eye colour only in case of biallelic mutations induced by Cas9, as previously observed in *C. capitata* (Meccariello et al., 2017).

This is the first report of successful CRISPR-based mutagenesis in the olive fruit fly. We anticipate that this technology will contribute to the generation of new molecular tools that will improve both our understanding of the olive fruit fly biology and the manipulation of its reproductive capacity via genetic control strategies for pest management.

TABLE 1 List of CRISPR/Cas9-based applications in Tephritidae

Insect	Delivery	Gene	Mutation	Reference
Bactrocera oleae	Protein	*white*	Knockout	Present study
Bactrocera tryoni	Protein	*white*	Knockout	Choo, Crisp, Saint, O'Keefe, and Baxter (2018)
Bactrocera dorsalis	Plasmid	*Bdmew*	Knockout	Zheng, Li, Sun, Ali, and Zhang (2019)
	Protein	*white*	Knockout	Bai et al. (2019); Sim, Kauwe, Ruano, Rendon, and Geib (2019)
	mRNA	*transformer*	Knockout	Zhao et al. (2019)
Ceratitis capitata	Protein	*white, paired MoY*	Knockout	Meccariello et al. (2017, 2019)
Anastrepha ludens	Protein	*white*	Knockout	Sim et al. (2019)
Anastrepha suspensa	Protein	*transformer-2*	Knockout	Li and Handler (2019)

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; mRNA, messenger RNA.

2 | MATERIALS AND METHODS

2.1 | Insect rearing

The experiments were carried out using the *B. oleae* "Demokritos" laboratory strain provided by Kostas Bourtzis, Carlos Caceres, and Ahmad Sohel (Insect Pest Control Laboratory, Seibersdorf, Austria) to Prof Saccone's Laboratory (Naples). Flies were maintained under constant laboratory rearing conditions at 25 ± 2°C, 65 ± 5% RH, and a photoperiod of L14:D10. Paraffin cones were used as oviposition substrates and laid eggs were collected by washing them with water, maintained on filter paper soaked in 0.3% propionic acid, and then transferred to artificial larval diet (Tzanakakis, 1989). Pupae were then collected and stored in petri dishes until adult emergence.

2.2 | *B. oleae white* gene identification

The *Bo-we* gene was identified by Basic Local Alignment Search Tool (BLAST) homology searches with the *C. capitata* orthologous gene. The gene structure was further curated manually at the *B. oleae* genomic database (https://i5k.nal.usda.gov/Bactrocera_oleae) on the Apollo platform of the i5k Workspace@NAL (Poelchau et al., 2014). Multiple sequence alignments of the complete amino acid sequences were performed with Clustal Omega (Madeira et al., 2019).

2.3 | Suitable single-guide RNAs synthesis and ribonucleoprotein complex preparation

Suitable single-guide RNAs (sgRNAs) were identified using the CHOPCHOP software (Labun, Montague, Gagnon, Thyme, & Valen, 2016). Two 20-bp sgRNAs (*Bo-white*_g1 and *Bo-white*_g2) were selected for double-sequence targeting of *Bo-we* gene, showing 53.04% and 73.79% predicted efficiencies, respectively. Each sgRNA including the protospacer adjacent motif (PAM) sequence was used as a query against *B. oleae* genome in Blastn searches to identify putative off-targets. Templates for sgRNA in vitro transcription were generated by annealing two complementary oligonucleotides (polyacrylamide gel electrophoresis-purified, Life Technologies) as previously described (Bassett et al., 2013), using:

(1) a specific forward primer for each sgRNA containing both the T7 promoter sequence and the respective *white* targeting sequence (underlined):
   (*Bo-white*_g1:GAAATTAATACGACTCACTATAGAGTTGTTTCCAGCCCGAACCgttttagagctagaaatagc)
   (*Bo-white*_g2:GAAATTAATACGACTCACTATAGAGAGTAGGTGAGATTATCCGgttttagagctagaaatagc).
(2) a common reverse primer encoding the remainder of the sgRNA sequence (AAAAGCACCGACTCGGTGC CACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC).

The template-free polymerase chain reaction (PCR) for primer extension and the sgRNA in vitro transcription was performed as described by Meccariello et al. (2017). The ribonucleoprotein (RNP) complex mix contained 1.8 μg/μl of purified Cas9 protein with 0.2 μg/μl of sgRNA in injection buffer solution (20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, 300 mM KCl, 1 mM dithiothreitol, 10% glycerol, and pH 7.5). The preassembly of the RNP for each sgRNA was performed by incubating the freshly prepared solution at 37°C for 10 min, and equal quantities of those generated RNPs were mixed before use and kept on ice upon injections.

2.4 | Embryo microinjection

Embryo microinjections were carried out at 22°C according to standard procedures with minor modifications. Eggs were collected within 30 min after egg laying (AEL) and aligned on a slide with double-stick tape. Before the injections, eggs were dehydrated for 20 min by placing the slide in a petri dish containing calcium chloride granules and covered after with Halocarbon oil 700 (Sigma-Aldrich, Munich, Germany).

Preblastoderm embryos were injected within 90 min AEL into the posterior pole through the chorion using commercially available open-ended needles filled with 1 μl of preloaded sgRNA-Cas9 mix for both targets. Injections were performed under an inverted compound microscope (Leica DM-IRB), using a Leica mechanical micromanipulator at injection pressures of 25–28 psi. After injection completion, excess oil was wiped off and embryos were placed on tomato juice agar plates and incubated at 26°C until larvae hatching. The surviving larvae were then transferred to artificial diet under the standard rearing conditions. For the phenotypic mutation analysis, individual G_0 flies were visually screened for red-white eye mosaicism under a Leica MZ10F stereomicroscope. Images were obtained using the camera Leica DFC295.

2.5 | Cloning and mutation analysis

Single fly genomic DNA was extracted using the NucleoSpin® Tissue kit (MACHEREY-NAGEL) according to the manufacturer's instructions and used as a template for genotyping. PCR of the target sequence that spans both the sgRNA sites was carried out using the primers Bo-white-F: CAATGAGCAGTCCTACGAGC and Bo-white-R: AGGCAATCGCATTCAGAAGTG and Q5®High-Fidelity DNA Polymerase (New England Biolabs) under cycling conditions of 98°C 3 min, 35 cycles of 98°C 15 s, 58°C 20 s and 72°C 25 s, and 72°C 2 min.

PCR products were visualized on 1% agarose gel and all amplicons were purified separately using the NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL) and subcloned in the pJET 1.2 vector using the Thermo Scientific™ CloneJET™ PCR Cloning Kit (Thermo Fisher Scientific) following the manufacturer's protocols. Clones were subsequently sequenced using Sanger method (CeMIA SA) and the results were analyzed with Geneious® 8.0.5 (Biomatters Ltd.).

3 | RESULTS AND DISCUSSION

To explore the feasibility of CRISPR/Cas9 system as a gene-specific mutagenesis method in the *B. oleae* genome, we chose to disrupt the *white* gene through embryo injections of RNP complexes. Initially, we identified the *B. oleae white* (*Bo-we*) gene based on BLASTp homology searches with the *C. capitata* orthologue (94% amino acid identity; XP_014094495.1). The identified NCBI GenBank gene sequence (XM_014239020.1) was partial, missing the first exon and the long first intron (16,025 bp). The intact gene structure (JAMg_model_4806.1) was identified by manual curation at the i5k Workspace@NAL based on gene models and supporting data on our newest *B. oleae* genome assembly (GCA_001188975.3). *Bo-we* spans a 18,503 bp genomic locus and consists of seven exons (2,040 bp total transcript length) that code for a 679 aa protein (Figure 1a). A multiple alignment analysis of Bo-we protein with relative species revealed its high similarity with *B. tryoni* (98%), *B. dorsalis* (97.9%), but also *D. melanogaster* (84%). The characterization of the *Bo-we* structure facilitated the identification of the exonic target regions for producing a loss-of-function phenotype. We applied a simple protocol of CRISPR mutagenesis, by designing specific sgRNAs to direct the Cas9 RNPs, after injections into embryos, to the desired gene regions, encoding the amino-terminal portion of the Bo-we protein.

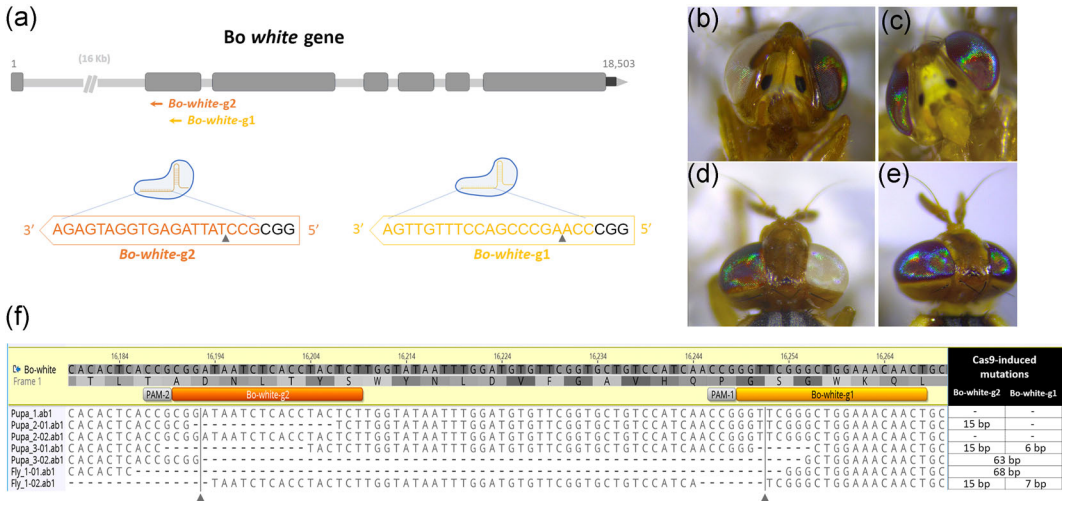

FIGURE 1 CRISPR/Cas9-mediated *Bo-we* gene editing. (a) A schematic representation of the *Bo-we* gene structure and the respective target sites of the sgRNAs *Bo-white*-g1 (yellow) and *Bo-white*-g2 (orange). The 20 nt sequence of each sgRNA is indicated in the respective boxes; PAM sequences are shown in black; vertical arrowheads below the sequence indicate the cleavage site. (b and d) Induced white eye somatic mutant and (c and e) wild-type eyes. (f) Sequencing results of *Bo-we* at the targeted sites. Pupa_1 was genotyped as a wild-type (only one 410-bp fragment was amplified in PCR). Presence or absence of indels in the cloned amplicons were found in Pupa_2, Pupa_3 and the emerged female adult (Fly_1). All sequences were aligned on the wild-type *Bo-we* genomic sequence. Dashes indicate gaps automatically introduced to show the Cas9 deletions. The right column shows the size of the induced deletions for both sgRNAs; vertical arrowheads indicate the expected cleavage site after Cas9 activity. CRISPR, clustered regularly interspaced short palindromic repeats; PAM, protospacer adjacent motif, PCR, polymerase chain reaction; sgRNA, single-guide RNA

Potential Cas9 target sequences were identified using the bioinformatics online software CHOPCHOP, using the coding sequence as an input. Two target sites were selected to induce either one or dual cleavage, leading to indels, frameshift mutations, and possibly also deletion of the intervening region (Figure 1a). The distance between the two target sites and their associated PAM sites is 62 bp within the *Bo-we* exon 2. A total of 900 preblastoderm *B. oleae* embryos were injected with the preassembled RNP complexes. A total of 170 larvae hatched, showing a hatching rate of about 18%, whereas the observed egg-to-pupa survival rate was very low (4/900). Among the four surviving pupae, only one emerged to a female adult, with an egg-to-adult survival rate of 1 of 900. Such low survival rates ranging from 0.8% to 6.5% have also been reported in previous genetic transformation studies that involved *B. oleae* embryo microinjections (Genc, Schetelig, Nirmala, & Handler, 2016; Koukidou et al., 2006). Moreover, "Demokritos" laboratory populations are highly inbred, a characteristic that even under standard rearing conditions negatively affects the egg-to-adult survival rate which ranges between 30% and 50%. Therefore, the observed very low survival rate is the likely result of a combination of causes. Technical parameters associated with the microinjection procedure itself could affect the survival efficiency. However, these were not solely associated with the low number of survived flies, since a satisfying hatching rate from egg-to-larva (18%) was observed. However, a high mortality rate was seen during larval development. This observation highlights the necessity for improvement of the synthetic larval diet used in the artificial rearing of the olive fly. Although several aspects of the artificial rearing of *B. oleae* have been improved through the years (Rempoulakis, Dimou, Chrysargyris, & Economopoulos, 2014), there are still many drawbacks that restrict the effective and consistent production of large number of flies mainly associated with larval development. Larval development possibly could be improved by introducing new ingredients and/or nutrients into the artificial synthetic diet.

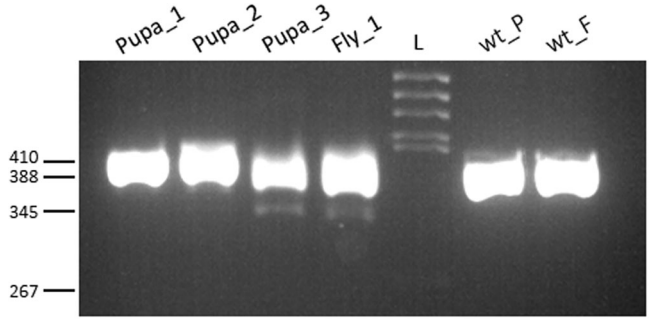

FIGURE 2 PCR amplification of the targeted *Bo-we* genomic region. The genomic DNA of the four G_0 individuals (three pupae (Pupa_1-3) and one female adult (Fly_1)) were used as templates in each PCR reaction, respectively. The resulting PCR products were analyzed by electrophoresis through 3% (wt/vol) agarose gel. Different size amplicon bands indicate putative deletion mutations. Each amplicon band was purified and cloned for subsequent sequencing. As positive controls (410 bp) were used DNA from wild-type single pupa (wt_P) and single female adult (wt_F), respectively; L: pBR322 DNA/BsuRI (*Hae*III) Marker (Thermo Fisher Scientific™). PCR, polymerase chain reaction

The CRISPR/Cas9 mediated disruption of the *Bo-we* gene, by indels and/or by deletion, was expected to induce red-white eye color mosaic phenotypes. However, only in the case of biallelic mutations, recognizable red-white color changes should be produced in the somatic clones, derived from a cell in which these mutational events were induced on the two homologous chromosomes. The phenotypic analysis of the *B. oleae* adult showed that one of the two eyes was completely white (Figures 1b and 1d). We extracted genomic DNA from this adult, which died within a day after eclosion, and from three pupae. We PCR-amplified genomic DNA fragments from the targeted *Bo-we* genomic region. Gel electrophoresis analysis showed that the PCR products contained, not only an expected 410-bp-long DNA fragments, but also DNA products having shorter length. This analysis suggested the presence of deletions of the intervening sequence between the two targeted sites. These amplicons were subcloned and sequenced (Figure 2). The genotyped-mutant individuals revealed heterogeneity in the generated indel mutations (Figure 1f), which is consistent with the random repair mechanism followed by the nonhomologous-end-joining (NHEJ) mechanism. Comparison of sequencing data to the reference wild-type sequence showed diverse indels and deletion patterns in proximity to the cleavage positions of both target sites, including large deletions between the sgRNA target sites. The identified NHEJ-induced indels ranged from 7 to 15 bp, whereas two longer 63-and 68-bp identified gaps resulted from the deletion of the whole region that spans the two sgRNA targets (Figure 1f). In the case of Pupa_2, the indel was generated only by the *Bo-white*-g2 sgRNA. This result could be explained considering the lower predicted efficiency of *Bo-white*-g1 compared to *Bo-white*-g2. Although somatic knockout of the *Bo-we* gene was clearly shown in this study, the efficacy of Cas9-induced germ-line disruption could not be determined, because the emerged G_0 fly died and no crosses could be performed. Future experiments should address whether the *white* mutations also occurred in primordial germ cells and could be inherited in successive generations. However, the molecularly identified somatic mutations in 75% of G_0 individuals (including pupae and one adult) demonstrate the effectiveness of the CRISPR/Cas9 system in inducing indel mutations and a large deletion using two sgRNAs.

4 | CONCLUSION

In conclusion, this study is the first application of in vitro assembled RNP complexes—formed by synthetic sgRNA components and a cost-effective lab-purified Cas9 nuclease—to induce a gene disruption in the olive

fruit fly. Diverse mutations in somatic cells were observed in the different individuals (indel mutations or large deletions) that survived the embryo injections, as a result of multiple Cas9 targeting. The next challenge will be to understand and/or reduce the high lethality observed at the later stages of development, which, however, could not be attributed solely to the experimental procedure but also to the low survival rate of this species in standard rearing. The development of the CRISPR/Cas9-mediated targeted mutagenesis in this insect is of fundamental importance for both basic and applied research. At a basic level, it enables the generation of desirable mutations in any physiological system that governs vital life traits. At an applied level, the ability to generate mutations in genes that control early embryogenesis or sex determination of Tephritidae (such as *MoY*, *tra*, or *dsx*) (Meccariello et al., 2019; Saccone, Salvemini, & Polito, 2011; Saccone, Salvemini, Pane, & Polito, 2002) acquires eminent importance, since such processes play a critical role in genetic sexing in mass rearing facilities performing the sterile insect technique. This provides also the means of future genome modifications via homology-directed repair insertions as successfully shown recently in *C. capitata* (Aumann, Schetelig, & Häcker, 2018).

Thus, such CRISPR-based strategies could provide powerful means to accelerate the development of alternative species-specific genetic control methods, which are still hindered in *B. oleae* by the unavailability of genetic sexing strains. This study suggests that the implementation of innovative, simple, and accurate genetic methods in the olive fruit fly is not far from being deployed, once the appropriate molecular tools will be generated.

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AUTHOR CONTRIBUTIONS

G. S. and K. D. M. initiated the project. K. T. T collected the embryos and prepared them for the injections, performed the molecular genotyping analysis, and drafted the manuscript; and wrote the manuscript with inputs from all authors. A. M. prepared the gRNAs and injected Cas9-gRNA mix into olive fly embryos. A. G. and P. P. contributed to the rearing and genetic crosses of the olive flies for embryo production. M. B. purified the recombinant Cas9 protein. All authors reviewed and approved the final version of the manuscript.

ORCID

Angela Meccariello http://orcid.org/0000-0001-9706-5764
Konstantina T. Tsoumani http://orcid.org/0000-0003-1890-3067
Andrea Gravina http://orcid.org/0000-0002-4580-5940
Pasquale Primo http://orcid.org/0000-0002-8700-8042
Martina Buonanno http://orcid.org/0000-0003-4419-4890
Kostas D. Mathiopoulos http://orcid.org/0000-0002-2875-5697
Giuseppe Saccone http://orcid.org/0000-0002-9835-3693

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SEX DETERMINATION

Maleness-on-the-Y (*MoY*) orchestrates male sex determination in major agricultural fruit fly pests

Angela Meccariello[1]*, Marco Salvemini[1]*, Pasquale Primo[1], Brantley Hall[2], Panagiota Koskinioti[3,4], Martina Dalíková[5,6], Andrea Gravina[1], Michela Anna Gucciardino[1], Federica Forlenza[1], Maria-Eleni Gregoriou[4], Domenica Ippolito[1], Simona Maria Monti[7], Valeria Petrella[1], Maryanna Martina Perrotta[1], Stephan Schmeing[8], Alessia Ruggiero[7], Francesca Scolari[9], Ennio Giordano[1], Konstantina T. Tsoumani[4], František Marec[5], Nikolai Windbichler[10], Kallare P. Arunkumar[11], Kostas Bourtzis[3], Kostas D. Mathiopoulos[4], Jiannis Ragoussis[12], Luigi Vitagliano[7], Zhijian Tu[2], Philippos Aris Papathanos[13,14]†, Mark D. Robinson[8]†, Giuseppe Saccone[1]†

In insects, rapidly evolving primary sex-determining signals are transduced by a conserved regulatory module controlling sexual differentiation. In the agricultural pest *Ceratitis capitata* (Mediterranean fruit fly, or Medfly), we identified a Y-linked gene, *Maleness-on-the-Y* (*MoY*), encoding a small protein that is necessary and sufficient for male development. Silencing or disruption of *MoY* in XY embryos causes feminization, whereas overexpression of *MoY* in XX embryos induces masculinization. Crosses between transformed XY females and XX males give rise to males and females, indicating that a Y chromosome can be transmitted by XY females. *MoY* is Y-linked and functionally conserved in other species of the Tephritidae family, highlighting its potential to serve as a tool for developing more effective control strategies against these major agricultural insect pests.

Tephritidae is a dipteran family comprising 5000 species, dozens of which are invasive and highly relevant pests of fruit crops. *Ceratitis capitata* (Medfly) is one of the most destructive members of this taxon, affecting more than 200 plant species (*1*). Besides pesticides, the most successful method to control Medfly is the sterile insect technique (SIT) (*2*), which involves the continuous mass-release of biofactory-reared, sterilized males that suppress wild populations by mating with wild females. A key determinant to the success of SIT programs has been the translocation of selectable traits to Medfly Y chromosome in genetic sexing strains that enable male selection on a massive scale (*2*). However, the development of similar strains in other Tephritidae pest species using classical genetics has been difficult. Identifying the male-determining factor (M factor) in Medfly and in related pests holds great promise for the development of novel genetic sexing strains using modern genetics (*2*) or even for transforming females into males, thereby increasing the efficiency of insect biofactories.

In insects, widely divergent primary signals of sex determination act via the conserved genetic switch *transformer* (*tra*), which was first characterized in *Drosophila* as a gene regulated by and operating through sex-specific alternative splicing (*3*–*6*). In females, two doses of the X chromosome result in an early zygotic transcriptional burst of the master gene *Sex-lethal* (*Sxl*), which promotes female-specific splicing of *tra* and female differentiation. In males, a single X leads by default to a *tra* transcript that encodes a short nonfunctional TRA polypeptide and male differentiation occurs. In Medfly, as in other non-Drosophilidae species, *Sxl* is not involved in sex determination (*3*). Unlike *Drosophila*, maternal deposition of *Ceratitis capitata tra* (*Cctra*) in developing embryos initiates its positive autoregulatory female-specific splicing, similar to the housefly (*5*, *6*), leading to female differentiation. In Medfly XY embryos, a Y chromosome–linked M factor either directly or indirectly represses *Cctra* function, thus promoting male development (*5*). However, the molecular identity of this Y-linked M factor has remained unknown (*7*, *8*).

We conducted the search for this M factor by: (i) focusing on transcripts in the 4 to 8 hours after egg laying (AEL), the period when male sex determination is first established (*9*); (ii) producing an XX-only embryonic RNA sequencing dataset as a reference; (iii) developing a long-read PacBio sequencing–based male genome assembly from the *Fam18* Medfly strain (*8*) that bears a shorter Y chromosome; and (iv) searching for conservation of putative M factors in another Tephritidae, the olive fruit fly, *Bactrocera oleae*. We identified 19 M candidates that expressed transcripts in mixed-sex embryos but not in XX-only embryos and that were predicted to be Y-linked on the basis of the ratio of genomic reads mapping from female versus male samples (chromosome quotient, CQ) (*10*) (Fig. 1A, table S1, and supplementary text S1). Seven of these transcripts did not map to the *Fam18* male genome assembly and were excluded from further analysis. Sequence similarity searches by nucleotide basic local alignment search tool (BLASTn) showed that 3 out of the remaining 12 transcripts had hits to XY but not to XX embryonic transcripts from *B. oleae*. Furthermore, one of these three Medfly transcripts (DN40292_c0_g3_i1) corresponds to a 0.7-kb sequence we previously identified in a preliminary screen (supplementary text S2). This transcript mapped to a predicted 12-kb long Y-linked contig in the *Fam18* genome assembly. Functional analysis (below) confirmed that this gene is the Medfly M factor and was thus named *Maleness-on-the-Y* (*MoY*). *MoY* is located on the long arm of the Y chromosome in proximity to the centromere (Fig. 1B) in a genomic region that contains nine other transcription units (Fig. 1C and supplementary text S3). *MoY* expression begins at 2 to 3 hours AEL, before embryonic cellularization, peaks at 15 hours, and is undetectable starting at 48 hours until adulthood (Fig. 1D and data S1).

Embryonic RNA interference (eRNAi) by injecting double-stranded RNA (dsRNA) into embryos 0 to 1 hours AEL resulted in loss of male-specific *Cctra* transcripts in 8-hour AEL embryos (Fig. 2, A to C, and tables S2 and S3). We observed a switch to the female-specific *Cctra* splicing in 3-day-old XY larvae from injected embryos and in XY adult intersexes (fig. S1). Among adults, 38% (14 of 37) of the molecularly karyotyped XY individuals displayed complete phenotypic feminization, and 19% (7 of 37) were intersex (Fig. 2D and fig. S2). Individuals were phenotypically classified as intersex if they displayed a mix of male- and

[1]Department of Biology, University of Naples "Federico II," 80126 Napoli, Italy. [2]Department of Biochemistry, Virginia Tech, Blacksburg, VA 24061, USA. [3]Insect Pest Control Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, A-1400 Vienna, Austria. [4]Department of Biochemistry and Biotechnology, University of Thessaly, 41500 Larissa, Greece. [5]Institute of Entomology, Biology Centre of the Czech Academy of Sciences, 370 05 České Budějovice, Czech Republic. [6]Faculty of Science, University of South Bohemia, 370 05 České Budějovice, Czech Republic. [7]Institute of Biostructures and Bioimaging (IBB), CNR, 80134 Naples, Italy. [8]Institute of Molecular Life Sciences and SIB Swiss Institute of Bioinformatics, University of Zurich, 8057 Zurich, Switzerland. [9]Department of Biology and Biotechnology, University of Pavia, 27100 Pavia, Italy. [10]Department of Life Sciences, Imperial College London, South Kensington Campus, London SW7 2AZ, UK. [11]Centre of Excellence for Genetics and Genomics of Silkmoths, Laboratory of Molecular Genetics, Centre for DNA Fingerprinting and Diagnostics, Hyderabad 500 039, India. [12]Department of Human Genetics and Bioengineering, McGill University and Genome Quebec Innovation Centre, Montreal, QC H3A 0G1, Canada. [13]Section of Genomics and Genetics, Department of Experimental Medicine, University of Perugia, 06132 Perugia, Italy. [14]Department of Entomology, The Robert H. Smith Faculty of Agriculture, Food and Environment, Hebrew University of Jerusalem, Rehovot 76100, Israel.
*These authors contributed equally to this work.
†Corresponding author. Email: giuseppe.saccone@unina.it (G.S.); mark.robinson@imls.uzh.ch (M.D.R.); p.papathanos@mail.huji.ac.il (P.A.P.)

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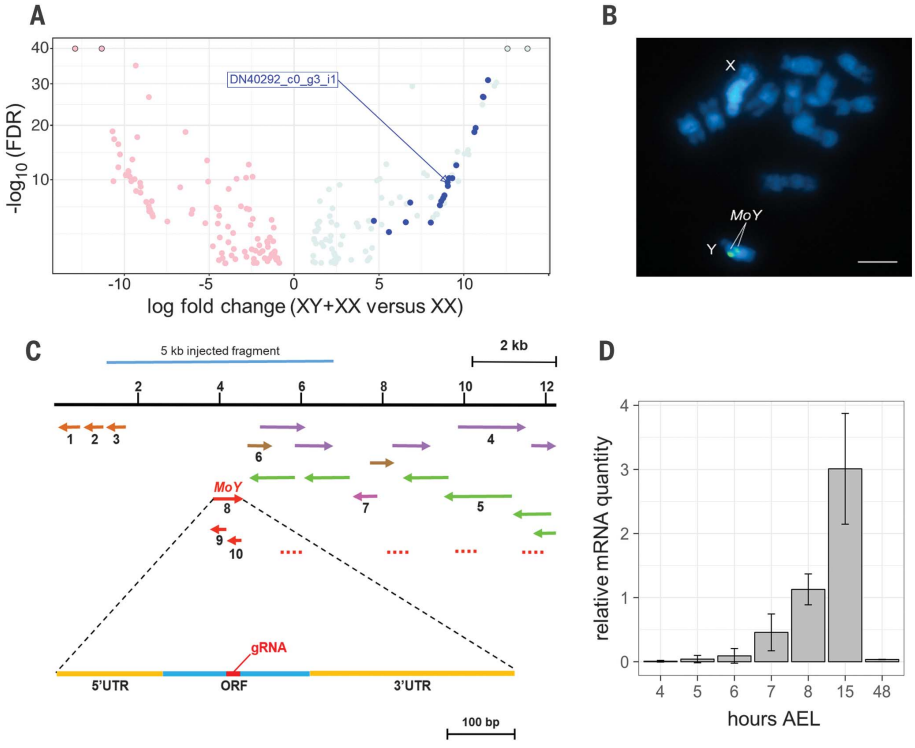

Fig. 1. *MoY* gene is Y-specific and transiently transcribed in embryos. (**A**) Volcano plot of 195 differentially expressed transcripts at 4 to 8 hours AEL. Pink dots indicate log fold change (mixed XX and XY samples versus XX samples) less than 0 (biased in the XX sample), light-blue dots represent log fold change > 0 (biased in the mixed sample), and dark-blue dots indicate 19 transcripts with a CQ value of 0 (putatively Y-linked). The transcript corresponding to *MoY* (DN40292_c0_g3_i1) is also shown. (**B**) Fluorescence in situ hybridization of *MoY* (green signals) on mitotic chromosomes stained with 4′,6-diamidino-2-phenylindole (blue); signals (one for each sister chromatid) locate *MoY* on the long arm of the Y chromosome near the centromere (scale bar, 5 μm). (**C**) A scheme of a 12-kb Y-linked genomic contig (contig00013010) containing the *MoY* transcript and other flanking transcriptional units (supplementary text S3). Also shown is the 5-kb region used for injections and the *MoY* guide RNA target site for Cas9. 5′UTR, 5′ untranslated region; 3′UTR, 3′ untranslated region; ORF, open reading frame. (**D**) Relative transcript expression of *MoY* during Medfly embryogenesis compared with housekeeping genes. For reference, Medfly cellularization occurs at ~9 hours AEL.

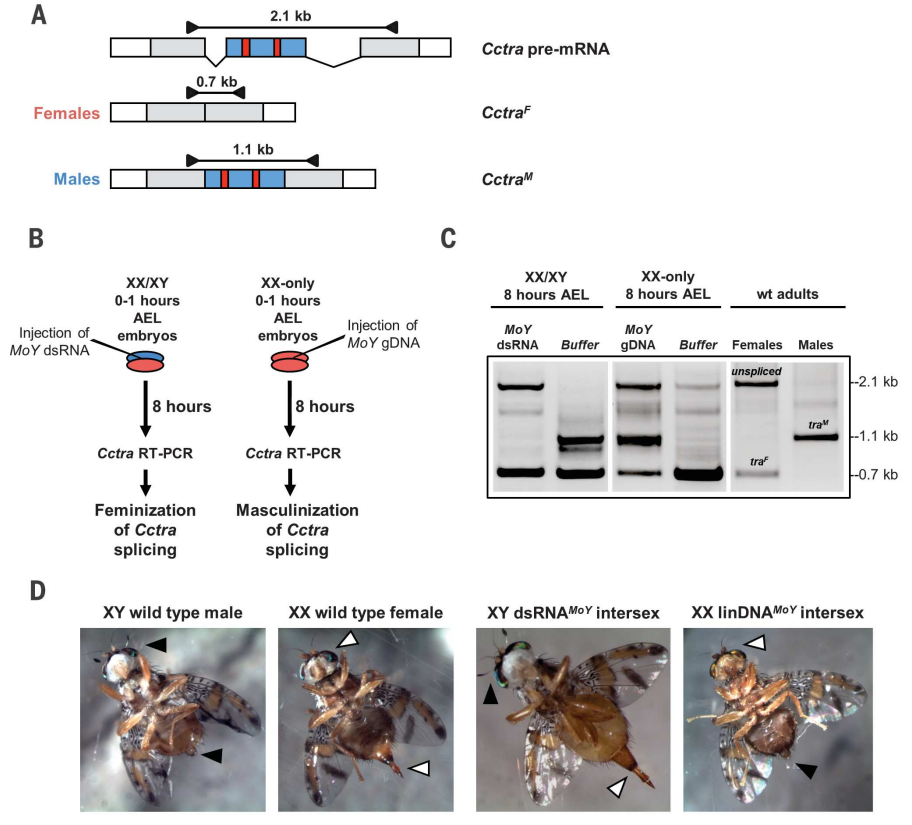

Fig. 2. *MoY* is necessary and sufficient for male sex determination. (**A**) Scheme of *Cctra* sex-specific transcripts. Coding regions, the male-specific exon, and stop codons are shown in gray, blue, and red, respectively. Black arrows indicate primers for reverse transcription polymerase chain reaction (RT-PCR). (**B**) Schematic overview of embryonic *MoY* injection experiments and expected effects on *Cctra* splicing after 8 hours. (**C**) RT-PCR analysis showing splicing patterns of *Cctra* in embryos injected at 8 hours AEL. (Left) Transient *MoY*-eRNAi (at 0 to 1 hours AEL) depletes mixed-sex embryos of the male-specific *Cctra* isoform at 8 hours AEL. (Middle) Injection of *MoY* gDNA is sufficient to instruct male-specific splicing of *Cctra* in XX-only embryos. The 2.1-kb *Cctra* amplicon is the unspliced transcript, detectable only in females (*5*). (Right) Wild-type male and female flies for reference; black and white colors inverted in the gel photo for clarity. (**D**) Representative photos of Medfly wild-type males and females and Medfly intersexes. The XY intersex from *MoY*-eRNAi has a female ovipositor (white arrowhead) and a male-like head with orbital bristles (black arrowhead). The XX intersex from *MoY* linear gDNA (linDNA; PCR fragment) injections has male genitalia (black arrowhead) and a female-like head, without orbital bristles (white arrowhead).

female-specific traits (Fig. 2D). To evaluate the fertility of XY females and to test whether a Y chromosome can be maternally transmitted, crosses were established to XX males that were generated by eRNAi targeting *Cctra* (*5*) (table S4 and fig. S3A). These crosses made it possible to demonstrate that the maternal transmission of the Y chromosome determined the male sex of the progeny. Among the 14 recovered XY females, one was fertile and transmitted the Y chromosome to one son. In a second experiment of *MoY*-eRNAi, using a strain displaying sex-specific pupal colors (*Vienna8*), we found another XY female that transmitted the Y chromosome (*2*) (table S2). This work demonstrates that the two

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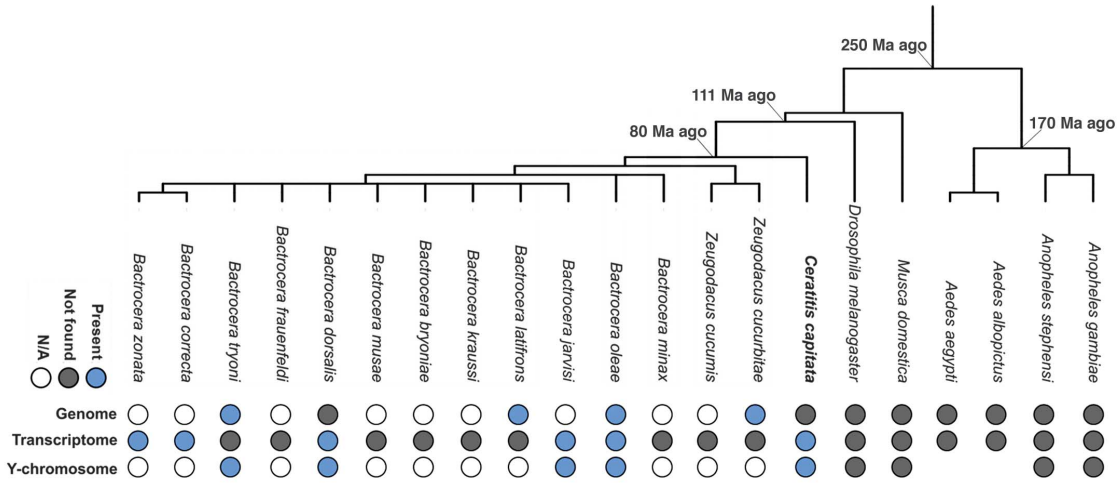

Fig. 3. *MoY* is conserved in Tephritidae species. A search of Medfly *MoY* orthologs was performed in 14 Tephritidae species and in 6 other dipteran species, in which primary sex determining signals have been molecularly characterized. *MoY* orthologs were discovered in the genomes and/or transcriptomes of eight Tephritidae species (blue circles). Y-linkage was confirmed by genomic PCR in four of these (Fig. 4A and supplementary text S4 to S12). The lack of detection of *MoY* (dark gray circles) in the remaining six Tephritidae species is likely the result of limited sequencing coverage. Divergence times are indicated as million years ago (Ma ago). N/A, not applicable.

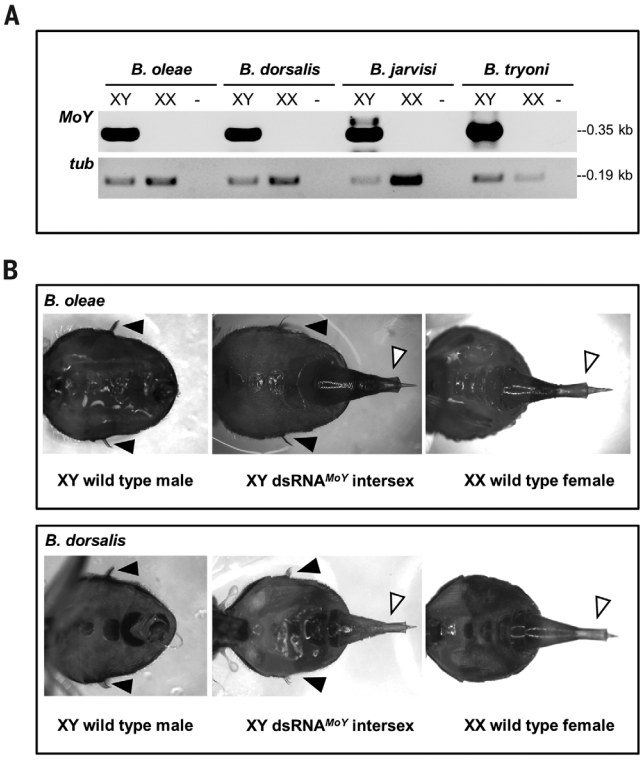

Fig. 4. *MoY* is Y-linked in *Bactrocera* species and functionally conserved. (**A**) PCR on male and female genomic DNA showing Y-linkage of *MoY* orthologs in *B. oleae*, *B. dorsalis*, *B. jarvisi*, and *B. tryoni*. Positive control: beta tubulin (*tub*). Black and white colors are inverted for clarity. (**B**) *B. oleae* (*Bo*) and *B. dorsalis* (*Bd*) male and female wild-type abdomens (left and right, respectively) and abdomens of intersex XY flies (middle), after *BoMoY*- and *BdMoY*-eRNAi (see also fig. S10). In both species, XY intersexes show the presence of female-specific characteristics (ovipositor, white arrowhead) together with male-specific characteristics (abdominal lateral bristles, black arrowheads).

sexes of an animal species defined by each karyotype can be reciprocally switched in both directions while maintaining fertility. This highlights, in both sexes, a resilience of Medfly somatic and gonadal development to perturbations in sex-determination signaling and suggests that the Y chromosome has no major detrimental effects on the development and fertility of XY females.

To further evaluate the role of *MoY* in sex determination, loss-of-function alleles were generated using Cas9 ribonucleoproteins (*11*) targeting the MOY coding sequence (table S2 and fig. S4A). Indels near the single-guide RNA target site were induced in the genomes of four G_0 XY larvae and three G_0 XY adult intersexes (fig. S5A). 50% (7 of 14) of the XY individuals (table S2) were transformed either into phenotypic females (2 of 7) or intersexes (5 of 7) (figs. S4, B to D, and S5B). One XY female crossed to XX males was fertile and produced female-only G_1 offspring composed of 3 XY and 18 XX flies (table S5 and figs. S3B and S4, D and E). Two of these XY G_1 females were analyzed and both showed *MoY* frameshift-inducing deletions resulting in truncated MOY proteins (fig. S5A).

Next, we investigated whether *MoY* is sufficient for male sex determination. A 5-kb genomic fragment, encompassing the *MoY* locus and flanking regulatory regions (Fig. 1C), was injected as a linear polymerase chain reaction (PCR) product or as circular plasmid into embryos (table S2). Male-specific *Cctra* splicing was induced in XX individuals at embryonic, larval, and adult stages (Fig. 2C and fig. S6) and led to partial or full masculinization of up to 75% of XX flies (9 of 12) (table S2, Fig. 2D, and figs. S6 and S7). Similarly, microinjection of MOY recombinant protein into XX-only embryos led to partially masculinized flies (showing either male-specific orbital bristles or male genitalia) in 19% (6 of 31) of the emerged XX adults (table S2 and fig. S8).

Ceratitis MoY DNA and protein sequences showed no significant BLAST hits to the National Center for Biotechnology Information databases, suggesting novelty or high sequence divergence. In contrast, tBLASTn (basic local alignment search tool of translated nucleotide databases using a protein query) searches of available genomic or transcriptomic datasets from 14 Tephritidae species spanning 111 million years of evolution (*12*) identified putative MOY orthologs in eight of them (Fig. 3), with an average amino acid sequence similarity to *Ceratitis* of 41 to 57% (supplementary text S4 to S12). The most conserved portion is located in the N-terminal region, where a consensus hexapeptide KXNSRT occurs (fig. S9). We confirmed by PCR of male and female genomic DNA (gDNA) the Y-linkage of *MoY* orthologs in four out of the eight species, namely *B. oleae*, *B dorsalis*, *B. tryoni*, and *B. jarvisi*, whose fly samples were available (Fig. 4A). *MoY*-eRNAi orthologs in *B. oleae* and *B. dorsalis* led to feminization of XY flies (57 and 33%, respectively), confirming the functional conservation of *MoY* (table S2, Fig. 4B, and fig. S10).

Here, we demonstrate that *MoY* is the Y-linked M factor in *C. capitata* because it is both necessary and sufficient to initiate male development during embryogenesis. How *MoY* suppresses female-specific *Cctra* splicing and whether this regulation is direct or indirect remain unclear. MOY does not bear any similarity to known proteins, in contrast

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to M factors of *Aedes* and *Musca*, which are related to splicing factors (*13*, *14*). This finding suggests that *MoY* is a newly emerged M factor. *MoY* is functionally conserved in various Tephritidae species, in contrast to previous studies conducted on other insects whose M factors diverged rapidly, even within the same species (*13*–*16*). Our finding that masculinization through transient *MoY* misexpression in Medfly XX embryos does not lead to lethality as in other species (*15*, *16*) suggests that *MoY* is not involved in dosage compensation. However, it is not clear from the present data whether Medfly has such regulatory mechanisms to equalize expression of X-chromosome genes in both sexes. From a translational perspective, these features, including *MoY* conservation and its ability to fully masculinize XX individuals, make *MoY* a promising candidate for transferring to other important Tephritidae pests established genetic control strategies such as SIT, and for future development of emerging methods such as male-converting gene drive (*17*–*20*).

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SUPPLEMENTARY MATERIALS

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Materials and Methods
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References (*21*–*40*)
Data S1 to S4

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Science

Maleness-on-the-Y (*MoY*) orchestrates male sex determination in major agricultural fruit fly pests

Angela Meccariello, Marco Salvemini, Pasquale Primo, Brantley Hall, Panagiota Koskinioti, Martina Dalíková, Andrea Gravina, Michela Anna Gucciardino, Federica Forlenza, Maria-Eleni Gregoriou, Domenica Ippolito, Simona Maria Monti, Valeria Petrella, Maryanna Martina Perrotta, Stephan Schmeing, Alessia Ruggiero, Francesca Scolari, Ennio Giordano, Konstantina T. Tsoumani, Frantisek Marec, Nikolai Windbichler, Kallare P. Arunkumar, Kostas Bourtzis, Kostas D. Mathiopoulos, Jiannis Ragoussis, Luigi Vitagliano, Zhijian Tu, Philippos Aris Papathanos, Mark D. Robinson and Giuseppe Saccone

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Making males in a fruit fly pest

The Mediterranean fruit fly or Medfly (*Ceratitis capitata*) is a global and highly destructive fruit pest. Meccariello *et al.* identified the master gene for male sex determination on the Y chromosome of Medfly and named it *Maleness-on-the-Y* (*MoY*) (see the Perspective by Makki and Meller). Flies of each sex were transformed into the other sex by genetic manipulation, and crosses of transformed files generated male and female progeny. *MoY* is functionally conserved in the olive fruit fly and in the invasive oriental fruit fly. This discovery has potential for insect genetic control based on mass release of sterile males and future strategies based on gene drive.

Science, this issue p. 1457; see also p. 1380

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BMC Genetics

RESEARCH Open Access

Targeting the autosomal *Ceratitis capitata transformer* gene using Cas9 or dCas9 to masculinize XX individuals without inducing mutations

Pasquale Primo[1†], Angela Meccariello[1†], Maria Grazia Inghilterra[1], Andrea Gravina[1], Giuseppe Del Corsano[1], Gennaro Volpe[1], Germano Sollazzo[1], Serena Aceto[1], Mark D. Robinson[2], Marco Salvemini[1] and Giuseppe Saccone[1*]

Abstract

Background: Females of the Mediterranean fruit fly *Ceratitis capitata* (Medfly) are major agricultural pests, as they lay eggs into the fruit crops of hundreds of plant species. In Medfly, female sex determination is based on the activation of *Cctransformer* (*Cctra*). A maternal contribution of *Cctra* is required to activate *Cctra* itself in the XX embryos and to start and epigenetically maintain a *Cctra* positive feedback loop, by female-specific alternative splicing, leading to female development. In XY embryos, the male determining *Maleness-on-the-Y* gene (*MoY*) blocks this activation and *Cctra* produces male-specific transcripts encoding truncated CcTRA isoforms and male differentiation occurs.

Results: With the aim of inducing frameshift mutations in the first coding exon to disrupt both female-specific and shorter male-specific CcTRA open reading frames (ORF), we injected Cas9 ribonucleoproteins (Cas9 and single guide RNA, sgRNA) in embryos. As this approach leads to mostly monoallelic mutations, masculinization was expected only in G_1 XX individuals carrying biallelic mutations, following crosses of G_0 injected individuals. Surprisingly, these injections into XX-only embryos led to G_0 adults that included not only XX females but also 50% of reverted fertile XX males. The G_0 XX males expressed male-specific *Cctra* transcripts, suggesting full masculinization. Interestingly, out of six G_0 XX males, four displayed the *Cctra* wild type sequence. This finding suggests that masculinization by Cas9-sgRNA injections was independent from its mutagenic activity. In line with this observation, embryonic targeting of *Cctra* in XX embryos by a dead Cas9 (enzymatically inactive, dCas9) also favoured a male-specific splicing of *Cctra*, in both embryos and adults.

Conclusions: Our data suggest that the establishment of *Cctra* female-specific autoregulation during the early embryogenesis has been repressed in XX embryos by the transient binding of the Cas9-sgRNA on the first exon of the *Cctra* gene. This hypothesis is supported by the observation that the shift of *Cctra* splicing from female to male mode is induced also by dCas9. Collectively, the present findings corroborate the idea that a transient embryonic inactivation of *Cctra* is sufficient for male sex determination.

Keywords: iCRISPR, Sex determination, *Ceratitis capitata*, Epigenetics, Autoregulation, *Transformer*

* Correspondence: giuseppe.saccone@unina.it
†Pasquale Primo and Angela Meccariello contributed equally to this work.
[1]Department of Biology, University of Naples Federico II, 80126 Naples, Italy
Full list of author information is available at the end of the article

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Background

In the last few decades, the Mediterranean fruit fly *Ceratitis capitata* (Tephritidae, Medfly) has become a major invasive agricultural pest worldwide, following its spread from Africa and its globalization [1]. For the local suppression of this invasive species, alternatives to the use of pesticides are genetic control strategies. One of them is the Sterile Insect Technique (SIT), which has been applied successfully over the last six decades in various countries [2].

The prerequisites of SIT include a method to mass rear the target species in a cost-effective way and a method to sterilize them with a low impact on their fitness once released. As the released sterile females contribute to the fruit crop mechanical damage with the ovipositor and consequent infections, and the sterile males tend to mate with the released females rather than with the wild ones, it is highly preferable to develop a method of sexing and only release sterile males [3]. A number of strategies have been developed, including transgenic approaches for sexing, which allow the mass rearing of the two sexes, and sorting the males at the expanded last generation before the release. These strains can be based on the expression of a conditional female-lethal dominant gene [4] or on the transformation of genotypic female individuals into males by manipulating a gene involved in female sex determination [5]. Molecular genetics studies on Medfly sex determination have been useful for this aim, uncovering a cascade of regulatory genes widely conserved in the Tephritidae family (Fig. 1) [6–10]. This taxon includes many other invasive agricultural pests, such as species of the *Bactrocera* and *Anastrepha* genera [11, 12]. This fundamental knowledge is not only interesting and valuable per se [13], but also useful to develop novel sexing strategies necessary to improve the applicability of SIT. Evolutionary conservation of homologous genes and the use of transgenesis and/or CRISPR/Cas9 potentially will enable the realization of additional versatile sexing methods that can be applied in different species [8, 9, 14–16].

The sex determination of Medfly is based, as in *Drosophila melanogaster*, on sex-specific alternative splicing

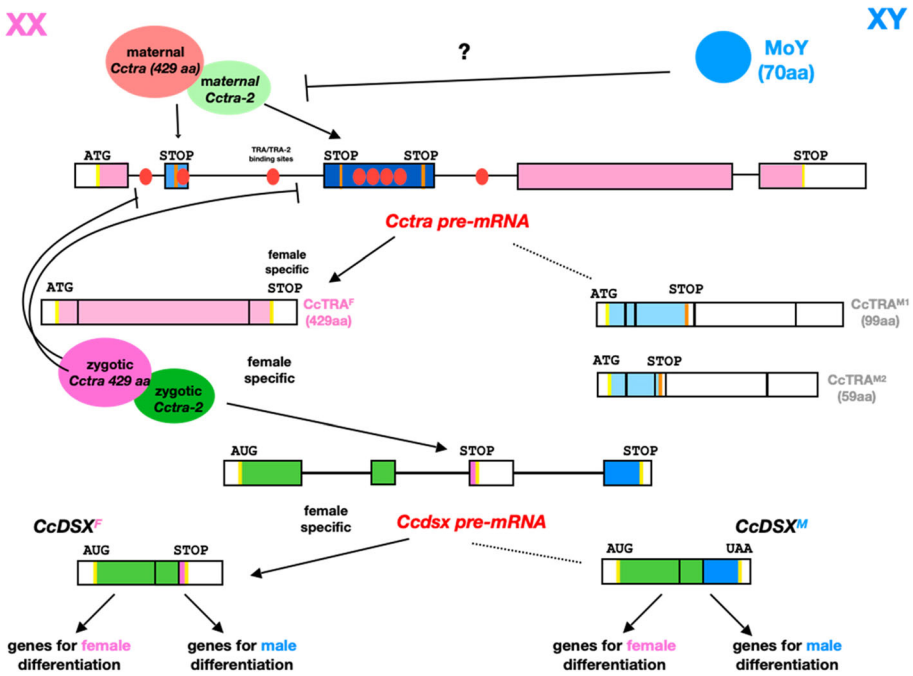

Fig. 1 Genetic pathway of sex determination in *Ceratitis capitata*. *Cctra* and *Ccdoublesex* (*Ccdsx*) pre-mRNAs exon-intron structures and sex-specific transcripts are shown. Female-specific and male-specific *Cctra* exons are indicated as pink and dark blue boxes, respectively. *Cctra* female-specific transcript on the left contains a 429 aa long ORF. *Cctra* male-specific exons introduce premature stop codons in male-specific longer transcripts (orange vertical bars). CcTRA M1 and M2 male-specific isoforms contain truncated CcTRA ORFs represented by azul regions. In XX embryos, maternal CcTRA (orange circle) and CcTRA-2 (green circle) proteins promote female-specific splicing of newly transcribed *Cctra* pre-mRNA, suppressing male-specific splicing by binding to TRA/TRA-2 *cis* regulatory elements (red spots). Female-specific *Cctra* mRNA encodes zygotic CcTRA (violet circle) that maintains (together with zygotic CcTRA-2; dark green circle) the *Cctra* autoregulation induced by the maternal contributions by a feedback loop. Both CcTRA and CcTRA-2 proteins promote also female-specific splicing of the downstream *Ccdsx* pre-mRNA, producing mRNAs that include a female-specific exon (pink) and encode CcDSXF isoform inducing female sexual differentiation [6]. In XY embryos the Y-linked *Maleness-on-the-Y* gene (*MoY*) induces male-specific *Cctra* splicing and, hence, the collapse of the positive feedback loop [7]. By default, male-specific splicing of *Ccdsx* leads to male-specific splicing and CcDSM isoform inducing male sexual differentiation [6, 8]

of key regulatory genes, including *transformer* (*tra*), *transformer-2* (*tra-2*) and *doublesex* (*dsx*) orthologues (Fig. 1) [6, 7, 17–20]. *Cctra* is a sex determining genetic switch, which is set to ON in XX embryos and to OFF in XY embryos during a narrow temporal window at 5–6 h from oviposition [8]. In contrast to *Drosophila*, in XX Medfly embryos, *Cctra* and the auxiliary *Cctransformer-2* (*Cctra-2*) maternal mRNAs are also required to establish a stable activation of *Cctra* by female-specific splicing which relies on a positive feedback loop [6, 19]. The female-specific *Cctra* mRNA encodes a full-length 429 aa protein arising from the translation of an evolutionarily conserved ORF contained in the first, fourth and fifth exons (Fig. 1). In males, two longer alternative *Cctra* RNA isoforms, containing all five exons, encode for two truncated CcTRA proteins called CcTRA M1 (59 aa) and CcTRA M2 (99 aa) (Fig. 1). Embryonic transient RNA interference targeting mRNAs of either genes led to XX males, which are fertile even in the absence of the Y chromosome [6, 19]. As in *Drosophila*, *Cctra* and *Cctra-2* are required for the female-specific splicing of *Ccdoublesex* (*Ccdsx*). The observation that some XX individuals are transformed in gynandromorphs (showing male-specific antennae and ovipositor or no antennae and male gonads) suggests that sex determination is cell-autonomous, as in *Drosophila* [6]. A TRA/TRA-2 binding element (13 nt long) is present in multiple copies in the *Ccdsx* female-specific exon, permitting a positive regulation by CcTRA/CcTRA-2, which leads to the use of this exon in *Ccdsx* transcripts of XX individuals, similarly to *Drosophila* [7, 18]. In contrast to *Drosophila*, multiple copies of this splicing regulatory element are also present in *Cctra* locus, within and in proximity to the male-specific exons. In this other novel case, these cis elements mediate, by CcTRA/CcTRA-2 binding, exon skipping in XX individuals leading to CcTRA-encoding female-specific mRNAs [6]. In XY embryos, *Maleness-on-the-Y* (*MoY*) encodes a novel short protein, MOY, of still unknown biochemical function, that leads, either directly or indirectly, to male-specific splicing of *Cctra* and exons inclusion, at 5–6 h from oviposition [8]. The presence of male-specific exons introducing stop codons in the 429 aa long *Cctra* ORF leads to two major RNAs encoding truncated polypeptides (respectively 59 and 99 aa long) and hence considered to be non-functional [6].

We planned to experimentally confirm this deduction, by Cas9-induced mutations in the *Cctra*, which would impact both the male- and female-specific ORFs. Furthermore, the availability of an efficient single RNA to induce mutations in a female-determining gene would open the future possibility to develop a gene drive strategy aimed at manipulating the sex ratio and hence the reproduction rate of this harmful species [4]. CRISPR/Cas9 has been used in the Medfly genome to target autosomal genes having two copies for each cell [16, 21, 22]. After targeting the *white eye* Medfly gene by injecting into early embryos in vitro pre-assembled and solubilized Cas9 ribonucleoprotein complexes (RNPs), containing sgRNA, adults showed partial mutant phenotypes caused by somatic mosaicism [16]. The most extreme mutant phenotype consisted of a fly mosaic with one of the two eyes fully colorless. While biallelic mutations were observed only in somatic clones of the fly, the germ line transmission rate was very high, reaching 100% in one case. On the contrary, targeting a Medfly single copy gene, as the Y-linked *MoY,* in XY individuals by Cas9 ribonucleoproteins injections, 70% of mutant G_0 individuals showed intersexual phenotype and 30% were transformed into XY mutant G_0 females [8].

We reasoned that introducing loss-of-function frameshift mutations in the first *Cctra* exon with CRISPR/Cas9 would lead to mutant alleles coding for truncated CcTRA proteins. The expected truncations would affect not only the 429 aa long female-specific ORF, but also the carboxy-terminal ends of two male-specific 59 and 99 aa ORFs. A masculinization of XX individuals by permanent loss-of-function mutations of *Cctra* altering also the male-specific CcTRA polypeptides would support the previous suggestion that these products are indeed non-functional.

Since in *Drosophila tra* and *tra-2* mutant alleles are recessive, we reasoned that also in the Medfly the presence in the same cell and in its clonal descendants of only monoallelic indel (insertion/deletion) mutations in *Cctra* (+/− heterozygous state) would be insufficient to masculinize XX cells. Thus, in the somatic mutant clones of these XX individuals, the CcTRA protein expressed from the wild-type allele would lead to female-specific splicing of *Cctra* pre-mRNAs from both wild-type and mutant alleles, leading to female sexual phenotype. Hence, only biallelic loss-of-function *Cctra* mutations (Fig. 2; $Cctra^{1-}/Cctra^{2-}$) in the same XX cell and its cellular descendants would lead to a male-specific *Cctra* and the downstream *Ccdsx* RNAs, causing a partial (mosaicism) or full (very early and high biallelic mutagenesis) masculinization of XX adults.

Results

Cas9-RNP injections targeting *Cctra* lead to fully masculinized XX flies in the G_0 progeny

With the aim of inducing indels leading to frameshift mutations in *Cctra*, we used Cas9 RNPs injections as a delivery method. In particular, a CRISPR/Cas9 *Cctra* target site was chosen within the coding region of exon 1 on the antisense strand to design a single guide RNA, named sgtraEx1 (Additional file 1: Fig. S1A). The targeted 20 bp long sequence is about 20 bp upstream of the first donor splicing site and upstream to the female-

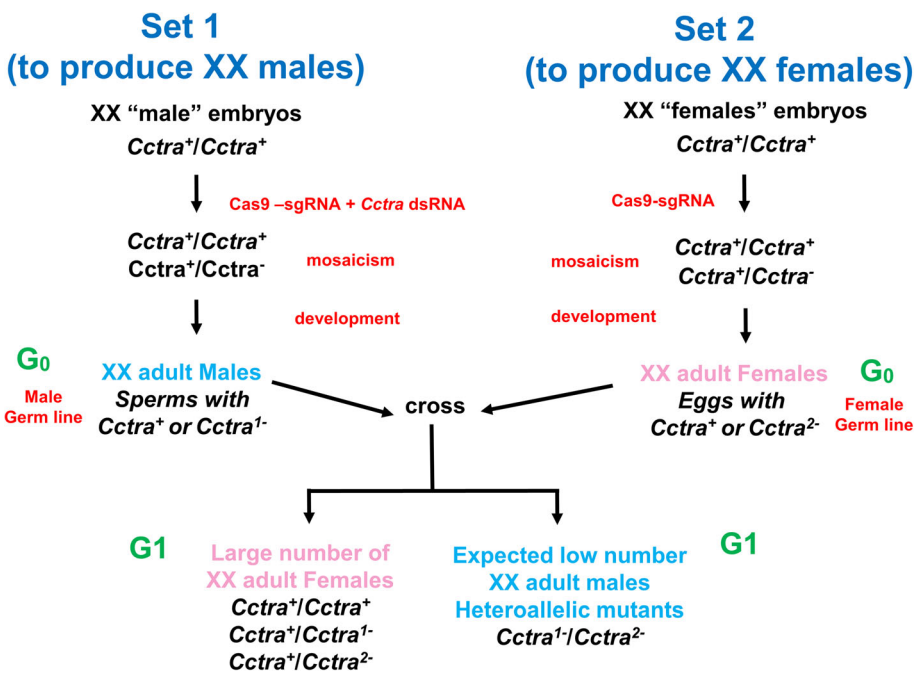

Fig. 2 Experimental design to produce G_1 XX males bearing heteroallelic mutation of *Cctra*. Two sets of parallel Cas9-RNP injections to target *Cctra* into XX embryos can lead to both G_0 XX males and XX females. The G_0 adults from the two sets can bear some *Cctra* monoallelic independent mutations (mosaicism) both in the soma and in the germ line. In set n. 1, the injection mix contains also *Cctra*-dsRNA to induce full masculinization of XX embryos (Pane et al., 2002). Hence, the G_0 XX males from set n. 1 can be crossed with G_0 XX females from set n. 2, to produce a G_1 XX progeny, expected to be composed mostly of XX females (carrying either none or monoallelic mutations) and of few G_1 XX males, carrying heteroallelic mutations of *Cctra* ($Cctra^{1-}/Cctra^{2-}$) inherited from the respective parents

specific long open reading frame encoding the CcTRA protein (Additional file 1: Fig. S1B). As described above, previous literature data showed that Cas9 RNPs applied to Medfly embryos led to a relatively low somatic biallelic mutation rate overall but a high rate in the germ line [16]. Based on this study, we speculated to observe masculinization of XX *Cctra* mutant individuals only at G_1 after crossing G_0 XY males and G_0 XX females developed from Cas9-injected. However, the discrimination of rare $Cctra^{1-}/Cctra^{2-}$ heteroallelic mutant XX males among the 50% of the G_1 progeny being XY males would have been tedious and challenging. We simplified their identification by planning to detect them among a G_1 female-only XX progeny, obtained crossing XX males and females obtained from RNP-injected embryos (Fig. 2). Among these G_1 XX females, $Cctra^{-1-}/Cctra^{2-}$ XX males would be easily detected even if very few, with the respect of the majority of XX females carrying either none or only one *Cctra* mutant allele (Fig. 2).

To produce a larger number of XX males (Fig. 2, set 1), from Cas9-injected XX embryos, a mix of Cas9-sgtraEx1 RNP and *Cctra* dsRNA, to efficiently masculinize XX, was injected into 370 XX embryos (Table 1, set n. 1). The aim was to obtain XX fertile males potentially carrying Cas9-induced *Cctra* monoallelic mutations also in the germ line. However, no pupae and adult flies emerged from this set of injections. When Cas9-sgtraEx1 RNP alone targeting *Cctra* were delivered into 400 XX embryos, few adults developed (3%; 12/400) (Table 1, set n. 2). It is likely that the co-presence of dsRNA+Cas9/sgRNA molecules could have a combined higher lethal effect for unclear reasons. Injections of dsRNA-*Cctra* alone resulted in 78 adults out of 200 XX embryos injected, with 73 XX being masculinized individuals and 5 being intersexes (Table 1, set n. 3). A similar survival rate was obtained when we injected sgtraEx1 RNA molecules but no effect (1 μg/μL), at a 5 times higher concentration being more susceptible to RNA degradation (70 females out of 160 injected embryos; Table 1, set n. 4).

Although upon Cas9-sgtraEx1 RNP injections we observed a very limited survival rate, the XX adults displayed some interesting features (Table 1, set n. 2). Indeed, the G_0 progeny was composed not only of six unaffected females, as we expected (Fig. 2 set 2), but also six XX males. These findings are suggestive of an unusually high rate of biallelic mutations. It is also worth noting that no intersexes were observed, suggesting an all-or-none effect on *Cctra* female-specific function of the Cas9 + sgtraEx1 injection.

Considering the apparently high efficiency of the ribonucleoprotein injections in set n. 2 (Table 1) in masculinizing 50% of the G_0 XX individuals, we reasoned that 1) also the XX females contained at least some somatic

Table 1 XX-only embryonic injection sets

Injection set	Cas9 delivery / []	sgRNA name / []	dsRNA name / []	XX embryos	XX Larvae	XX Females	XX Males	XX intersexes	*Cctra* male-specific in XX
1	Cas9 Protein / 1,8 μg/μL	sgtraEx1 / 200 ng/μL	dsRNA-Cctra / 0.5 μg/μL	370	48	0	0	0	–
2	Cas9 Protein / 1,8 μg/μL	sgtraEx1 / 200 ng/μL	–	400	60	6	6	0	yes in XX males
3	–	–	dsRNA-Cctra / 0.5 μg/μL	200	83	0	73	5	–
4	–	sgtraEx1 / 1 μg/μL	–	160	75	70	0	0	no
5	Plasmid-dCas9 / 1 μg/μL	sgtraEx1 / 1 μg/μL	–	290	37	7	0	3	yes
6	Cas9 Protein / 1,8 μg/μL	sgtraEx1 / 200 ng/μL	–	40	–	–	–	–	yes
7	Plasmid-Cas9 / 1 μg/μL	sgtraEx1 / 1 μg/μL	–	40	–	–	–	–	yes
8	Plasmid-dCas9 / 1 μg/μL	sgtraEx1 / 1 μg/μL	–	40	–	–	–	–	yes
9	buffer	buffer	buffer	40	–	–	–	–	no
10	–	sgtraEx1 / 1 μg/μL	–	40	–	–	–	–	no
11	Cas9 Protein / 1,8 μg/μL	–	–	40	–	–	–	–	no
12	Plasmid-Cas9 / 1 μg/μL	–	–	40	–	–	–	–	no
13	Plasmid-dCas9 / 1 μg/μL	–	–	40	–	–	–	–	no

clones bearing monoallelic *Cctra* mutations, though with no phenotypic effect, and that 2) a high mutagenic rate could also be present in the germ lines of these XX female adults as in the XX males. Assuming a 20–50% transmission rate of mutant alleles for each G_0 parent to the next progeny as previously observed in the Medfly [16], the probability to observe a double mutant individual in the G_1 progeny (Fig. 2) would be in a range of 4–25%. When we crossed among them the six XX males and six XX females from injection set n. 2 (Table 1), all 100 individuals of G_1 were females, indicating that the six XX masculinized fathers were fertile. However, the absence of XX males in the G_1 progeny indicated that, if any *Cctra* mutation was induced by Cas9 in the parental germ lines (male and female ones), the transmission rate was lower than 10% for each parent as the expected G_1 heteroallelic mutants frequency would be less than 1% of the progeny (hence not detectable among a number of 100 individuals).

Lack of indels in the targeted *Cctra* region in most cDNA clones from XX G_0 males and in all genomic DNA clones from G_0 XX females

The six females and six XX males, which composed the G_0 progeny of set n. 2, were analyzed respectively by RT-PCR and genomic PCR, to investigate *Cctra* splicing and DNA sequence of the targeted site.

The six reverted XX G_0 males showed only male-specific *Cctra* transcripts, as expected for adult flies having a full fertile male phenotype (Fig. 3). These data, together with the absence of female-specific *Cctra* mRNAs in all six males, suggested the presence of biallelic mutations in most, if not all, of the somatic cells of these XX G_0 males. Shotgun plasmid cloning of the RT-PCR reactions from the six XX G_0 males, followed by PCR colony screening of 30 clones (five colonies A-E, for each of the six XX males) led us to arbitrarily select 13 clones for sequencing (two or three cDNA clones for each male) (Additional file 1: Figure S2 and Figure S3)(Additional file 2). The splicing isoforms detected in the six XX males corresponded mostly to the know M1 and M2 *Cctra* male-specific isoforms. Out of 13 cDNA clones from the six XX males, seven correspond to male-specific *Cctra* isoform M1 (59 aa), two correspond to the male-specific isoform M2 (99 aa), one to a new splicing male-specific isoform (Male 1D encoding a 44 aa long protein isoform, named M3), one to the female-specific isoform (429 aa) and one to an unspliced longer isoform (male 5C) (Additional file 1: Figure S3; Additional file 2) [6]. Very surprisingly, despite the observed *Cctra* male-specific full shift, 11 cDNA clones showed

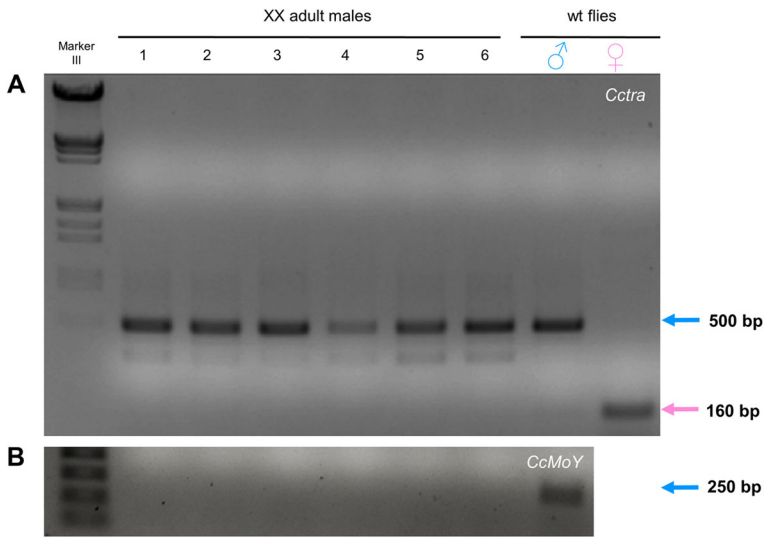

Fig. 3 RT-PCR analysis of reverted XX males. **(A)** The six XX males from injection set n. 2 (Table 1) showed only male-specific *Cctra* transcripts (500 bp) and shorter less abundant splicing variants. No traces of female-specific transcripts were visible in the gel electrophoresis (160 bp) **(B)** RT-PCR with *MoY*-specific primers indirectly confirmed the absence of the Y chromosome in these six fully reverted XX males and its presence in XY flies (positive control)

only wild-type sequences (Additional file 1: Figure S3). In one XX male, we have found a cDNA showing a 16 bp long deletion (cDNA 3E), in addition to a wild type cDNA clone (cDNA 3B) (Additional file 1: Figure S1D). The mutated cDNA encodes a 35 aa long CcTRA truncated protein. Another XX male contained two wild type (5B and 5D) cDNA clones and a mutated one lacking of 5 bp (5C) (Additional file 1: Figure S1C). This cDNA Male 5C encoded a truncated CcTRA protein of only 42 aa (Additional file 1: Figure S1C). The male 5C cDNA from unspliced *Cctra* RNA seems to correspond to a previously described RNA only present in adult females [6]. The female specificity of this unspliced product could be due to the binding of female-specific CcTRA/CcTRA-2 complex to the *Cctra* pre-mRNA required for the autoregulation. Interestingly, the *Cctra* unspliced isoform we found in one XX male contains also the Cas9-induced 5 bp deletion. We speculated that the 5 bp long deleted region of *Cctra* could be involved in enhancing the recognition of the 5′ donor site, which is only 20 bp downstream to the deletion by the spliceosome. We have found that these 5 bp are perfectly conserved in other Tephritidae *tra* orthologues, suggesting their requirement for proper *Cctra* male-specific default splicing (Additional file 1: Figure S1E). On the other hand, the cDNA 3E containing even a larger deletion (16 bp) of the same *Cctra* region performed a male-specific splicing, indicating that this sequence (14 bp out of 20 bp targeted sequence conserved in 5 distantly related Tephritidae species; Additional file 1: Figure S1E) is not strictly required to perform this alternative splicing but could be involved in performing the female-specific one. Collectively, these data showed intriguingly that the six XX males had a full switch from female-specific to male-specific splicing of *Cctra*, even in the presence of a very low (hence, mostly monoallelic) or even zero number of mutant *Cctra* alleles.

As the six XX females showed a normal female phenotype, female-specific *Cctra* splicing was expected to be found, even if they were carrying monoallelic *Cctra* mutations (Fig. 2). Hence, *Cctra* genomic DNA (rather than RNA or cDNA) was analyzed and eight plasmid clones showed only *Cctra* wild-type sequences (Additional file 1: Figure S4; Additional file 3). The lack of *Cctra* mutations in the DNA clones from all six G_0 females and in most of the cDNA from the six XX males is consistent with the absence of XX mutant heteroallelic $Cctra^{-1}/Cctra^{-2}$ males we observed in their G_1 progeny.

Embryos co-injections of dCas9 and sgtraEx1 lead to partially masculinized XX embryos and adult flies, indicating a long-lasting effect in the absence of mutations

We speculated that this Cas9 ribonucleoprotein complex was able to bind but not efficiently cut the *Cctra* DNA target site. The dead Cas9 (dCas9) is a mutant Cas9 which lacks of only the endonuclease activity and it can be used to perform transient transcriptional repression, named CRISPR interference, or iCRISPR [23–25]. We co-injected the sgRNA sgtraEx1 (1 μg/μl) with a plasmid bearing a dCas9 transgene under a *Drosophila* actin promoter, expecting that dCas9 would be produced after

transcription and translation and would bind the available sgRNA [24] (Table 1, set n. 5). A higher concentration of sgRNA (1 μg/μl; see mat. & meth.) was used in the injected mix because we expected that these RNA molecules being not pre-assembled with Cas9 before injections, could have been more exposed to degradation. This high concentration of sgRNA alone had no effect on *Cctra* female-specific splicing in injected XX embryos and in developed XX adults (Table 1, set n. 10; Fig. 4, B). Three out of ten XX adult females showed malformations of the gonadal apparatus, which suggested a mild masculinization effect (Additional file 1: Figure S5A). RT-PCR analysis of *Cctra* on RNA from these three XX adult flies confirmed the presence of also male-specific RNAs, indicating that the ovipositor malformations are likely the result of a partial masculinization during development (Additional file 1: Figure S5B). These data indicated that the dCas9 can induce a partial masculinization of XX embryos and a stable shift toward male-specific splicing of *Cctra* likely in some somatic clones.

We investigated the sex-specific splicing pattern of *Cctra* in XX-only embryos, injected at 1 h after egg laying, and developed for additional 14 h (set n. 6–13). Injections of Cas9 recombinant protein+sgtraEx1 RNP (set n. 6), or co-injections of sgtraEx1 with plasmids encoding either Cas9 (driven by E1 early promoter [23]) or dCas9 (set n. 7 and set n. 8) led to the appearance of additional male-specific *Cctra* transcripts (Table 1, Fig. 4A). Injections of only buffer, sgtraEx1, Cas9 protein, Cas9-encoding and dCas9-encoding plasmids had no effect (Fig. 4B). These data suggest that, as Cas9, also dCas9 can induce male-specific splicing of *Cctra* during the first hours of development. Hence, no *Cctra* mutations are required for this splicing change.

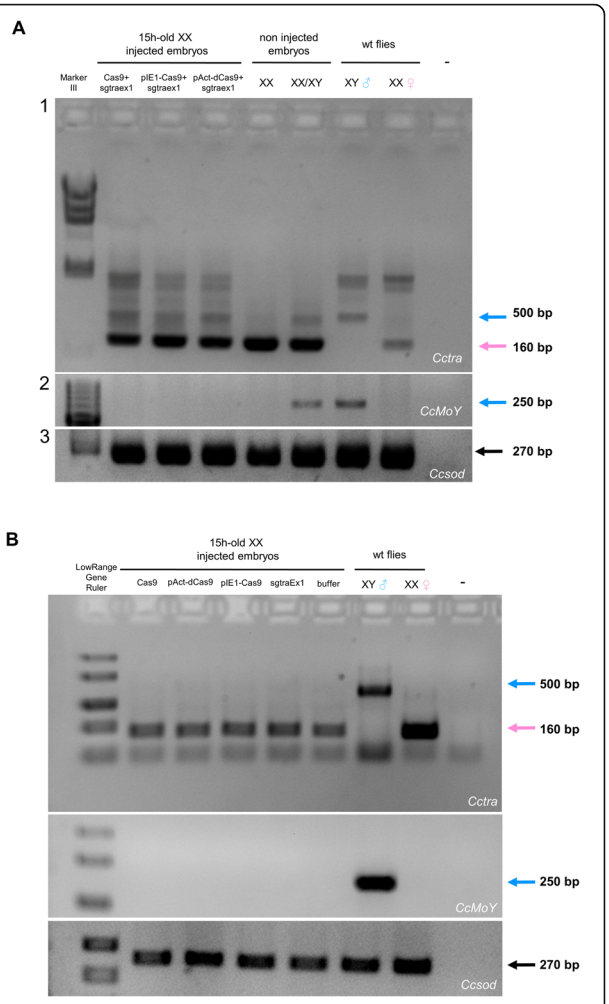

Fig. 4 Molecular partial masculinization of XX embryos by dCas9 targeting *Cctra*. **(A)** RT-PCR analysis of *Cctra* sex-specific transcripts in 15 h old XX embryos, following injections of various samples (Table 1, set n. 6–8) at 1 h after egg laying (Panel 1). The XX embryos were injected respectively with Cas9 protein+sgtraEx1 (first lane), with sgtraEx1 + Cas9-encoding plasmid (pIE1-Cas9; second lane) and with dCas9-encoding plasmid (pAct-dCas9) (third lane). Male-specific *Cctra* transcripts were detected (500 bp cDNA fragment) in all three samples of injected XX embryos, in addition to the female-specific transcripts (160 bp cDNA fragment). RT-PCR analysis of the Y-linked *MoY* gene indirectly confirmed the absence of Y chromosome in all 3 samples and in XX females, and its presence in a mixed XX/XY embryos sample and in adult XY flies (Panel 2). *CcSOD* positive control is shown in panel 3. **(B)** Negative controls. RT-PCR of *Cctra* sex-specific transcripts in 15 h old XX embryos, following injections at 1 h after egg laying (Table 1, set n. 9–13). The XX embryos were injected respectively with Cas9 protein, Cas9-encoding plasmid, dCas9-encoding plasmid, with sgtraEx1 and with buffer alone. Only female-specific *Cctra* transcripts were detected (160 bp) in all samples of injected XX embryos (Panel 1). In Panel 2 and 3, the control of the karyotype and positive controls, conducted as in A, are shown

Discussion

The Cas9-sgtraEx1 RNPs injected into XX Medfly embryos led to a masculinization of 50% of XX individuals. All six XX males showed by RT-PCR and cDNA sequencing analyses mostly male-specific *Cctra* isoforms, concordant with the observed male phenotype. Unexpectedly, no indel mutations were detected in cDNA fragments from the Cas9 targeted site in four out of six males. Two XX males showed a mix of wild type and mutated cDNA fragments, suggesting the presence of monoallelic mutations in some cellular regions. XX males and XX females from injected embryos produced a G_1 consisting of only XX females, which indicated absence of homozygous individuals for mutant *Cctra* alleles. We concluded that very low mutation rate, if any, was reached not only in the soma but also in the germ line of injected G_0 individuals. In contrast, Meccariello et al. [16], observed that mutant mosaic G_0 flies

mutagenized by Cas9 in the *white-eye* gene transmitted germ line mutations to the progeny at a very high rate (up to 100%). Hence, the Cas9-sgtraEx1 is likely very inefficient in mediating Cas9 gene disruption but surprisingly very efficient in masculinizing XX individuals. These data suggested that the Cas9 ribonucleoprotein promoted masculinization by inducing male-specific *Cctra* splicing in 50% of XX embryos which developed into adults by a mechanism different than gene disruption.

We reasoned that the sgRNA designed for the CRISPR experiment and/or the *Cctra* targeted region structural features posed limitations in the second step of the Cas9 action, namely the endonuclease activity, but not in the first step, the binding to the *Cctra* genetic locus. Indeed, a two-state model for Cas9 binding and cleavage was recently proposed: a seed match triggers binding but only extensive pairing with target DNA leads to cleavage [26]. Target sequence mismatches can induce the Cas9-RNP complex to bind also off-target sites without DNA cleavage, because the transition to the active conformation is prevented [26, 27]. Co-injections of sgtraEx1 and a plasmid encoding dCas9 into XX embryos induced a partial shift of *Cctra* splicing toward male-specific pattern after few hours of development and the development of XX females (three out of ten) with malformed ovipositor. These females were found to be molecular intersexes, as they showed both female-specific and male-specific *Cctra* isoforms. Also, these malformed females showed a mix of male and female-specific *Cctra* transcripts and, hence, correspond to partially masculinized individuals (intersexes) [6]. Similar malformed ovipositors were observed by Pane et al. [6] following embryonic RNA against *Cctra*. The lack of fully masculinized XX individuals in the progeny of ten individuals suggests a reduced efficiency of dCas9 in masculinizing XX individuals. However, this could be due to the different delivery methods of Cas9 and dCas9. In the first case, purified recombinant Cas9 ready to act was injected, while in the second case a dCas9 encoding transgene was transcribed from a *Drosophila* actin promoter, after embryos injections of a plasmid. Lower efficiency is to be expected in the second delivery method, due to the transcription and translation steps required to express dCas9. The transient binding of Cas9 RNPs to the 5′ *Cctra* DNA region could have reduced the *Cctra* zygotic transcription during the first hours of embryogenesis and, hence, the accumulation of female specifically spliced *Cctra* mRNA, promoted by the maternal *Cctra* contribution in XX embryos. This transient reduction of newly transcribed *Cctra* female-specific mRNA and of the encoded CcTRA protein in the XX embryos could have blocked the establishment of *Cctra* female-specific autoregulation leading to a negative epigenetic effect on *Cctra*. Similarly, a transient depletion of *Cctra* mRNA by embryonic RNAi led to a collapse of its positive autoregulation and to obtain XX fertile males expressing male-specific *Cctra* RNAs [6].

Conclusions

The question if CcTRA male-specific ORFs are required for male-specific *Cctra* splicing remains still open, as biallelic *Cctra* mutant XX males were not obtained. The second question if the chosen sgtraEx1 guide RNA is suitable for future gene drive strategy aimed to efficient mutagenesis by Cas9 had a conclusive and negative reply, as very low mutagenesis rate was observed. However, these data support the hypothesis that the transient binding of the Cas9-sgtraEx1 ribonucleoprotein complex on the first *Cctra* exon during the first hours of embryogenesis led to a repression of the establishment of the *Cctra* female-specific autoregulation in XX embryos even in the absence of induced mutations. Our study raises new general issues concerning the use of CRISPR/Cas9 method. We serendipitously uncovered a novel problem of unplanned stable changes in the expression of genes able to autoregulate, which calls for further investigation. If a Cas9 + sgRNA binds to off target sequences of autoregulating bistable genes, this event can provoke long lasting epigenetic effects even in the absence of DNA mutations.

Methods

Rearing of *Ceratitis capitata*

Wild type (WT) and transgenic Medfly lines were maintained under standard rearing conditions. The WT *Benakeion* strain, which has been reared in laboratories for more than 20 years, was obtained from P. A. Mourikis 30 years ago (Benakeion Institute of Phytopathology, Athens, Greece). The strains were reared in standard laboratory conditions at 25 °C, 70% relative humidity and 12:12 h light–dark regimen. Adult flies were fed yeast/sucrose powder (1:2).

RNP complex assembly and injections

Cas9 was expressed as his-tagged protein and purified from bacteria [16, 19]. sgRNA was designed using the CHOPCHOP online software [28]. The lack of SNPs within this 20 nt long sequence in three different *Ceratitis* lines (Benakeion, used in this study, ISPRA [22] and FAM18 *Ceratitis* [8]) suggested that no Cas9-resistant *Cctra* alleles would be already present in individuals of these lab strains. Template for sgRNA in vitro transcription were generated by annealing two complementary oligonucleotides (PAGE-purified, Life Technologies) as previously described [16, 21], using the primers F-sgtraEx1 and Reverse-Crispr from Life Technologies (Additional file 4). sgRNA was synthesized according to

instructions of the Megascript® T7 kit (Ambion) with 1 μg of DNA template and a 5′ flanking T7 promoter. After RNA synthesis, the template was removed by incubating with TurboDNase® (Ambion) for 15 min at 37°. Prior to the injection, the RNP complex was prepared by mixing 1.8 μg/μL of purified Cas9 protein with approximately 200 ng/μL of sgtraEx1, containing 300 mM KCl [16]. The mix was incubated for 10 min at 37 °C. A glass needle was filled with the pre-loaded sgtraEx1-Cas9 mix and the injection was performed into the posterior end of embryos collected 45 min after egg laying as described for RNA interference in *Ceratitis capitata* [6]. When injecting sgRNA alone, a 5 times higher concentration (1 μg/μL) was used in the injected mix, because we expected that these RNA molecules, being not pre-assembled with Cas9 before injections, could have been more exposed to degradation.

RNAi and XX-only progeny production

A *Cctra c*DNA 800 bp long fragment was PCR amplified using RNA from female adults of *C. capitata* and longer 164+/900- primers, introducing a T7 promoter sequence at each extremity. In vitro transcription of *Cctra* dsRNA was performed using the Ambion MEGAscript® RNAi kit T7 RNA polymerase, following manufacturer instructions. Embryonic RNAi (0.5 μg/μL dsRNA solution) was used to repress *Cctra* in XX/XY embryos and to produce male only progeny. Single males from this progeny were crossed with three females in small cages and the crosses having XX males were identify by Y-specific PCR (Y-specific primers) [8] on a small sample of laid embryos.

dCas9 encoding plasmid and injections

Plasmid expressing dCas9 under the control of the *Drosophila melanogaster* actin promoter was kindly provided to GS by Lenny Rabinow (Perrimon's lab, Harvard, USA) [23]. A mix containing 1 μg/μL of pAct-dCas9 plasmid and 1 μg/μL of sgtraEx1 transcribed in vitro into the posterior end of embryos. We used 5 times higher concentration of sgRNA because we expected that these small RNA molecules being not pre-assembled with Cas9 before injections, could have been more exposed to degradation.

RNA extraction and RT-PCR

Total RNA was extracted from pools of embryos, intersexes, male and female adults using TRIzol® Reagent (Invitrogen®) following manufacturer instructions. Oligo-dT-primed cDNA was prepared from DNAse-treated total RNA using EuroScript® m-MLV reverse transcriptase (Euroclone®). RT-PCR expression analysis was performed with the following primers: *Cctra* 164+/320-, *CcSOD+/CcSOD-* and *CcMoY A+/CcMoY A-* (Additional file 4). For XX malformed females obtained from XX only embryos with dCas9 and sgtraEx1 *Cctra* RT-PCR was performed using *Cctra* 164+/900- primers pair, annealing on the same exon as *Cctra* 320- primer (Additional file 4). Gel electrophoresis diagnostic amplicon run was performed using Marker III (Lambda genomic DNA digested with EcoRI/HindIII) or 100 bp ladder from Thermo Scientific®.

DNA extraction and molecular analysis

DNA extraction was performed, with minor modifications, according to the protocol of Holmes and Bonner et al. [29]. Adult XX female flies G_0 were placed in a 1.5 ml tube and manually crushed with a pestle in 200 ml Holmes Bonner buffer (Urea 7 M, 281 Tris-HCl 100 mM pH 8.0, EDTA 10 mM pH 8.0, NaCl 350 mM, SDS 2%). Subsequently, DNA was purified by phenol/chloroform extraction, followed by chloroform extraction and ethanol precipitation. The pellet was resuspended in 30 μl water containing RNase A.

cDNA and gDNA cloning and sequencing

PCR cDNA fragments from XX adult males were cloned into pGEM-T Easy Promega® vector according manufacturer instruction. PCR colony screening was carried out using 164+ and 320- primers. Positive colonies were used to extract plasmid DNA which was sequenced using Applied Biosystem® Big Dye v 3.1. Genomic DNA from the six G_0 XX females was used as template to amplify the region encompassing the target sites, using the primers *Cctra* 164+ and *Cctra* 164-Rev (Additional file 4). DreamTaq (Life Technologies) polymerase was used for PCR amplifications according to the manufacturer's instructions. The PCR products were purified with StrataPrep PCR Purification Kit (Agilent Technologies) and subcloned using StrataClone PCR cloning Kit (Agilent Technologies). Positive clones were sequenced by Sanger method and ABI 310 Automated Sequencer (Applied Biosystems) using the primer *Cctra* 164+ (Additional file 4).

RNA extraction from injected embryos after 15 h of development

The pools of 40 embryos injected with various mixes (Table 1) were let develop for 15 h at 25 °C, 70% relative humidity. The embryos were then detached from the cover slip using heptane, which dissolves the glue, and collected in a 1.5 mL tube. They were then washed three times with 1X PBS to remove heptane before RNA extraction was performed using TRIzol® Reagent (Invitrogen®) following manufacturer instructions. The RNA samples were analyzed for *Cctra* splicing pattern by RT-PCR using the primers *Cctra* 164+ and *Cctra* 900- (Additional file 4).). The cDNA and genomic sequences

were deposited at the GenBank database with the following accession numbers: MW200161 to MW200180.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12863-020-00941-4.

Additional file 1.
Additional file 2.
Additional file 3.
Additional file 4.
Additional file 5.

Abbreviations

SIT: Sterile Insect Technique; *ORF*: Open Reading Frame; *CRISPR*: Clustered Regulated InterSpaced Palindromic Repeats; *RNP*: Ribo-Nucleo-Proteic Complex; *dsRNA*: double strand RNA; *sgRNA*: single guide RNA; *iCRISPR*: interference CRISPR; *dCas9*: dead Cas9; *SNP*: Single Nucleotide Polymorphism; *RNAi*: RNA interference; *WT*: Wild-Type

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Authors' contributions

AM, AG, MGI, GDC, performed set n. 1–4 embryonic injections (Table 1) and the embryonic injections of *Cctra* dsRNA+Cas9 + sgtraEx1, which led to 100% lethality. PP performed PCR, cloning and sequencing analyses of the 6 XX males and 6 XX females from of injection set n. 2 (Table 1). PP and GSO performed injection set n. 5 and RT-PCR of the 3 XX malformed females. PP and GV performed injection set n. 6–9 and GV performed RT-PCR analyses on the 15 h-old injected embryos. PP performed the cross of G_0 males and females from set n. 2 and visually screened the G_1 progeny. AM, PP, AG, MGI, GDC, GSO and GV maintained the Medfly strains, preparing larval and adult food, performing crosses and collecting embryos. GSA wrote the paper, with the inputs of MDR, SA, MS, and AM. GS prepared the figures, with contributions of AM, MS and PP. All authors contributed with minor corrections/editing of the manuscript, especially AG. All the authors have read and approved the final version of the manuscript.

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Availability of data and materials

The cDNA and genomic sequences were deposited at the GenBank database with the following accession numbers: MW200161 to MW200180.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare absence of competing interests.

Author details

[1]Department of Biology, University of Naples Federico II, 80126 Naples, Italy. [2]Department of Molecular Life Sciences and SIB Swiss Institute of Bioinformatics, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland.

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