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SECTION: MOLECULAR SYSTEMATIC

XXV CYCLE

**Research and molecular and functional characterization of
genes of the sex determination in the *Ceratitis capitata* and
Aedes aegypti dipterans**

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ABSTRACT

Il meccanismo di determinazione del sesso nel dittero *Ceratitis capitata* è caratterizzato dalla presenza di un fattore M (non ancora isolato molecularmente) sul cromosoma Y che influenza in maniera diretta o indiretta lo splicing maschio-specifico del gene chiave *Cctransformer* (*Cctra*) in embrioni XY fino a stadio adulto. In embrioni XX, *Cctra* viene maturato mediante splicing femmina-specifico controllato dalla proteina CcTRA che forma un complesso con la proteina CcTRA-2. Tale complesso attiva lo splicing femmina-specifico dei geni a valle *Ccdoublesex* (*Ccdsx*) e *Ccfruitless* (*Ccfro*). Per quanto riguarda il meccanismo di determinazione del sesso del dittero *Aedes aegypti*, non si è conoscenza di un segnale primario che regoli il sesso e l'unico gene che è stato isolato è l'omologo del gene *dsx* (*Aeadsx*), presente a valle di tale cascata. L'attività di ricerca svolta durante il dottorato è stata incentrata sulla ricerca di geni che fossero coinvolti nella determinazione del sesso di entrambi i ditteri *C. capitata* e *Ae. aegypti*. Per quanto riguarda *C. capitata*, è stato analizzato il clone BA11, ottenuto dall'analisi di sottrazione molecolare e Real-Time PCR, prodotti su campioni di RNA estratti da embrioni misti XX-XY e soli XX a 8-10 ore dopo la deposizione. L'analisi bioinformatica e molecolare ha portato ad ipotizzare la presenza di due paraloghi in *Ceratitis*, di cui uno solo Y-linked, mentre un secondo che produce un trascritto espresso in entrambi i sessi. L'analisi funzionale del clone Y-linked, mediante RNA interference, non ha validato tale ipotesi.

Successivamente, sono stati prodotti due sequenziamenti mediante tecnologia illumina a partire da RNA di embrioni misti XY-XX e soli XX a 8-10 ore. I dati grezzi di sequenza sono stati assemblati de novo usando il software Trinity ed operata una sottrazione in silico che ha identificato il clone 10393, perfettamente identico al clone BA11, ma con una sequenza più lunga sia all'estremità 5' che 3'. L'analisi molecolare ha identificato il trascritto maschio-specifico che verrà usato per la produzione di un mRNA sintetico per iniezione in embrioni di *C. capitata*.

Per quanto concerne l'analisi di possibili geni coinvolti nella determinazione del sesso di *Aedes aegypti*, è stato identificato l'omologo del gene *fruitless* (*Aeafro*). Sono state effettuate analisi di RT-PCR sul gene *Aeafro*, usando l'RNA totale estratto a diversi stadi, da embrioni fino ad adulti, al fine di analizzare il pattern di espressione. Tali analisi hanno evidenziato la presenza di trascritti che sono maturati in maniera sesso-specifica.

Infine, studi bioinformatici su un gruppo di geni di *Ae. aegypti*, che presentavano un dominio RNA-binding di tipo RRM, hanno portato all'identificazione di un gene, *AeaF-SR1*, ipoteticamente coinvolto nella determinazione del sesso di *Aedes*. Le analisi di RT-PCR hanno evidenziato la presenza di un trascritto femmina-specifico a partire da stadio larvale di terzo e quarto instar. Tale trascritto verrà usato per esperimenti di RNA interference al fine di valutare un ipotetica funzione all'interno della cascata di determinazione del sesso di *Ae. aegypti*.

1 INTRODUCTION

Sex determination is the mechanism that allows the embryo to develop in male or female. In living organisms there are two main mechanisms of sex determination: genetic sex determination and environmental sex determination. In the latter case the control of sexual development is due to particular environmental signals, such as temperature (as alligators and turtles), local sex ratio (as with some tropical fish), or population density (as with mermithid nematodes). In the first case, males and females have chromosomal differences, different alleles or even different genes that specify their sexual morphology. In most of mammals, including humans, males have two distinct sex chromosomes (X and Y, heteromorphic chromosomes), while females have two identical XX sex chromosome (homomorphic).

The great variability of sex determination genetics are well represented by insects. In this class of arthropod, there are all kinds of genetic sex determination known to date.

1.1 SEX IN INSECTS

With around one million species and possibly several times that number still to be classified, insects are a group of living organisms in which we can investigate different molecular genetic mechanisms of sex determination. Insects can be found in almost all terrestrial and freshwater habitats and few species are even marine. Thanks to this huge variability insects have attracted researchers interested in evolutionary studies of the different mechanisms of sex determination. Indeed, insects are convenient organisms to study this topic, because these mechanisms evolve very quickly and offers a unique opportunity to understand the molecular dynamics that lead to the evolution of hierarchies of regulatory genes involved in a developing sex determination.

It is useful also to compare them with the mechanism of sex determination of dipteran *Drosophila melanogaster*, which represents the model system of sex determination in insects.

During the last decades, a number of genetic studies have been conducted on sex determination in different insect species belonging to different orders; many of this species have a strong economic impact, because are harmful to the agriculture or are disease vectors.

1.2 CERATITIS CAPITATA

Ceratitis capitata, the Mediterranean fruit fly, or medfly, belongs to the Tephritidae family, a group of about 4,000 species, and represents one of the world's most destructive fruit pests. It is a highly polyphagous species whose larvae develop in a very wide range of unrelated fruits (Fig. 1).

C. capitata originates in tropical Africa, from where it has spread to the Mediterranean area during the early 19th century and from there to the rest of world, thanks to its ability to adapt to cooler climates better than most other fruit flies species (Fig. 2). This pest affects more than 260 different fruits, flowers, vegetables and nuts, producing a devastating impact on trade and the economy of the producing companies and even States. The adults of *Ceratitis capitata* are 4 to 5 mm long and have a pronounced sexual dimorphism. Females are larger than males and can be identified by a pointed ovipositor (about 1.2 mm long) which is used to deposit eggs within the host fruit (Fig. 3). Males have more brightly colored eyes and a pair of supra-fronto-orbital bristles.

The adult body is protected by an exoskeleton made of chitin and the general color is yellowish with a tinge of brown, especially the abdomen, the legs and some of the markings on the wings. The oval shaped abdomen is clothed on the upper surface with scattered black bristles and has two narrow, transverse, light colored bands on the basal half. The wings are broad, translucent and embellished with patterns of brown, yellow, black and white. The head of the male bears a pair of spatulate appendages which have sharp-pointed ends and the color of the spatulate sections is black. The time required for the Medfly to complete its life cycle is 21-30 days, under tropical conditions (Fig. 4). Eggs of *C. capitata* are slender, curved, smooth and shiny white. They are deposited inside the fruit and often females may use the same hole to lay more than 75 eggs clustered in one spot.

The eggs after 2-3 days develop into the first instar larvae and continue their development for 10 days inside the host fruit. These larvae reach a length of 7-9 mm, changeable in relation to the food availability. The color of larvae are white-yellowish, at the front of the head show sensory structures and the mouthparts. At the stage of third instar, the larvae get out of the fruit and jump into the soil to become pupae. After about 10 days at the pupal stage, the fly comes out of the same and is ready to start its biological cycle. The average life of *C. capitata* is about 30 days.



Figure 1. Damages to fruits by *C. capitata*.

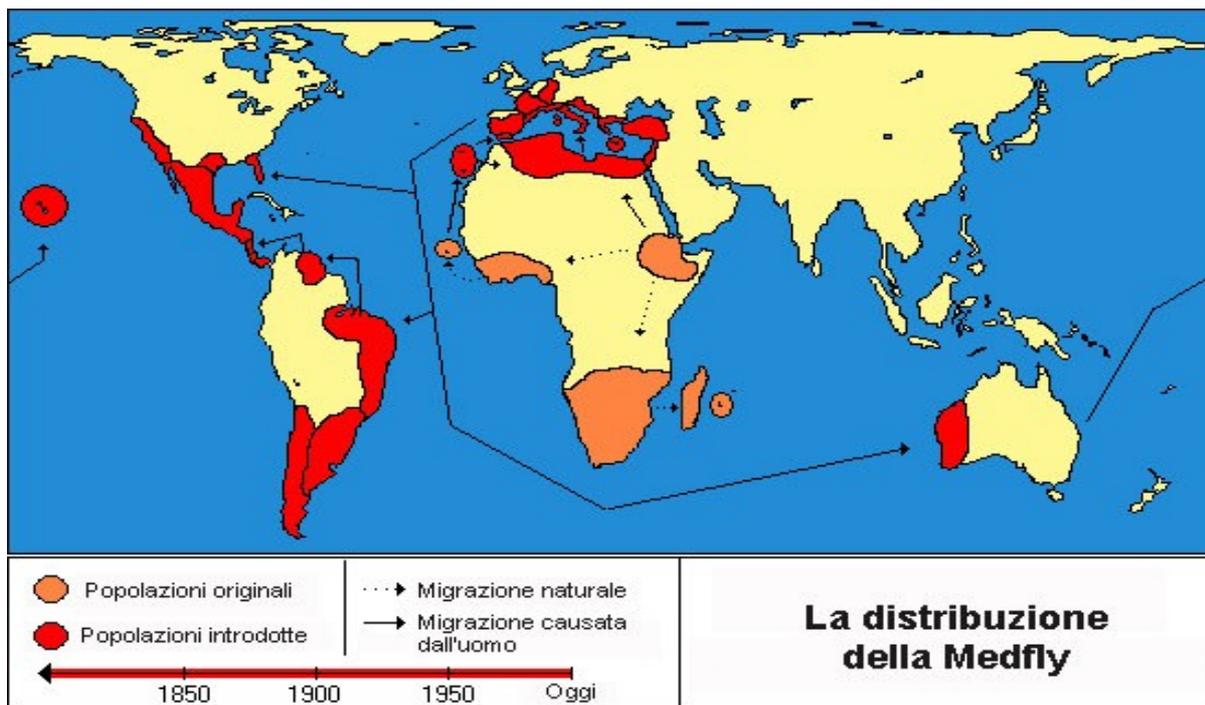


Figure 2. World-wide distribution map of *Ceratitidis capitata* Pest.

Ceratitis capitata

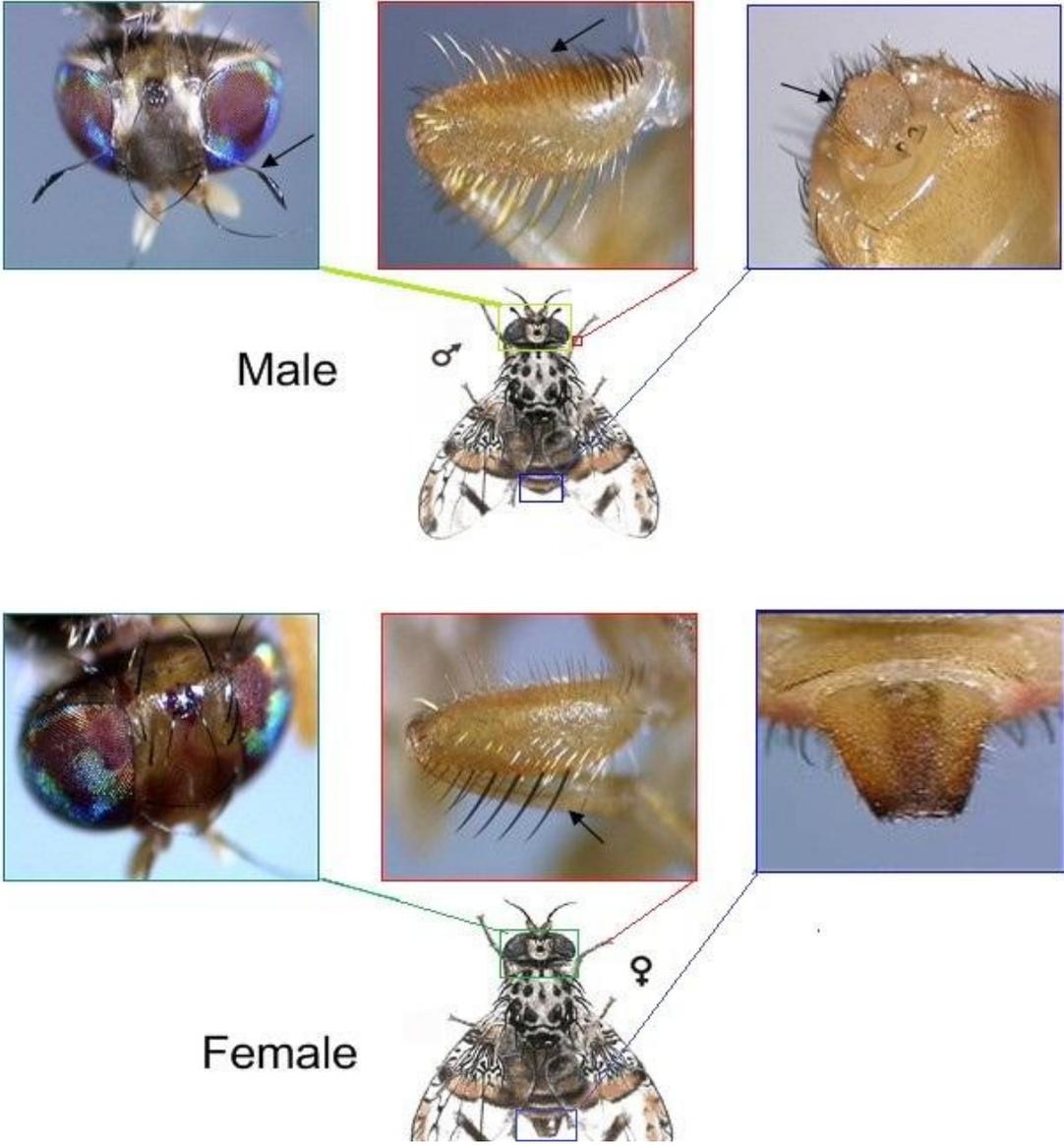


Figure 3. Sexual dimorphism of *C. capitata*.

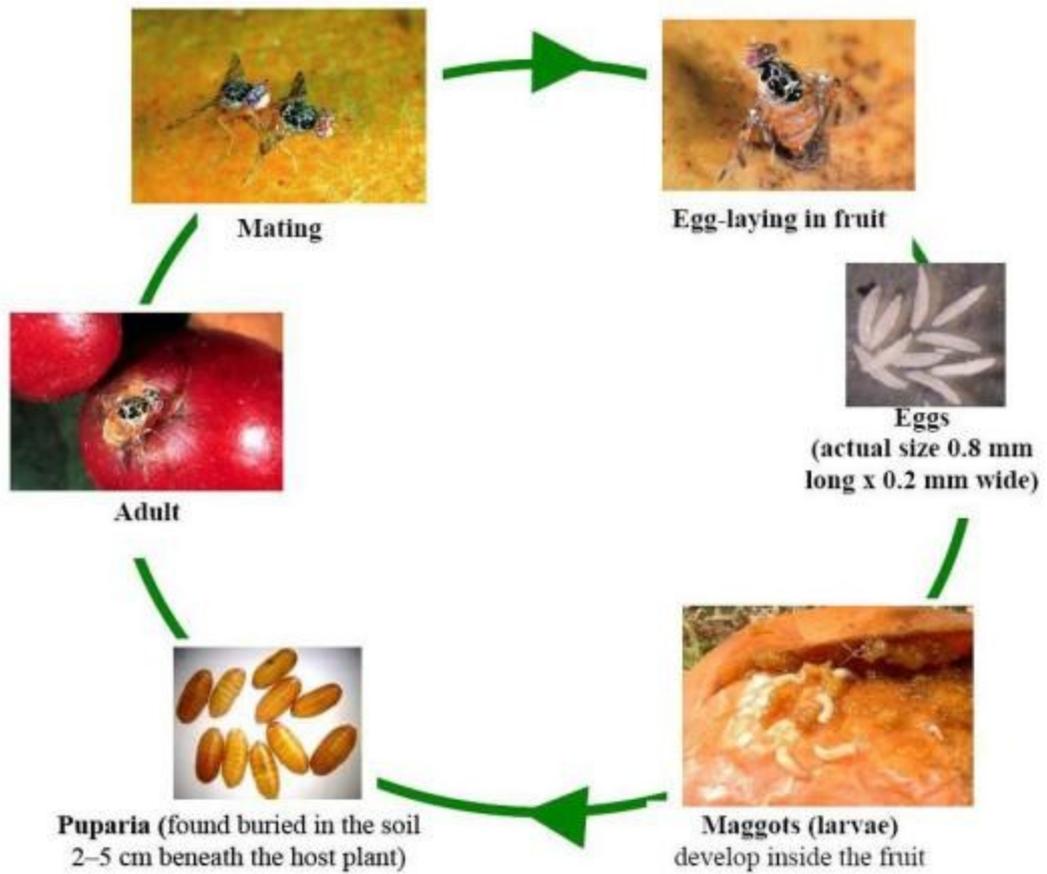


Figure 4. Life cycle of *C. capitata*.

1.3 SIT

The sterile insect technique (SIT) is the first method involving insect genetics for population control of pest species, such as *Ceratitis*. It is a program that can be applied only to pest species that reproduce sexually and that can be mass reared artificially. The target pest species is adapted to the lab, then industrially mass-reared, sexed, sterilized and released within reasonable proximity of all native females, not only limited to selected zones. This leads to the decrease of progeny by competition of sterilized males with wild type males for wild type females. The idea of controlling economically relevant insect species was understood in the late 1930s by an American entomologist, Dr Edward F. Knipling. A similar concept was conceived independently by the Soviet geneticist Serebrovsky (1940). Knipling and his colleague Bushland were working on screwworm fly problem, a serious enemy of warm-blooded animals, including humans, in an area extended from Argentina to the southern USA and were trying to find an efficient way to eliminate the entire screwworm population. Bushland researched also chemical treatment of screwworm-infested wounds in cattle, Knipling developed the theory of autocidal control-breaking the life cycle of the pest itself. In 1954, the technique was first successfully used in the field to control the screwworm fly in Curacao (Netherlands Antilles). However, a method to induce sexual sterility had already been devised during the 1920s by Herman J. Muller at the University of Texas. He had used a dentist's X-ray machine to induce mutations in the genes and chromosomes of the *Drosophila melanogaster*.

Since then, SIT was used to control and eradication of others pest species in many countries, for example, it has been used against the Mediterranean fruit fly in Mexico and California, melon fly (*Bactrocera cucurbitae*), tsetse fly (*Glossina* species), and so many other insect of different genera. The application of SIT against medfly focused initially on the concept of eradication (Hendrichs et al., 2002). The first SIT program was initiated in southern Mexico in 1977, to prevent the spread of medfly, with the construction of a 500 million per week sterile fly mass rearing facility in Tapachula. Until 1980, the medflies were controlled by applying malathion-bait sprays.

However, the use of high dose of radiation in SIT program produced low competitiveness of flies. Andrewartha and Birch (1960) suggested that the efficiency of the SIT could be improved through the release of only sterile males insects and Whitten (Whitten et al., 1969) was the first to propose the genetic sexing strains (GSS) using male-linked translocation. In *Lucilia cuprina* a pupal color mutation was used to develop a sexing method; this early studies paved the way for the development of this method in medfly for SIT programs. Two sexing systems were produced which are based on male-linked translocations, one using a pupal colour mutation, *white pupae* (*wp*), and

the second using a *temperature sensitive lethal* mutation (*tsl*) (Robinson, Franz and Fisher, 1999). The *tsl* is used to eliminate the females by raising the temperature during egg incubation. Following development of transgenesis in medfly (Loukeris et al., 1995; Zweibel et al., 1995), novel sexing methods and control-strategies have been developed.

Another system for insect pest *C. capitata* is transgenic embryonic lethality system that causes complete reproductive sterility (Schetelig et al., 2009). This system is based on the use of an early embryonic lethal transgene combination and when transgenic males carrying this system are mated with wild type females, all progeny die during embryogenesis.

The RIDL system (Release of Insects Carrying a Dominant Lethal Gene) consists of introducing a lethal dominant gene (tTA protein activating its own transcription by a positive feedback loop) under control of a female-specific promoter such as the vitellogenin gene *yp* (Thomas et al., 2000) or of a female-specific spliced intron (Fu et al., 2007). The expression of the female-specific lethal transgene could be inactivated by adding the tetracycline as antidote, which is removed from the system when is required the separation of males and females, causing the death of all females.

This system has been shown to be functional in *Drosophila* and *Ceratitidis* transgenic flies with 100% of females died when tTA is over expressed.

New testes-specific genes have been identified and isolated in lepidopteran and dipteran species to develop transgenic sexing strains, based on automatic sorting of male only larval progeny (Franz and Robinson, 2011; Catteruccia et al., 2005). Improving of large scale expressed sequence tagged sites (ESTs) for *C. capitata* potential sex-specific genetic functions have been identified.

SIT was advanced and promoted by the International Atomic Energy Agency (IAEA) and the Food and Agricultural Organization of the United Nations (FAO). To date the sterile insect technique has been one of the major subjects of IAEA and FAO to be world-wide promoted, involving both applied research to improve the technique and to develop it for new pest species so that they can benefit from improved plant, animal and human health, cleaner environments, increased crop and animal production in agricultural systems, and accelerated economic development.

1.4 SEX DETERMINATION IN *DROSOPHILA MELANOGASTER*

The sexual differentiation in *D. melanogaster* is a process that involves the specific morphology of the two sexes, the physiology, the behavior, the dosage compensation and the sex-specific development of the germline. These differences occur through the activation of a series of genes ordered in a hierarchy of regulation. The control of their expression is mainly post-transcriptional and is realized through alternative splicing that produces different transcripts in the two sexes (Fig. 5).

In *D. melanogaster* chromosomal signal determines sex; diploid flies with two X chromosomes are female and those with one X are male. The primary signal is due to the ratio of the number of numerators genes on X chromosomes to the number of denominators genes on autosomes, called X:A ratio. Generally, when the X:A equals or greater than 1.0 leads to a female, while the X:A equals or less than 0.5 leads to a male (Keyes et al., 1992). Recent evidences point to the number of X chromosomes rather than the X:A ratio as the primary signal (Erickson and Quintero, 2007). The X chromosome encodes several involved transcription factors, like *runt*, *sisA* and *sisB*, which are indicated as *X-linked signaling elements* (XSE), that in female transcriptionally activate the *Sxl* gene. In male the single X chromosome results in the absence of the SXL protein. SXL protein contains two highly conserved RRM-type RNA binding domains and is involved in three different levels of regulation: selection of the developmental pathway, the maintenance of a heritable commitment to the pathway and his expression (Salz H. et al, 1989). Genetic and molecular studies have shown that *Sxl* controls the activity state of genes downstream in the sex determination pathway and dosage compensation system. *Sxl* has, in his gene organization, 10 exons and 9 introns and is transcribed from two alternative promoters, which are turn on in two different stages of embryonic development. In the early stages of XX embryonic cells (2 hours from oviposition), the primary signal active the transcription of *Sxl* from the early promoter P_E , that yields a transcript spliced to encode a functional early SXL protein. This splice pattern depend on the use of the 5' splice site from the early exon E1, while in XY embryos in later stages is used the 5' splice site of late exon 2. (Zhu et al., 1997). The use of a different promoter results in XY embryos in the default exclusion of exon 3, that includes stop codons, in the early *Sxl* transcript. In XX embryos, the early SXL promotes female-specific splicing of *Sxl* pre-mRNA and enables the production of a functional late SXL protein. SXL is important to maintain female-specific *Sxl* splicing by auto regulation during all development (Cline, 1984).

SXL regulates also female-specific splicing of *transformer* (*tra*) pre-mRNA by binding to a polypyrimidine tract in the first *tra* intron to use the female-specific 3' splice site in exon 3 instead

of the non-sex-specific 3' splice site in exon 2 (Sosnowski et al., 1989). This *tra* transcript encodes a functional TRA protein in XX embryos, which interacts with Transformer2 (TRA-2) protein, which is non-sex-specific, to form a heterodimer, via its arginine-serine (RS) domains. TRA along with TRA2 binds *doublesex* (*dsx*) pre-mRNA through *dsx* repeat element (*dsxRE*) situated in the middle of exon 4; *dsxRE* contains six copies of the 13 nucleotide sequence TC(T/A)(T/A)C(A/G)ATCAACA (Inoue et al., 1992). Moreover, there is a purine-rich enhancer element (PRE) required for the specific binding of TRA2 to the *dsxRE*. TRA/TRA2 binds these sites to produce the female-specific splicing of *dsx*, retaining exon 4 in the *dsx* pre-mRNA, and the production of a female-specific DSX protein. DSX protein belongs to the *Dmrt* family, which is a structurally and functionally conserved group of zinc-finger proteins.

TRA/TRA2 also regulates the female-specific splicing of *fruitless* (*fru*) by alternative splicing, through common cis elements TRA/TRA-2 binding sites present in multiple clustered copies only in female-specific exons, which encode a female specific portion of the FRU protein (Burtis and Baker, 1989, Heinrichs et al., 1998, Hoshijima et al., 1991, Ryner et al., 1996).

DSX and FRU protein, placed at the bottom of cascade, are transcriptional regulators responsible for the development of sex-specific somatic traits and behavioural traits.

In XY embryos no early SXL protein is synthesized and transcription of *Sxl* pre-mRNA from the late promoter is male-specifically spliced by default, producing a truncated non-functional SXL protein. This event produces the default splicing of the *tra* pre-mRNA and production of a nonfunctional short TRA peptide. The absence of TRA leads to the default male-specific splicing of both *dsx* and *fru* pre-mRNA, generating male-specific DSX and FRU protein.

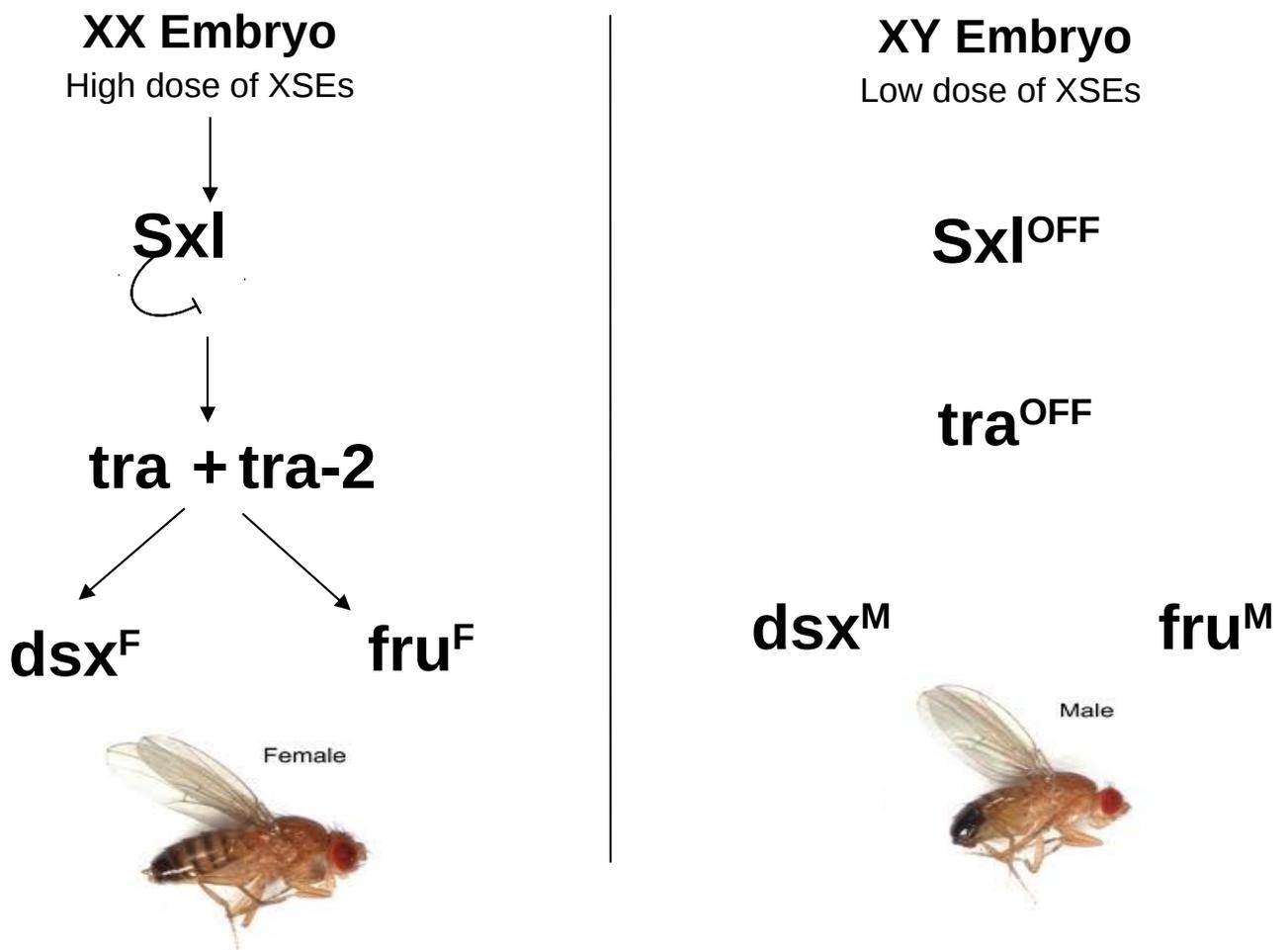


Figure 5. The sex determination cascade in *D. melanogaster*.

1.5 SEX DETERMINATION IN *CERATITIS CAPITATA*

C. capitata diverged from *D. melanogaster* about 100 million years ago, but there is a partial conservation of the sex determination cascade (Fig. 6). Unlike *D. melanogaster*, in *C. capitata* the choice between male or female development is based on the presence or absence of the Y-linked male-determining factor (M-factor), still not isolated (Willhoeft and Franz, 1996). *Sxl* homologous gene (*CcSxl*) has been isolated in *C. capitata* and it is expressed in both XX and XY embryos and does not function as a key switch gene in the sex determination process (Saccone et al. 1998). *transformer* orthologue plays an essential role in *Ceratitis* sex determination and is regulated by sex-specific splicing resulting in the production of a functional RS protein only in females (Pane et al., 2002; Saccone et al., 2011). In XX embryos, a maternal *Cctra* mRNA provides full-length CcTRA protein that initiates a positive feedback regulation and this protein drives a female-specific splicing of zygotically transcribed *Cctra* pre-mRNA so that new CcTRA protein can be produced. The newly synthesized protein controls the maintenance of *Cctra* autoregulation. TRA associates with the Transformer-2 protein (TRA-2), via its arginine-serine (RS) domains, forming a protein complex that produces female-specific splicing of *dsx* pre-mRNA binding to target *dsx* pre-mRNA repeat elements (*dsxRE*). Presumably TRA/TRA-2 complex protein promotes the female-specific splicing of *Ccfru* pre-mRNAs also. *CcDsx* and *CcFru* drive the female somatic development and female sexual behavior (Fig. 6).

In XY fertilized eggs, *Cctra* autoregulation is repressed by the male determining M factor (still to be isolated). The M factor could prevent the action of maternal CcTRA and/or CcTRA/CcTRA-2 proteins, leading finally to male specific *Cctra* mRNAs (that contains stop containing male-specific exons) and hence to truncated non-functional CcTRA protein in XY embryos, and impairing the initiation of the autoregulatory loop. In the absence of CcTRA, *CcDsx* and *Ccfru* pre-mRNAs are spliced in the male specific manner by default, producing the CcDSXM and CcFRUM isoforms, which in turn induce male somatic development and male sexual behavior (Fig. 6).

Cctra gene has very low identity at the protein sequence level and length when compared to *D. melanogaster* (Pane et al., 2002). TRA protein is an example of a protein showing high sequence divergence but conserved molecular genetic function (Saccone et al., 2011). Indeed CcTRA expressed in *D. melanogaster* produces feminization of XY individuals and can rescue female somatic and germline differentiation of *tra* mutant XX individuals (Pane et al., 2005). RNAi against *Cctra* causes transient exhaustion of *Cctra* mRNA in embryos producing a complete sex reversal of XX animals during development into adult fertile males. When crossed with wild type XX females, they give rise to only female XX progeny thanks to absence of the Y-chromosome in XX males,

which is hence not required for male fertility (Pane et al. 2002). The autoregulatory property of *Cctra* was suggested by RNAi studies and by the presence of TRA/TRA2 putative binding sites located in the region where *Cctra* splicing is differentially regulated.

The experiments of RNAi against *Cctra-2* also results in a complete sex reversal of XX individuals and molecular analysis showed that *Cctra-2* is required for female-specific splicing of *Cctra*, *Ccdsx* and *Ccfra* (Salvemini et al. 2009). The presence in adult XX pseudo-males of male-specific transcript of *Cctra*, *Ccdsx* and *Ccfra* indicate a permanent change in their splicing pattern caused by a transient embryonal RNAi against *Cctra-2*, resembling hence an artificially provoked epigenetic event. Hence most likely in XX embryos maternal CcTRA is transiently required to establish zygotic *Cctra* activation, leading to the start of the positive feedback loop. For this reason it has been proposed to refer to these two genes like *Cctra^{ep}* (epigenetic) and *Cctra-2^{aux-ep}* (auxiliar epigenetic) (Salvemini et al., 2009). Moreover, the presence of the TRA/TRA-2 binding sites within the male-regulated genomic region of *Cctra* strongly support the model of an autoregulatory mechanism for *Cctra* splicing.

This result suggests a model of splicing regulation in which CcTRA and CcTRA-2 operate on *Cctra* pre-mRNA splicing promoting skipping of male-specific exon (Pane et al. 2002; Ruiz et al. 2007; Salvemini et al. 2009).

Regarding the explanation of how autoregulation of *Cctra^{ep}* begin in XX-only embryos is suggested by the detection of *Cctra^{ep}* female-specific mRNAs in unfertilized eggs (Pane et al. 2002). This transcripts in eggs can be used for female-specific processing when *Cctra^{ep}* is zygotically transcribed. In Pane et al. (2002) have been proposed three models of how *Cctra* is regulated: 1) the M factor could prevent the translation of maternal *Cctra^{ep}* transcripts in the zygote; 2) M factor could inhibit the function of the translated protein or 3) repress *Cctra^{ep}* transcription initiation in the zygote.

Recent studies showed that *Cctra* female-specific maternal transcripts are present in XX and XY embryos but it is difficult to discriminate between maternal and zygotic contribution. This results suggested that the M factor inhibits the CcTRA protein activity, so that it does not reach the threshold concentration required for female-specific splicing of all the *Cctra^{ep}* transcripts (Gabrieli et al. 2010).

Another novelty for sex determination of *C. capitata* is the observation that *Cctra-2* is required for skipping the two male-specific exons in the *Cctra* pre-mRNA of XX embryos, and during all development, leading to female-specific *Cctra* mRNA which encodes a full length CcTRA female protein (Salvemini et al. 2009). CcTRA protein, along with CcTRA-2, support *Cctra* female-specific activation and promotes female-specific splicing of *dsx* and *fru*.

Ultimately, the function of *Cctra* and *Cctra-2* seems like to be analogous to the *D. melanogaster Sxl* epigenetic autoregulatory function (Cline 1984, Cline2005; Saccone et al., 2010).

Ccdsx gene, at the bottom of cascade, has shown the conservation of two *Drosophila* regulatory elements, a weak polypyrimidine tract at the 3' acceptor splice site before the female-specific exon and four conserved putative TRA/TRA2 binding sites in the female-specific 3' untranslated region of *Ccdsx* (Saccone et al., 2008). most likely the *Ccdsx* female-specific splicing is regulated by a conserved alternative splicing mechanism in which the male-specific mode is the default state and female-specific mode needs the positive action of the CcTRA/CcTRA-2 splicing complex (Pane et al., 2002). *Ccfru* sex-specific expression seems to be under the control of CcTRA/CcTRA-2 proteins, although it is still to be shown in this gene the presence of conserved TRA/TRA-2 binding sites (Salvemini et al., 2009).

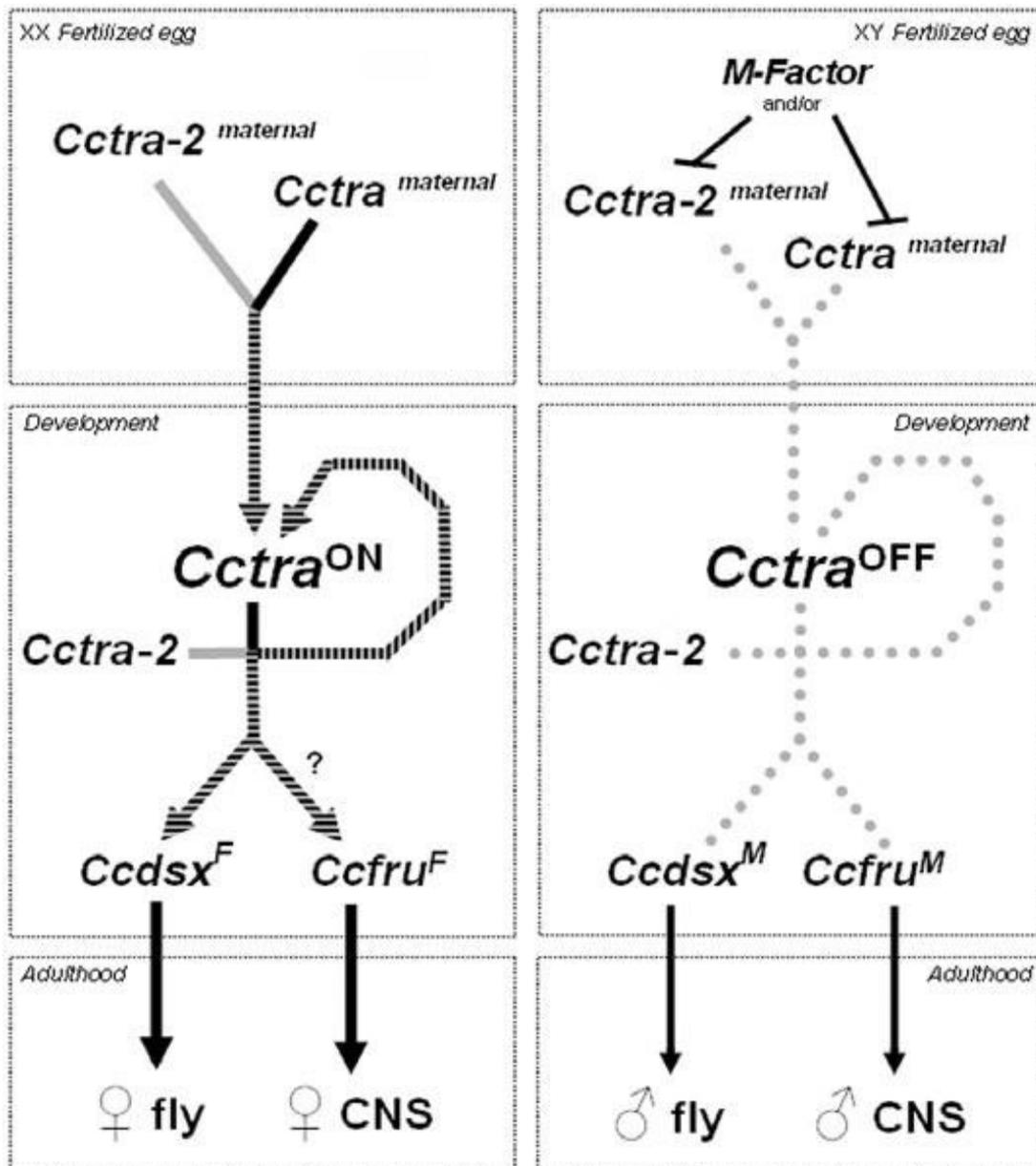


Figure 6. Sex determination mechanism in *C. capitata*.

1.6 SEX DETERMINATION IN *Aedes aegypti*

Aedes aegypti is an arthropod vector of pathologies as the yellow fever and dengue viruses. For many years have been used different types of pesticides to eradicate the presences of *Ae. aegypti* and prevent the spread of the diseases. To date, with genetic engineering, there is the possibility to produce a transgenic insect strains, in which to insert genes that are able to induce conditional lethality in the offspring. An example is represented by “OX513A” strain of *Ae. aegypti*, produced by British company OXITEC ltd. This strain contain a construct that has the promoter of the gene that encodes the *heat shock 70 protein* of *D. melanogaster* and the gene *tTAV*, that encodes a transcriptional activator. The protein tTAV amplify its own expression by binding the site *tetO*, located at downstream of the promoter. This protein is toxic at high concentration, then the individuals die at the late larval stages (Phuc et al., 2007). The tetracycline is an antibiotic that binds the tTAV protein to prevent the interaction with tetO site, so that there is not accumulation of the toxin and the larvae not die.

Two years ago the males of this strain have been released by “Oxitec” in the british island Cayman to observe the competition with wild type males for coupling with females. Open field trials are currently also underway in Malaysia and Brazil (Lacroix et al., 2012).

Understanding of the mechanisms of sex determination in *Ae. aegypti*, could offer a good opportunity to improve the control systems.

To date, the *Aedes* genome and transcriptome sequences have been partially determined (Nene et al., 2007) but the sex determination is still to be clarified. In *Ae. aegypti*, differentially from *An. Gambiae*, heteromorphic sex chromosomes are absent and sex is controlled by an autosomal locus that carries a masculinizing factor, known as the M factor (Craig et al., 1967).

The *tra* homologue seems to be absent in *Ae. aegypti* (Gailey et al., 2006; Nene et al., 2007; Salvemini et al., 2013). The *Aedes dsx* orthologue has been recently isolated and investigated in structure and regulation (Salvemini et al., 2011; Salvemini et al., 2013). *Aeadsx* gene shows at least 8 exons and 7 introns; the first 4 exons are not sex-specific and encode a common region at the N-terminal of 248 aa, OD1 domain. There are two female-specific exons, 5a and 5b; the exon 6 encoding for a male-specific domain at the C-terminal, while in the female becomes a 3' UTR. The exon 7 encoding a 3' UTR sequence in both sexes. Bioinformatic analysis have shown the presence of putative regulatory sites of splicing of *Aeadsx* pre-mRNA between female-specific exons 5a and 5b. Have been found five copy of the TRA/TRA2 binding sites of *D. melanogaster* and two PRE (purine rich element) element in the female-specific exon 5b. A cluster of four conserved *Nvdsx*REs in the female-specific exon 5a, although is not clear their presence. Moreover, there are the presence

of two cis-acting elements, the RBP1 binding site and the TRA-2-ISS element (Salvemini et al., 2010). RBP1 in *D. melanogaster* is an SR protein involved in the activation of the female-specific splicing of *dsx* gene, while the ISS sequence binds a specific isoform of TRA-2, that in the male germline represses the splicing of an intron M1 in the *tra-2* pre-mRNA (Qi et al., 2007). Sequences analysis have revealed that the 3' and 5' splicing sites flanking exon 5a (“strong”) are recognized from the spliceosome complex more than of the 3' and 5' splicing sites flanking exon 5b (“weak”). In *Aedes* genome are present four TRA-2 paralogues and most probably they have evolved different sequence binding specificity and novel functions. The presence of these regulatory sites in *Aeadsx* gene, suggest the existence of an SR protein, that has substituted, most likely, the *tra* function, which, along with RBP1 and TRA-2, would control the sex-specific splicing of *Aeadsx* pre-mRNA. The sex-specific regulation of *Aeadsx* could be governed by a splicing competition mechanism (Fig. 7). The action of the sex-specific splicing factor would repress the use in males of *dsx* 5a exon 3'female-specific ss, rather than promoting it in females. Moreover, the hypothetic presence of a male-determining gene of *Aedes* could produce this splicing repression (Salvemini et al., 2013). The identification of sex-specific transcripts and novel putative cis-acting elements of *Aeadsx* could be helpful to understand the sex determination process toward the isolation of upstream regulators in *Ae. aegypti*. Moreover, this knowledge will be useful to develop new approaches for vector-targeted control of disease.

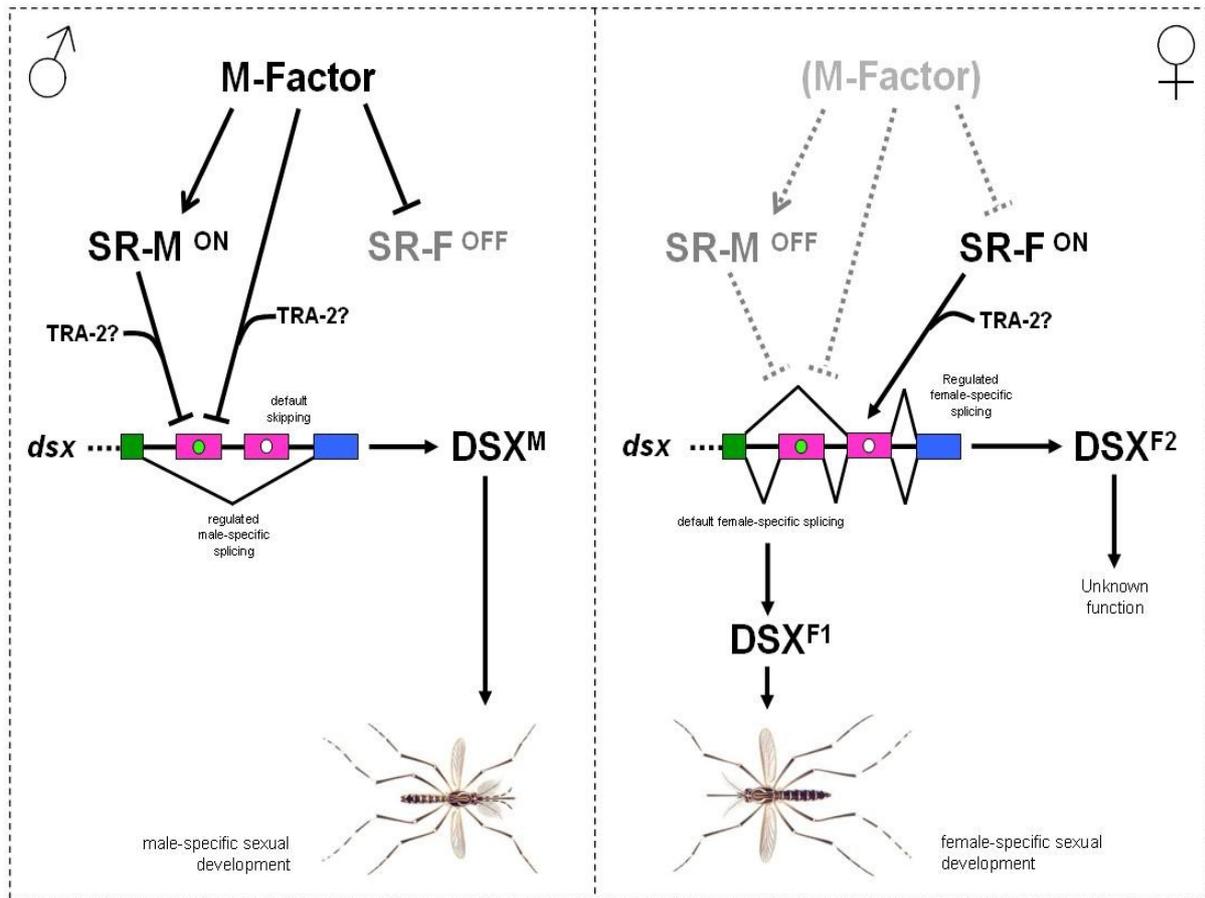


Figure 7. Sex determination in *Aedes aegypti*.

1.7 EVOLUTION OF SEX DETERMINATION GENES IN OTHER INSECTS

In the last two decades is increased the interest to study the mechanisms of sex determination in different insect species, like agricultural pests and disease vectors. Knowledge of sex determination genes in *D. melanogaster* has served as a reference to identify the orthologues in different Drosophilidae species.

The gene *dsx*, at the end of the cascade, has been characterised in different species of insect; this gene is highly conserved in its structure, expression and possibly also in its function. *dsx* homologues in *Megaselia* (Sievert et al., 1997), *Bactrocera* (Sherman and Frommer, 1998), *Musca* (Hediger et al., 2004), *Anastrepha* (Ruiz et al., 2005; 2007b) and *B. mori* (Ohbayashi et al., 2001; Suzuki et al., 2001) express male- and female-specific transcripts and the exon-intron structure is largely conserved.

Structural analysis of these *dsx* orthologues show the presence of sequences that are involved in female-specific splicing: a weak 3' acceptor splice site preceding the female-specific exon and conserved *dsxRE* elements in the female-specific 3' untranslated region (Saccone et al., 2011). *DSX* protein belong to a family of transcription factors which contain a highly conserved zinc finger DNA binding motif.

dsx homologous has been isolated also in the silkmoths *B. mori*, where has been found additional female-specific ORFs and differences in the *dsx* splicing pattern (Shukla and Nagaraju 2010).

The *fruitless* gene of *D. simulans*, *D. yakuba*, *D. pseudoobscura*, *D. virilis* and *D. sukuzii* (Billeter et al., 2002) has been characterised and, in all the cases, showed a conserved molecular structure and the male-specific FruM protein arise by sex-specific splicing, which is controlled by *transformer* (Sanchez, 2008).

dsx and *fru* orthologues gene isolated in the wasp *Nasonia vitripennis* present novel 8 bp long cis regulatory elements putatively involved in the splicing regulation, which exhibit a different consensus with respect to TRA/TRA-2 binding sites in dipteran (Bertossa et al. 2009). The same putative regulatory sequences have been found in orthologues *dsx* and *fru* gene of *Apis mellifera* (Bertossa et al. 2009).

The first *tra* orthologue has been identified in *C. capitata* (Pane et al. 2002) and then, outside the drosophilids, has been characterized in *Bactrocera oleae* (Lagos et al., 2007) and *Anastrepha obliqua* (Ruiz et al., 2007a). In these orthologues *tra* gene have been found Tra-2 specific binding intronic splicing silencer sites (ISS) in the splicing regulatory region but not in *dsx* pre-mRNA of *Anastrepha* species (Ruiz et al., 2007). Hence, these sites may be specifically involved in exon-skipping of male sequences of *tra* pre-mRNA in females (Ruiz et al., 2007). In *Lucilia cuprina tra^{ep}*

the Tra/Tra-2 binding elements are clustered in an intronic region closer to the common exon 2 (Concha and Scott, 2009). In *Lucilia* there is the presence of female variant (probably maternal) of *Lctra^{ep}* in both male and female embryos after fertilization, while the male variants is produced from the first larvae stage. In *tra^{ep}* orthologues of *Musca domestica* there is the same genomic organization and exon skipping splicing mechanism as Tephritidae *tra^{ep}* (Hediger et al., 2010). In *Musca domestica* the gene *Mdtra^{ep}* plays a key role for female sex determination. Its maternal product is needed to activate the zygotic function of *Mdtra^{ep}*, which show autoregulation (Dubendorfer and Hediger, 1998).

A *tra* orthologue has not yet been identified in Lepidoptera; in *B. mori* there is not the presence of dsxRE or PRE binding sites on *Bmdsx* (Suzuki et al., 2001; Suzuki et al., 2008).

Female-specific splicing of all *tra* gene, isolated in insects except *D. melanogaster*, involves an autoregulatory loop and with TRA protein required for female-specific splicing of *tra* pre-mRNA (Pane et al., 2002; Salvemini et al., 2009; Concha et al., 2009; Lagos et al., 2007; Gempe et al., 2009; Hediger et al., 2010; Verhulst et al., 2010).

These studies in different taxonomic groups of the genes involved in the sex determination mechanisms showed a particular conservation of downstream *dsx* and *fru* genes and in part about the *tra* gene, while the primary signal in these species is different, in agreement with the model of Wilkins (1995), which proposed that the evolution of sex-determining cascades were built bottom up, with the genes at the bottom more conserved than the more upstream genes in the cascade.

1.8 THE PRIMARY SIGNALS

Sex chromosomes provide the primary signal in the sex-determining pathway and have evolved independently many times in plants and animals and some of these appear to be well conserved, while other are more short-lived.

The sex chromosomes are designated X and Y when males are the heterogametic sex and W and Z in female-heterogametic systems. In general there is a particular gene/s present on one of the two sex chromosomes that contribute to the primary sex-determination signal and acts to regulate a cascade of genetic switches for the development of an embryo into a female or male individual.

In eutherians the SRY is the primary switch for testis differentiation and some of its functions is to activate SOX9 to the development of Sertoli cells and the male type of gonad development. The presence of SRY on mouse Y chromosome and experimental evidences of XX mice converted to males by the introduction of Sry, confirmed the role of this gene in the sex-determining function

(Koopman et al., 1991; Gubbay et al., 1990).

The *D. melanogaster doublesex* and *Caenorhabditis elegans male abnormal (mab)-3* genes are known to control sexual development in these animals (Erdman et al., 1993; Raymond et al., 1998). These two genes encode proteins containing a zinc finger-like DNA-binding motif called DM domain. This domain has been found in the gene *DMRT1* of different vertebrates and is involved in sexual development. In mouse, *DMRT1* is essential for postnatal testis differentiation (Raymond et al., 1999; Raymond et al., 2000). In chicken and turtle, *DMRT1* expression is connected to testis formation in undifferentiated gonads (Nanda et al., 1999; Nanda et al., 2000; Kettlewell et al., 2000). Chicken shows a ZZ/ZW system and *DMRT1* is located on the Z chromosome, suggesting that gene dosage may induce male development (Nanda et al., 1999; Nanda et al., 2000).

In the teleost fish *medaka* has been isolated *DMY/dmrt1bY* genes, which is a coorthologue of *DMRT1*, that acts as sex-determining genes triggering formation of the testis, leading to male sexual development (Koopman et al., 1991; Matsuda et al., 2002; Nanda et al., 2002; Matsuda et al., 2007). A female-specific W-linked gene, called DM-W, a paralogue of *DMRT1*, has been isolated in the African clawed frog *Xenopus laevis* (Yoshimoto et al. 2008), supporting conclusions that females are heterogametic. This is the first master gene of primary sex determination identified in amphibians.

Among insects, there are different sex determination mechanisms discovered in the various taxa.

Sxl homologues were found in *Ceratitis* (Saccone et al., 1998), *Chrysomya* (Muller-Holtkamp, 1995), *Megaselia* (Sievert et al., 1997), *Musca* (Meise et al., 1998), *Bactrocera* (Lagos et al., 2005) and *Sciaridae* (Ruiz et al., 2003; Serna et al., 2004), but do not appear to play a key discriminatory role in the control of sex determination of these Dipteran insects, though the *SXL* protein were found in both sexes of the same size. Hence, the structure of *Sxl* is very conserved, but its developmental function is not.

The housefly *Musca domestica* has a Y chromosome that carries a male-determining factor, M (Schutt e Nothiger, 2000). In some *Musca* populations, the male factor is located on one of the autosomes. In still other populations there is the presence of a dominant female-determining gene (FD) that suppresses the M factor (Hilfiker-Kleiner et al., 1994). As we know, *D. melanogaster* has been the first insect in which has been identified the primary signal of sex determination, which is based on the concentration of *X-linked signal elements* (XSE), as described above. In other species, including *Megaselia scalaris*, there is a polymorphic chromosomal position of the sex determination gene (Willhoeft and Traut, 1990), but the nature of M is still unknown. In *Chironomus tentans* there are non-homologous Y chromosomes that coexist and each of which is sufficient to start male development.

The blowfly *Chrysomya rufifacies* provides an example of maternal sex determination in which the sex of the zygote is exclusively determined by the genotype of the mother: gynogenic females produce only female offspring, while androgenic females produce male offspring. The gynogenic female is heterozygous for a gene that encodes a maternal factor (F), while in the androgenic female is homozygous for the recessive allele *f*.

In other species like *Ceratitis*, *Bactrocera* and *Anastrepha* the heteromorphic sex carries the male determining factors on the Y chromosome. In the case of lepidopterans (butterflies and moths) the male is the homomorphic sex (ZZ) and the female the heteromorphic sex (ZW). The lepidopteran *B. mori* has epistatic female determinant/s on the W chromosome, while *Lymantria dispar* has Z-linked male determinant (M) and maternally inherited female determinant factor (F).

In *Sciara*, there is the differential elimination of sex chromosomes in which all zygotes start with the 3X;2A constitution; the loss of either one or two paternal X chromosomes determines whether the zygote becomes female (2X;2A) or male (X0;2A) (Sanchez, 2008).

Insect groups like thrips, beetles and Hymenoptera are characterized by haplo-diplo sex system: males are haploid, develop from unfertilized eggs and inherit a maternal genome, whereas females are diploid, develop from fertilized eggs and inherit a paternal and a maternal genome. The primary signals in haplodiploid species is represented by the homo/hetero allelic composition of the *csd* locus (complementary sex determination), which encodes a RS-like protein with short but significant sequence similarity with CcTRA but not DmTRA (Beye et al. 2003). Females are heterozygous while males hemizygous at this locus and diploid embryos homozygous for *csd* become sterile males. The *csd* gene has not different expression in both sexes. *Fem* locus isolated in *Apis* (Hasselmann et al. 2008) appears to be a duplication of *csd* but produces sex-specifically spliced mRNA, as the dipteran *tra* orthologues. Most likely, *fem* is more ancestral and *csd* arose from a duplication event of *fem*. FEM shares with TRA of *C. capitata*, and of other dipteran species, a 30-amino acid motif recently named as TRA^{CAM}. This finding suggests that *Apis* FEM is an orthologue of CcTRA rather than of DmTRA.

Instead, in *N. vitripennis* *csd* is not the primary signal, females regulate the sex of the offspring by providing a feminizing effect by maternal input of *Nvtransformer* (*Nvtra*), while at the same time preventing zygotic expression of *Nvtra* in haploid offspring (Verhulst et al., 2010). Finally, as regards the mosquitoes, such as *Anopheles gambiae* and *Aedes aegypti*, there is the hypothetical presence of a dominant male-determining factor that control the splicing of downstream genes, *Aeadsx* and *Aeafru* (Salvemini et al., 2011; Salvemini et al., in press).

In general, we can say that the understanding of the different primary signals in insects, except for *Drosophila*, still remains at the level of the chromosome and we know little about the molecular

nature.

1.9 AIMS OF THE STUDY

The primary signal of sex determination in *C. capitata* is still unknown and the unique information is that this factor is mapped on the long arm of the Y chromosome (Willhoeft and Franz, 1996). Recent evidences show that the ipotetic M factor could be active during a temporal window that spans from 5 to 10 hours after oviposition (Pane et al., 2005; Gabrieli et al., 2010). To date, there are not information about the transcripts present in this temporal window of development of insects, also for *D. melanogaster*. During this early period in *C. capitata* occurs the transition from genetic maternal information to zygotic genetic information and the choice of the sex of the embryo, through the integration of maternal signals and genomic sex-specific signals. Is unknown also the molecular nature of M factor, that could be a coding gene or a non coding transcript with regulative function.

The first part of my Ph.D. experimental work was focused on the identification of the Y-linked gene/s that are important in male sex determination of *C. capitata* by molecular and functional analysis of transcripts obtained by molecular and *in silico* subtraction techniques from mixed XY/XX and XX-only embryos at 8-10 hours (Salvemini et al., unpublished).

Regarding the sex determination in *Ae. aegypti*, as mentioned above, there is not information about neither the primary signal of sex determination nor about the splicing regulator, which produce a sex-specific splicing of the downstream *Aeafu* and *Aeadsx* genes.

For this purpose, the second part of Ph.D. experimental work is focused on the identification of an hypothetic key gene involved in the sex determination of *Ae. aegypti*. In addition to this, we performed the developmental expression analysis of the *fruitless* gene of *Ae. Aegypti* (*Aeafu*) (Salvemini et al, 2013).

2 RESULTS

2.1 MOLECULAR SUBTRACTION

In our laboratory molecular subtraction experiments by Suppression Subtractive Hybridization (SSH) have been performed between two different RNA samples extracted respectively from mixed XY/XX and XX-only embrionics populations at two different stages, 8-10h and 23-25h from oviposition (Salvemini, Saccone et al., unpublished). Two subtracted libraries were obtained for each stage and differential screening was performed only on 8-10h stage sample by cDNA dot blots of PCR products. From this experiment 26 clones were isolated and sequenced and BlastX analyses revealed that 18 clones encode putative protein domains with significant homology with known proteins of various functions. Quantitative Real Time PCR analyses has been performed and neither of the 26 clones resulted expressed only in males but 8 out of 26 clones were male-biased (Fig. 8).

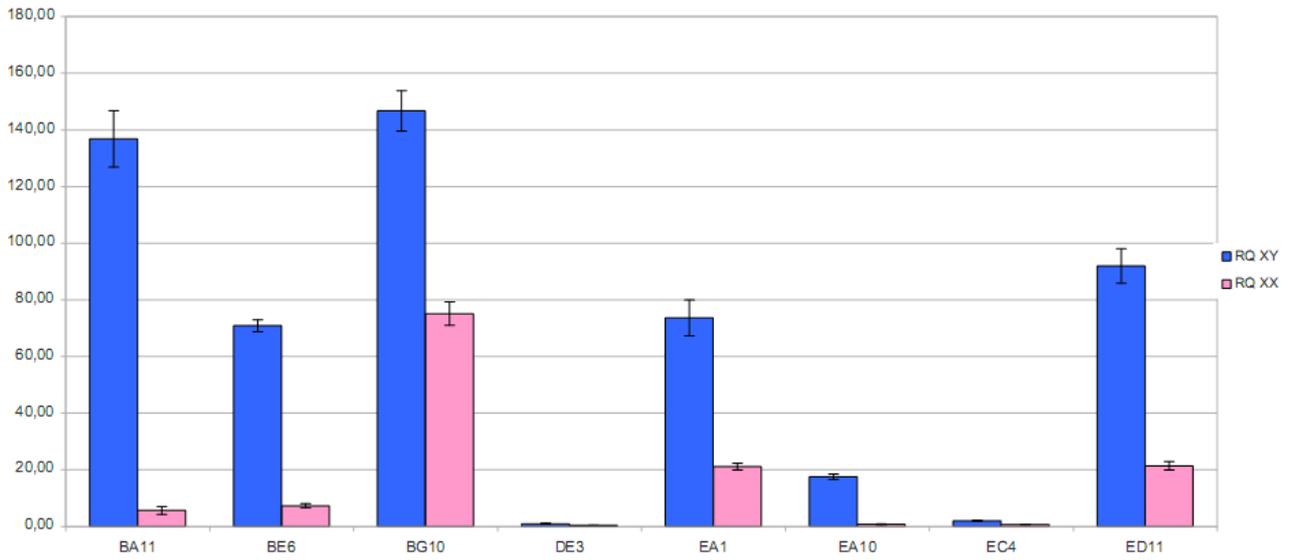
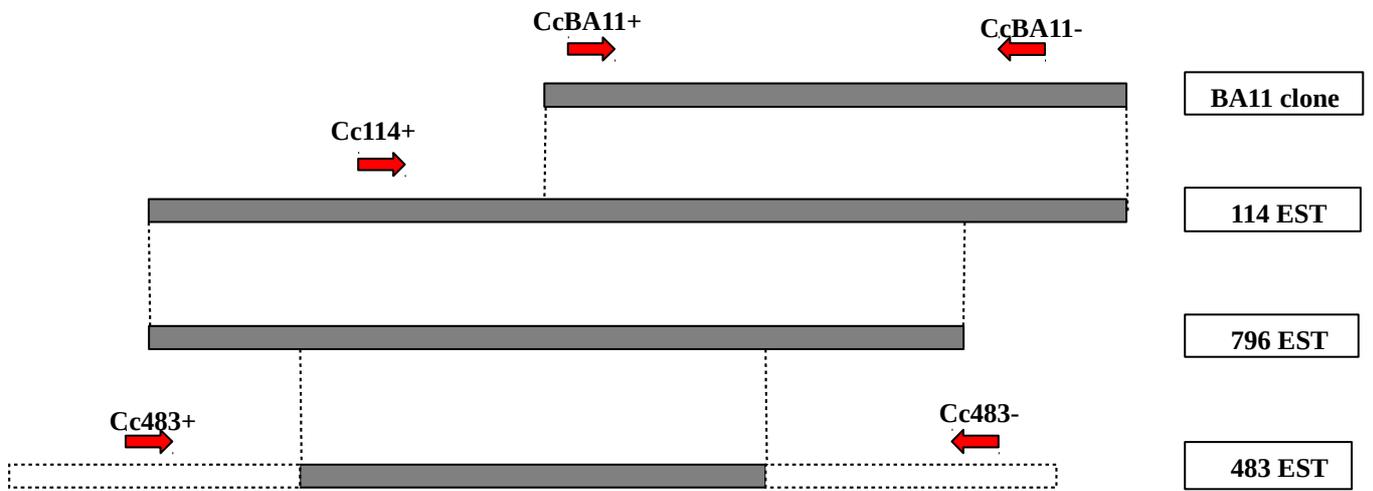


Figure 8. Validation of the male biased expression of 8 cDNA clones by Real Time PCR.

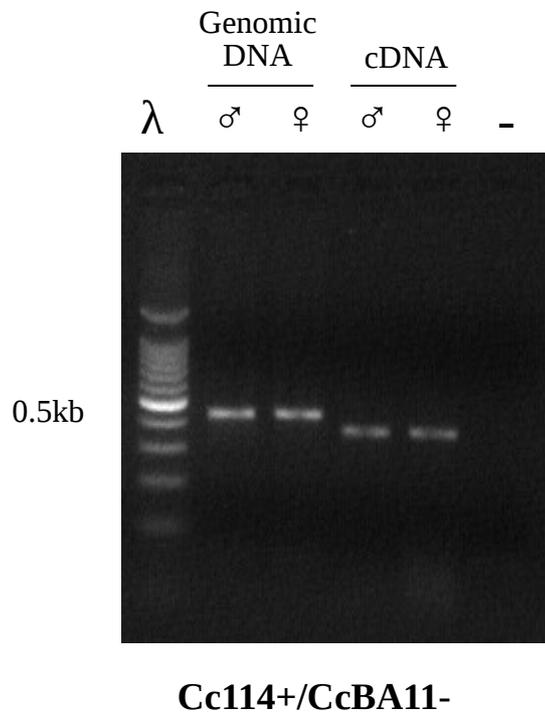
2.2 BIOINFORMATIC AND MOLECULAR ANALYSIS OF THE CLONE CcBA11.

We selected the BA11 clone which showed the higher expression in the sample XX/XY versus XX-only (15 fold in males). We used BlastX algorithm to search for homologous hits of clone CcBA11 in NCBI database; we identified a *Drosophila* RNA-binding protein (CG10803), of unknown function, showing an identity of 54% at the aminoacid level with CcBA11. BlastN analyses using the Expressed Sequence Tags (EST) database of *C. capitata* identified three partially overlapping ESTs (named EST 114, EST796 and EST 483). The EST 114 produced a full overlap with clone CcBA11, as for the EST 796. EST 483 produced an overlap of only about 160 nucleotides with BA11 (fig. 9A). These analyses suggested us the presence of two different transcripts. We designed specific primer (named CcBA11+, CcBA11-, Cc114+,Cc483+ and Cc483-) and we investigated the expression pattern of the putative corresponding transcripts by molecular analysis through RT-PCR experiments on genomic DNA and cDNA of male and female adult of *C. capitata* Benakeion strain. We used the primers that amplify the *Rpp1* gene, constitutively expressed in *C. capitata*, as endogenous positive control (data not shown). After this control, we performed PCR amplifications with primers pairs Cc114+/CcBA11-, CcBA11+/BA11- and Cc483+/Cc483-, which showed the same pattern on male and female genomic DNA and cDNA (Fig. 9-B,9-C,9-D). With primer pairs Cc483+/CcBA11- we got one amplification signal in both sexes (CcGm1, a 1 Kb long band) and a shorter band only from male genomic DNA sample (CcGm2, 0.6 Kb long). Using the same pair of primers, a 0.5 Kb long band (CcDmale) was amplified only in males (Fig. 9-E). These three products were cloned, sequenced and then analyzed by bioinformatic tools (ClustalW and Blast). The alignment of CcGm1 with CcGm2 showed an overall 72% identity, indicating that they are derived from paralogous sequences (Table 1). The cDNA CcDmale is 100% identical to CcGm2 and are 56% identical to CcGm1. These findings suggest that CcGm2 is a gene transcriptionally active and that it is Y-linked, while CcGm1 could be an autosomal paralogue. We performed alignments of these two clones with the three ESTs previously identified. The alignments of EST 796 and EST 114 show 100% identity with the Y-linked CcGm2 gene, while EST 483 gave an identity of 67% with it and 100% with CcGm1 (Table 1).

A



B



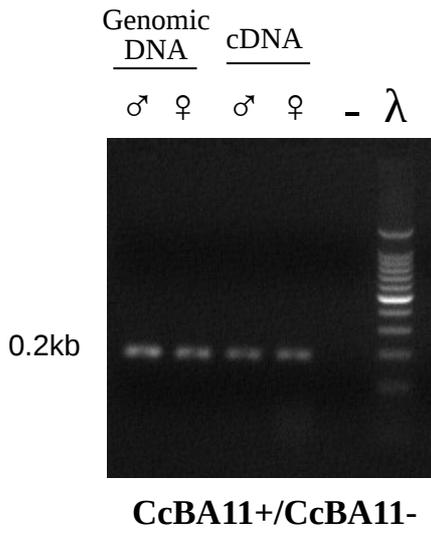
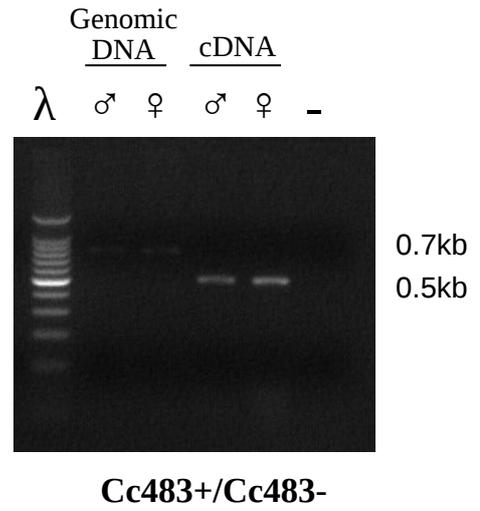
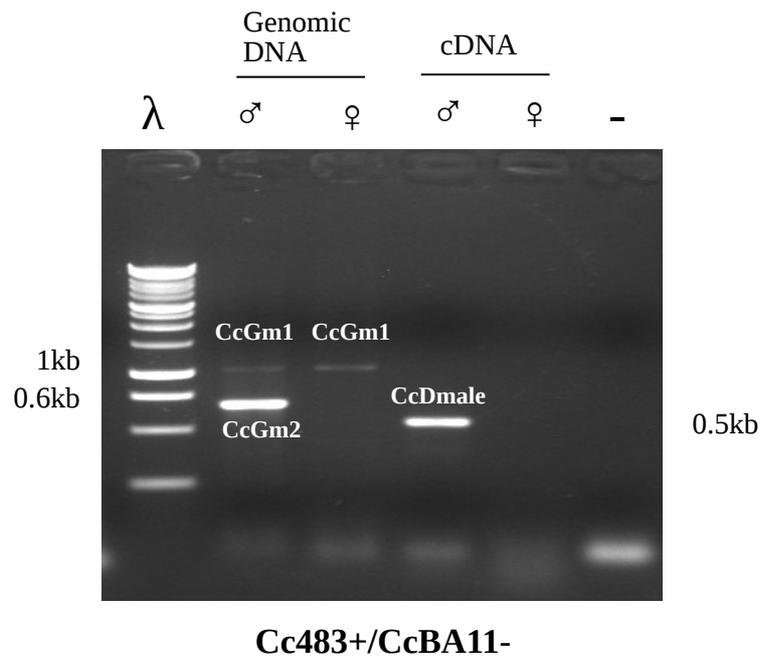
C**D****E**

Figure 9. Molecular analysis of CcBA11 clone.

TABLE 1

ESTs	CcGm1	CcGm2	CcDmale
EST 114	25%	77%	100%
EST 796	29%	77%	100%
EST 483	87%	53%	67%

2.3 RNAi IN EMBRYOS AND RT-PCR ANALYSIS OF THE CLONE CcGm2.

To understand if CcGm2 corresponds to the M factor, we performed a transient RNAi.

We designed a primer, named Cc483Y+, that utilized with the CcBA11- primer, specifically amplifies the sequence CcDmale, 0.5 Kb long. We used this amplified region to produce dsRNA for RNAi analysis in embryos of *C. capitata*.

If the hypothetic Y-linked transcript of BA11 constitutes the male determining factor, its silencing at embryonic stage will lead to a sex reversal of male in female. RNAi experiments was performed using dsRNA obtained with megascript RNAi kit of Ambion (see materials and methods). We resuspended dsRNA in a phosphate buffer at 1 µg/µl concentration and injected into pre-blastoderm embryos (30 - 45 minutes after oviposition) of *Benakeion C. capitata* strain (see materials and methods). Out of 900 injected embryos, we got 250 adults but we did not obtain only females, as we expected, or intersexes, but 100 males and 150 females, hence a sex distortion in favour of females. We got also 71 pupae that have not hatched (Table 2). Operating a Chi Quadro test on the number of males and females adults obtained, we found that this data is statistically significant ($p=0.01$). The normal ratio of two sexes should be 1:1, then we have hypothesized, as a possible explanation of this result, that 25 on 150 female were XY (16%). Hence, we analyzed 40 adult females developed from injected embryos to verify the presence of the XY karyotype by a genomic DNA PCR, using a primer pairs (CcY1/CcY2) that amplify a repetitive regions on Y chromosome.

In contrast to our expectations, none of the PCRs showed the presence of Y chromosome (data not shown). An alternative explanation of the female biased sex ratio observed could be a male-specific lethality at pupal stage induced by the RNAi injection against BA11 clone. Unfortunately we could not extract amplifiable genomic DNA from died pupae to test this alternative hypothesis.

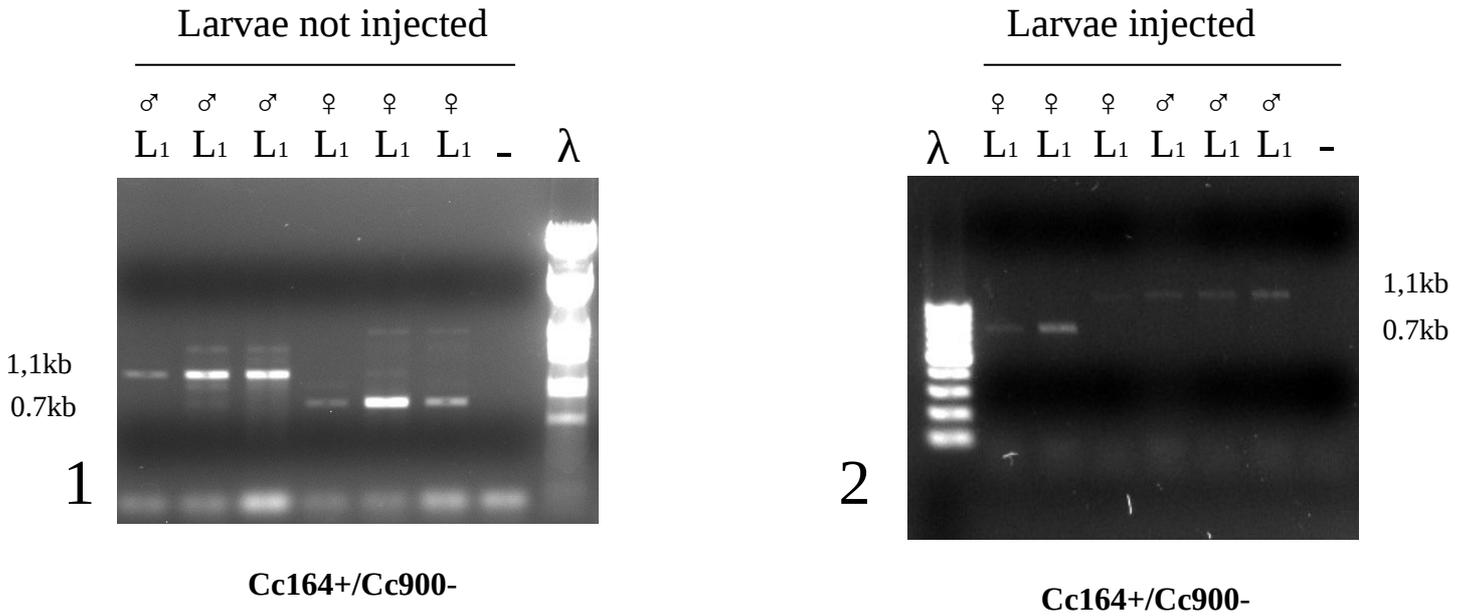
But why we did not observe a feminization of XY individuals? A possible explanation is that dsRNA of BA11 could have only a transient action unable to produce a stable reversion toward the female sex of the XY individuals, due to recovery of transcriptional activity of the BA11 gene during later embryogenesis or early larval stages after the disappearance of RNAi effect. In order to assess this hypothesis, we planned to verify if the CcDmale-RNAi has a transient effect of the male-specific pattern of *Cctra* and *Ccdsx* in XY individuals, as expected in the case that CcGm2 corresponds to an upstream regulator, such as an M factor. We extracted RNA from first instar larvae injected with dsRNA of BA11 at embryonic stage and, as a positive control, RNA from first instar larvae developed from uninjected embryos. First, molecular sexing of samples were performed through PCR with primers CcY1/CcY2 (data not shown), establishing the sex of larvae and afterwards we produced cDNA from three sample of RNA for each sex from injected and uninjected larvae. After PCR with primers of *Rpp1* gene (data not shown), as endogenous positive control, we carried out a *Cctra* sex-specific splicing RT-PCR analysis using the primers

Cc164+/Cc900- (fig. 10A.1,2). We obtained the normal sex-specific pattern for males and females uninjected larvae, while in one female injected larval sample we had a male specific product of *Cctra* gene. Also in the case of RT-PCR performed with primers Cc1400+/Cc1300-/Cc2000-, which amplify a sex-specific products of *Ccdsx* gene, we had a normal pattern for females and males larvae uninjected, while for the same female injected larval sample we had a male specific product (fig. 10B.1,2). These results suggested us to perform a further RNAi experiments using a more concentrated dsRNA of BA11. We have tried to inject at 2 µg/µl concentration of dsRNA. However we encountered technical problems in injecting the dsRNA at this concentration due to a too much viscous injecting solution which caused repeatedly occlusions of the injection needle.

TABLE 2
CcBA11 RNAi EXPERIMENT

Injected embryos	Larvae	Pupae	Adults	Males	Females	Pupae death
916	557	321	250	100	150	71
[inj. dsRNA]	% Embryonal survival rate	% Larval survival rate	% Adults survival rate	% Males	% Females	% Pupae death
1 µg/uL	60%	35%	27%	10.9%	16%	0.07%

A



B

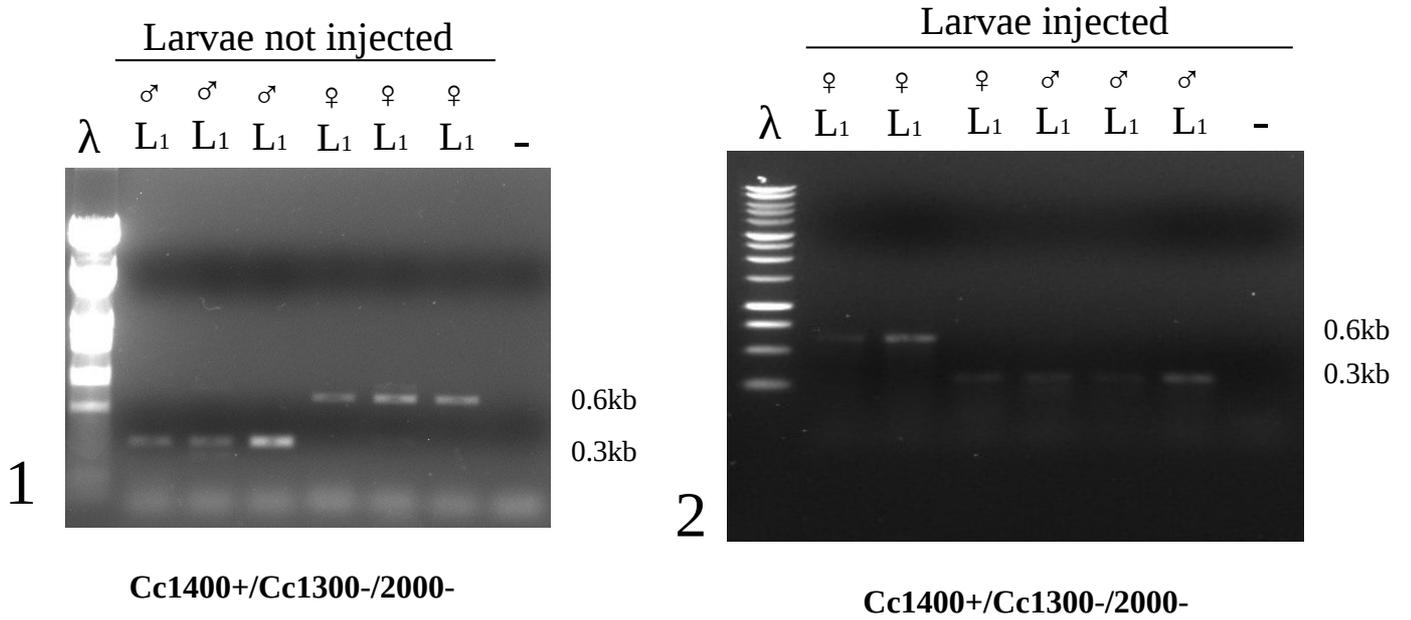


Figure 10. Molecular analyses of injected and uninjected larvae.

2.4 DE NOVO ASSEMBLY AND IN SILICO SUBTRACTION OF XY/XX AND XX-ONLY EMBRYONIC TRANSCRIPTOMES.

In addition to the previous experiments, in our laboratory an RNA-seq analyses on XX/XY and XX-only embryos at 8-10 hours from oviposition have been performed to find the MDF. We used the total RNA extracted from XX/XY and XX-only *C. capitata* embryos at 8-10 hours from oviposition, used in the previous experiments. This analysis has been conducted as a collaboration between my tutor Prof. G. Saccone and Dr. Nagaraju of CDFD, Hyderabad - India. They produced cDNA from total RNA and the libraries of each sample obtained was submitted for Illumina sequencing. About 40 million of paired-end 100 nucleotides long reads of XY/XX sample and about 35 million of reads of XX-only sample were produced. The assembly of reads for each sample was done using Trinity software (Grabherr et al., 2011), and resulted in 36427 transcripts and 33765 transcripts from XY/XX and XX-only samples respectively (Salvemini et al., unpublished).

After the assembly the two transcriptomes were used for an *in silico* subtraction with the RSEM and edgeR softwares (Li B. et al., 2011; Robinson MD. Et al., 2010) to quantify the expression levels of all transcripts and to identify those expressed exclusively in the XY/XX sample. From these analysis we obtained about 200 transcripts with different expression between the samples XY/XX and XX-only (Fig. 11). In particular, with the list of the putative male-biased transcripts we identified a clone, named Cc10393 that present a stronger expression in XY/XX respect to XX-only (50 times more) and corresponds to the Y-linked CcGm2.

2.5 BIOINFORMATIC AND MOLECULAR ANALYSIS OF CLONE Cc10393

Indeed using BlastX on *Drosophila* genome database to investigate the clone Cc10393, we found an identity of 34% with the same RNA-binding protein (CG10803) found previously in Blast searches using as query the CcBA11 transcript.

Hence, using ClustalW program we found a 100% identity between CcGm2 and Cc10393. Furthermore, Cc10393 shows longer sequence at 3' and 5' ends with the respect of CcGm2. CcGm1 showed a 44% identity with Cc10393.

These alignment results suggested the presence of a Y-linked transcript and of a putative paralogue, expressed in both male and female individuals.

We performed a BlastN search using as query the Cc10393 on the database of the novel transcriptomes of *C. capitata* XY/XX embryos obtained from RNA-seq, to search for additional longer overlapping transcripts derived from CcGm2 and for paralogues sequences. The output of the search contained an hit match with other two transcripts, Cc12258_3kb and Cc12258_1,4kb. The alignment of Cc12258_1,4kb with Cc10393 produce an hit with an identity of 95%, with different polymorphisms at the 3' end, while the alignment of Cc10393 with 12258_3kb an hit with 60% of identity.

The transcript Cc12258_3kb produced a perfect match with the EST 483 (hypothetic autosomal or X-linked paralogue, CcGm1). Cc12258_1,4kb align perfectly with the ESTs 796 and 114 (all derived from the putative Y-linked paralogue, CcGm2) (Table 3).

Using a database of genomic sequences of *C. capitata* not yet assembled in a genome draft but available for Blast searches (SRA, NCBI), we have tried to find the genomic sequences corresponding to the Y-linked CcGm2 gene and hence to gain more sequence informations of the flanking regions. We used as probe the sequence from the Cc10393 and Cc12258 cDNA clones.

We have found about 20 partially overlapping SRA sequences (450 bp long on average) and we gained a different portion of sequence at 3' end of each transcript but the same sequence extended at 5' end.

Based on these two new sequences we designed a forward primer (Cc10393+), that matches with both transcripts, Cc10393 and Cc12258_1,4kb, and two reverse primer (Cc10393- and 12258-), specific for each sequences. In addition we designed other two primers that amplify the Cc12258_3kb transcripts (Cc12258_3kb+ and Cc12258_3kb-). We performed PCR experiments using genomic DNA and cDNA of males and females *Benakeion C. capitata* strain. The PCR performed with primers Cc12258_3kb+ and Cc12258_3kb- produced a common amplification signal in both male and female samples of genomic DNA and of sexed cDNA (fig. 12A). The PCR with primers Cc10393+/Cc12258- failed to amplify a product, while using the primer pairs

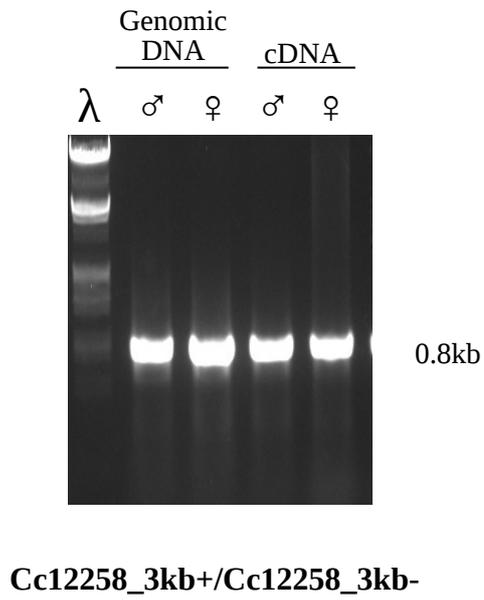
12258_1,4kb+/Cc12258- (Fig. 12B) we got an amplified genomic product of 0.8 Kb long band but no amplification products on cDNAs (fig. 12B). A PCR with primers Cc10393+/Cc10393- let to amplify a 1.6 Kb long band only in males from both cDNA and genomic DNA samples, suggesting that the CcGm2 Y-linked gene is intron-less (fig. 12C).

To evaluate if the ectopic expression of this putative M factor could promote maleness in XX embryos, we planned to inject an artificial RNA obtained from CcGm2.

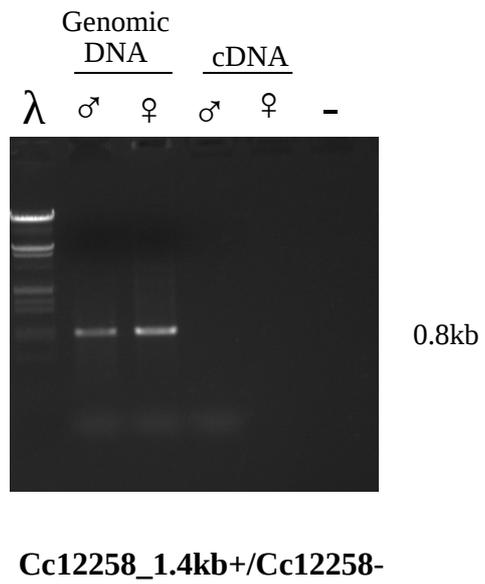
We prepared a synthetic capped RNA of the transcript obtained from the PCR with primers Cc10393+/Cc10393- on cDNA male sample (see materials and method; a kit kindly provided to Prof. G. Saccone by Prof. Campanella and Prof. Carotenuto).

We resuspended in phosphate buffer the synthetic RNA of clone Cc10393 at 250 ng/uL concentration and the injection in embryos are in progress.

A



B



C

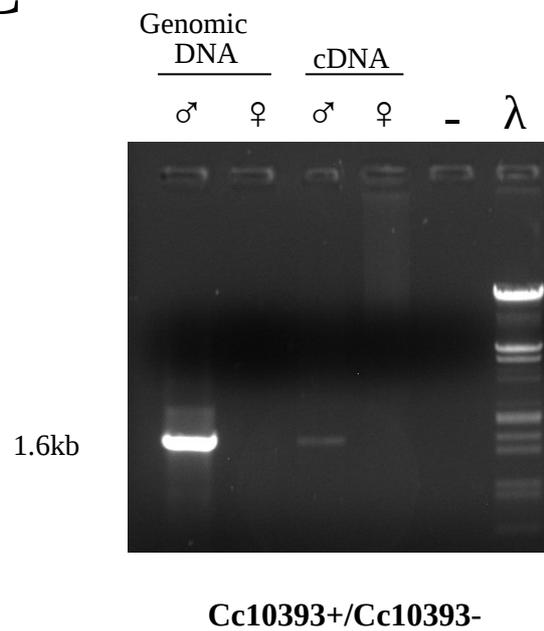


Figure 12. Molecular analysis of Cc10393 clone.

2.6 THE ORTHOLOGUE OF *D. MELANOGASTER FRUITLESS* GENE IN THE MOSQUITO *AEDES AEGYPTI*.

The fruitless gene of the mosquito *Aedes aegypti* (*Aeafru*) has been isolated combining a classical PCR-based approach with available bioinformatic and genomic tools (Salvemini et al., 2013). The *Aeafru* gene has eight exons and seven introns; compared with the *fru* gene of *D. melanogaster* and *An. Gambiae*, exhibit the highest conservation for the exon C1 and C2, while exons C3, C4 and C5 show a more variable size and amino acid content of the encoded domain. Highly conserved is also the exon Zc (zinc-finger encoding exon) and exon P1, which encodes a conserved male-specific N-terminal domain and a sex-specific alternative splicing regulation, as observed in *D. melanogaster* and *Ae. Gambiae* orthologues. Finally, *Aeafru* gene shows a wide divergence in intron length respect to *Drosophila* and *Anopheles* (Salvemini et al., 2013).

Through bioinformatic tools have been found, in the *Aeafru* P1 exon, nine putative type-B RBP1 binding sites and three putative TRA-2 ISS; these sequences highly conserved are involved in the control of alternative splicing of this *Aeafru* region.

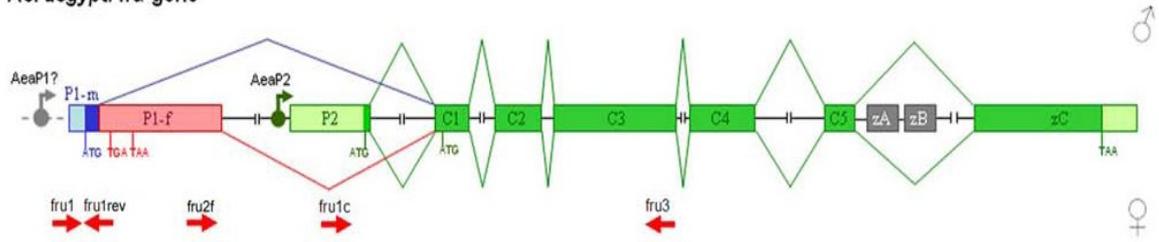
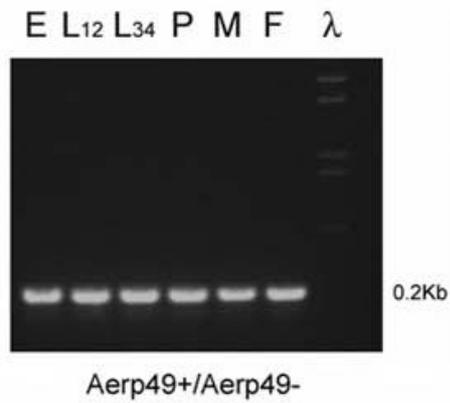
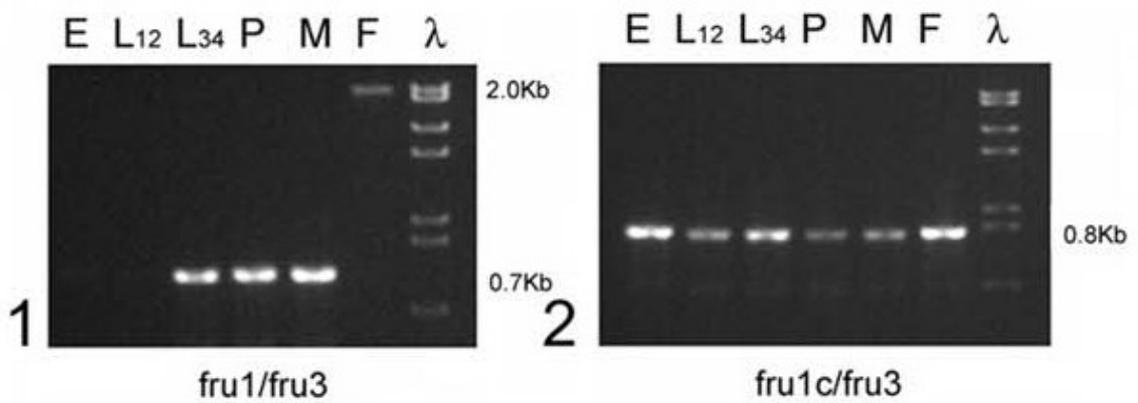
Moreover, there are three sequences showing only few similarities to the 13 nt long *Drosophila* TRA/TRA-2 binding site consensus (Salvemini et al., 2013). We performed RT-PCR analysis on *Aeafru* gene, using total RNA extracted from different stages, from embryonic stage till adulthood, to analyze the developmental expression pattern. We used primers designed on the P1 sex-specifically regulated exon, P2 exon and the common region on the *Aeafru* gene (fig. 13A). Before starting with RT-PCR we did a PCR, as a positive control, with the *rp49* gene, constitutively expressed in *Aedes* (fig. 13B).

RT-PCR with primers fru1/fru3 amplified cDNA fragment from 3rd instar larval stage till adulthood. These amplified fragments are putatively derived from the *Aeafru*^{P1-m-C} and *Aeafru*^{P1-f-C} transcripts spliced in a sex-specific manner in adult (Fig. 13C.1). To investigate the absence of the expected female-specific amplification signal in mixed larval and pupal sample we sexed single larvae and pupae using *dsx* sex-specific splicing RT-PCR analysis, after PCR with primer *rp49* (fig. 13D.1); afterward we performed on this sexed samples a further RT-PCR with primers fru1/fru3. The result of the PCR reveals a male-specific *AeaP1 fru* transcript in males larva and pupa (fig. 13D.2), while the RT-PCR on the same samples with primers fru2f/fru3, produced a female-specific transcript (fig. 13D.3). Finally, RT-PCR with primer fru1/fru1rev (fig. 13D.4), have shown the same transcript in both sexes.

These sex-specific fru transcripts share a common 5' exonic region, indicating that the P1 promoter is active in both sexes (Salvemini et al., 2013).

The second class of transcripts, obtained with primers fru1c/fru3, revealed the presence of non-sex-

specific transcript expressed from embryonic stage till adulthood (fig. 13C.2). This transcript corresponding to the *Aeafu*^{P2-C} mRNA and possibly derived from a different promoter respect to the P1 promoter of *Aeafu*.

A*Ae. aegypti fru* gene**B****C**

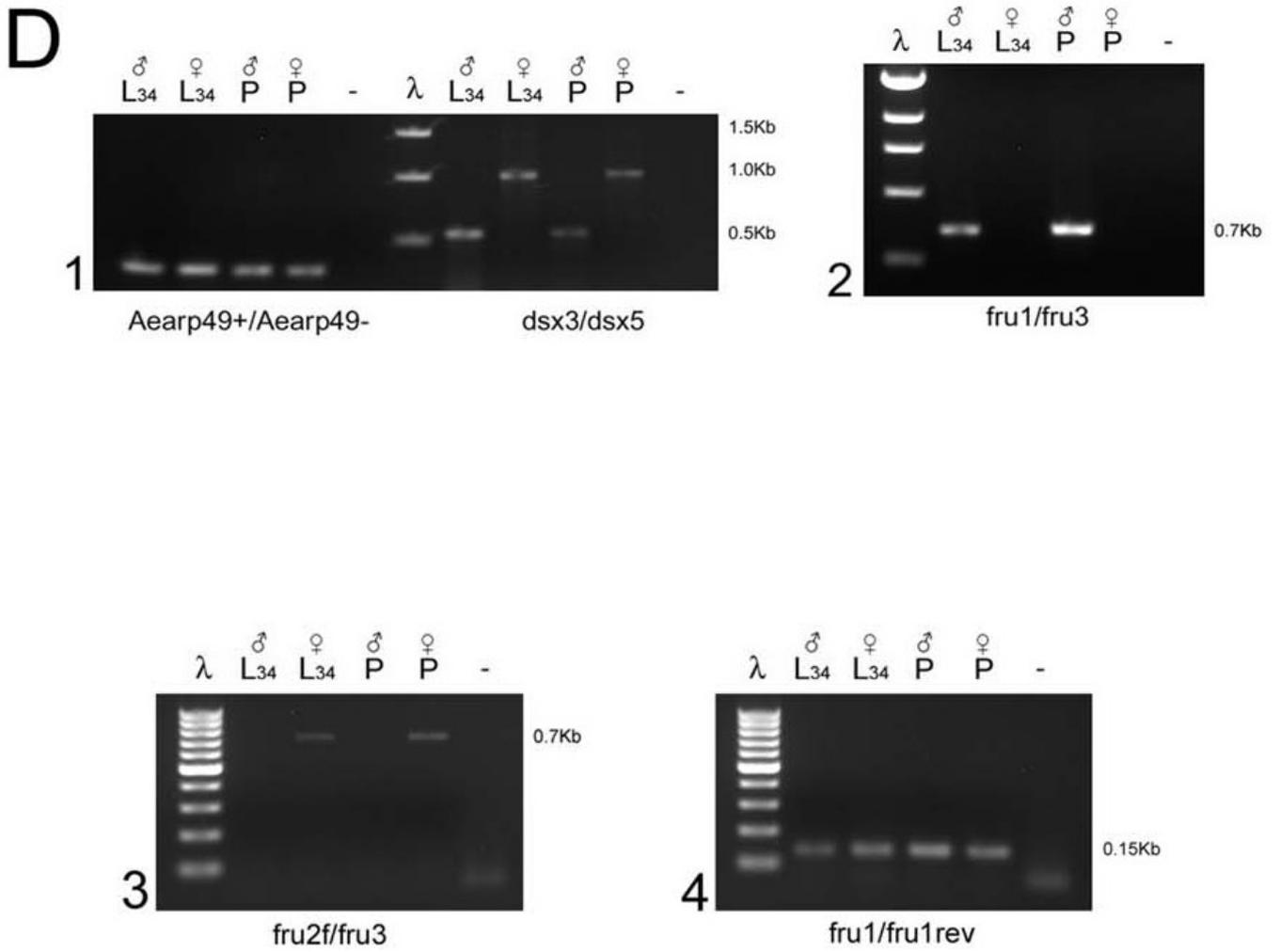


Figure 13. Developmental expression analysis of the Aeafru gene.

2.7 RT-PCR ANALYSIS OF THE *AeaF-SR1* GENE OF MOSQUITO *AEDES AEGYPTI*.

In the sex determination cascade of *Ae. aegypti* the primary signal that produce sex-specific splicing of the downstream genes *Aeadsx* and *Aeafnu* is unknown.

Recent bioinformatic studies conducted in our laboratory on a group of genes of *Aedes aegypti*, that share an RNA-binding domain of RRM type, have identified an hypothetic master gene of sex determination mechanism. This gene (AAEL012618) in the *Aedes* genome database shows three exons and we designed two primers *AeaRRM-A+* and *AeaRRM-A-* in the first and third exons respectively. We performed RT-PCR to verify the expression pattern from embryonic till adulthood stage. We did a PCR, as a positive control, with the *rp49* gene (Fig. 14A); the RT-PCR, with the primers designed, revealed a transcripts in 3rd and 4th mixed instar larval stage sample, in mixed pupae and finally in only female adult (Fig. 14B). We investigated the sex-specific expression of this gene in single sexed larvae and pupae using *dsx* sex-specific splicing RT-PCR analysis (fig. 14C). We obtained separately male and female of the 1st- 2nd instar larval stage, 3rd - 4th and pupae. On the same samples we performed RT-PCR using the primers *AeaRRM-A+* and *AeaRRM-A-* and obtained a transcript only in female samples of each stage (Fig. 14D).

Hence, it is possible that we have isolated a putative female gene that could be involved in the *Ae. aegypti* sex determination.

To obtain more information about its function we have produced a dsRNA, using a female product obtained from RT-PCR with primers *AeaRRM-A+* and *AeaRRM-*. The Dr. Derric Nimmo of the "OXITEC" ltd. UK company will inject this dsRNA in larvae of *Ae. aegypti*.

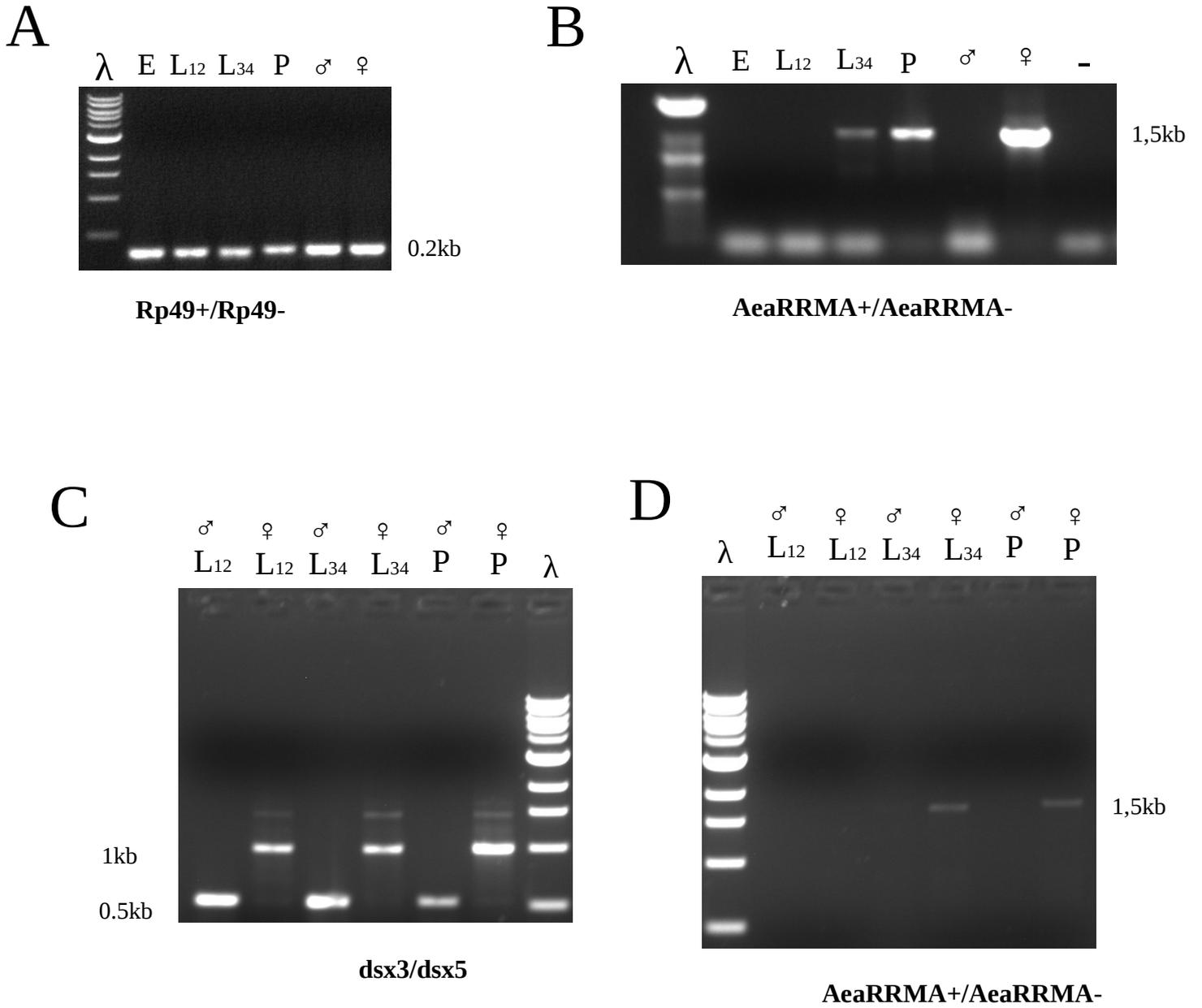


Figure 14. Developmental expression analysis of AeaF-SR1 gene.

DISCUSSION

The molecular and *in silico* subtraction experiments performed in our laboratory allowed us to identify some clones more expressed in the XY/XX respect to XX-only embryos RNA samples. In particular, both experiments identified the same clone, CcBA11 (or Cc10393). The molecular analyses suggested us that the CcBA11 clone could have two paralogues, *CcGm2* Y-linked and expressed exclusively in male, and *CcGm1* probably autosomic or X-linked, expressed in both male and female individuals. The functional analyses through RNAi experiments of the *CcGm2* failed but the molecular analyses through PCR on the first instar larvae injected with dsRNA of *CcGm2* at embryonic stage, revealed the presence of one female injected larval sample with *Cctra* and *Ccdsx* male-specific product. To overcome problems related to the RNAi injection we will perform the injection of synthetic capped RNA of the clone Cc10393, longer than the *CcGm2*. In this case the ectopic expression of this putative M factor could produce a male-specific splicing of *Cctra*, *Cctra-2*, *Ccdsx* and *Ccfru* downstream genes to promote maleness in XX embryos.

In addition, we will perform molecular and functional analyses of other male-biased transcripts derived from *in silico* subtraction.

Most probably *CcGm2* (or Cc10393) could be an intermediate regulatory factor upon which an hypothetical *MDF* could acts or there are several genes, included *CcGm2*, that together contribute to the male-specific splicing of downstream genes of sex determination cascade in *C. capitata*.

In *Ae. aegypti* *Aeadsx* and *Aeafru* genes shared a sex-specific splicing and most probably have a common sex-specific splicing regulator that is still unknown. From Blast search it has been reported that no significant putative orthologues of *transformer* are present in *Aedes* genome. Hence, the sex-specific splicing of *Aeadsx* and *Aeafru* could be achieved by a direct action of the *Aedes M* gene. In our laboratory, we have identified from bioinformatic studies a gene that shared an RNA-binding domain of RRM type. We have performed molecular analysis of this gene sharing a transcript only in females from third instar larval stage till adult. In collaboration with the Dr. Derric Nimmo of the "OXITEC" ltd. UK company the injection of dsRNA of this transcript in embryos and larvae of *Ae. aegypti* are in progress. Hence, it is conceivable that this is a putative gene involved in the *Ae. aegypti* sex determination mechanism.

Knowledge about the regulation of genes involved in sex determination mechanism of *Aedes* would contribute to the development of transgenic sexing strains in *Aedes aegypti*, which are useful for the male sterile insect technique.

MATERIALS AND METHODS

List of primers

RPP1+	TTGCGTTTACGTTGCTCTCG
RPP1-	AATCGAAGAGACCGAAACCC
Y1-SPEC	GCGTTTAAATATACAAATGTGTG
Y2-SPEC	TACGCTACGAATAACGAATTGG
Cctra164+	CAGCGGAAGTGGTTCGGTT
Cctra900-	TCCATGATGTCGATATTGTCC
Ccdsx1400+	TGAAGAGGCATCAAGGCGTAT
Ccdsx1130-	CTGGTGGTGACATCGTATCG
Ccdsx2000-	ACGACGGCATGACCTTTAAC
CcYF	GCTCGAAGACATGCATTGAA
CcYR	GACGGTAAGTGCCATTCGTT
CcSOD+	TGCTCCGAGAACG TTCACG
CcSOD-	TCATCGGTCAATTCGTGCAC
T7	TAATACGACTCACTATAGGG
SP6	ATTTAGGTGACACTATAGAA
CcFrw483	CCAGCAGTCGTTCCGGTAATAA
CcFrw114	TTAGGACATTTGCCATGGAAT
CcFrwBA11	CGTGGTAATCCTGAAAACAGC
CcRevBA11	CTTACGATCTTCCATGCTTCAC
CcRev483	TGTATCGGAATAACGCATCG
Cc483Y+	CAACAGCAGTCGTTCCGGCAAC
Cc483Y+T7	TAATACGACTCACTATAGGGCAACAGCAGTCGTTCCGGCAAC
CcBA11-T7	TAATACGACTCACTATAGGGCTTACGATCTTCCATGCTTCAC
Cc1197_F	GCGGTGGTGCTCGCGAAAAG
Cc1197_R	TGACCACTCTTACCGCAGCCG
Cc1547_F	GTCGCGTGGTAGTTCTGCCG
Cc1547_R	CCTACAGCAACAGCTTTTAGGCCA
Cc15591_F	GTGGACATGTGACGCGGCTG
Cc15591_R	ACTGCCCGGTGCCATTTGTGG
Cc1197_F+T7	TAATACGACTCACTATAGGGGCGGTGGTGCTCGCGAAAAG
Cc1197_R+T7	TAATACGACTCACTATAGGGTGACCACTCTTACCGCAGCCG
Cc1547_F+T7	TAATACGACTCACTATAGGGGTGCGGTGGTAGTTCTGCCG
Cc1547_R+T7	TAATACGACTCACTATAGGGCCTACAGCAACAGCTTTTAGGCCA
Cc15591_F+T7	TAATACGACTCACTATAGGGGTGGACATGTGACGCGGCTG

Cc15591_R+T7	TAATACGACTCACTATAGGGACTGCCCGGTGCCATTTGTGG
Cc10393+	AGTGGCAACTCCACAATTCC
Cc10393-	CCCCAACCAATTGCAAATAA
Cc12258-	TCAGTTCAACTTTCGTTTATGTATTTTC

REARING OF THE *C. CAPITATA* STRAIN

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

RNA AND GENOMIC DNA EXTRACTION

Total RNA from embryos, larvae, pupae and single adult of the *C. capitata* was extracted with “*Buffer Rosa*” protocol (Andres and Thummel, 1994). Genomic DNA extraction from adult of *C. capitata* (male and female) was performed with “*Holmes-Bonner*” buffer according to Maniatis et al. (1982).

REVERSE TRANSCRIPTION PCR

RT-PCR was performed using RNA (after Dnasei treatment) from different stages with Advantage® RT-for-PCR Kit (Clontech). Starting from a maximum concentration of 4 ug of RNA from each sample, add 1 µl of oligo (dT), incubate the sample at 70°C for 5 min. then the sample is mixed with 5X Reaction Buffer, dNTP mix, Rnase inhibitor, MMLV reverse transcriptase in the total volume of 20 µl. The mixture was incubated in a thermal cycle at 42°C for 1h and 94°C for 10 min. The primer pairs RpP1+/RpP1- was used as the positive control. The following PCR cycles were performed: 5 min at 94°C, 35 cycles with 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, 10 min at 72°C.

DNA SEQUENCING AND SEQUENCE ANALYSIS

Sequencing was performed using an automated 377 DNA sequencer (Applied Biosystem). Sequence comparison were performed using the BLAST search algorithm at the NCBI. Multiple alignments were performed using the MultAlin program.

RNA INTERFERENCE

CcDmaledsRNA was obtained and injected as described for *Drosophila* (Kennerdell and Carthew, 1998). A CcDmale fragment from position 1 to 528 was amplified with primers that introduced a T7 promoter sequence at each end. This template was used to produce dsRNA fragments by in vitro transcription with T7 RNA polymerase using the Megascript Kit (Ambion). The dsRNA was precipitated with ethanol and resuspended in injection buffer (Rubin and Spradling, 1982). Embryos were collected 30 minutes after egg laying, hand dechorionated and microinjected with 1 $\mu\text{g}/\mu\text{l}$ dsRNA. Injected embryos were allowed to develop at room temperature.

RT-PCR ANALYSIS OF INJECTED LARVAE

RT-PCR experiments to analyze *Cctra* and *Ccdsx* expression patterns in larvae injected with CcDmaledsRNA were performed by using the Advantage RT-for-PCR Kit (Clontech) with the following gene-specific primers: Cctra164+ (located in *Cctra* exon 1), Cctra900- (located in *Cctra* exon 2), CcdsxC 1400+ (located in *Ccdsx* common exon 3), CcdsxF 2000- (located in *Ccdsx* female-specific exon 4) and CcdsxM 1130- (located in *Ccdsx* male-specific exon 5).

SYNTHETIC MRNA

Cc10393 transcript was cloned in the pGEM T easy vector. Plasmid DNA was linearized through *ApaI* restriction enzyme downstream of the insert to be transcribed. This plasmid linearized was used to produce a capped synthetic RNA by mMMESSAGE mMACHINE Sp6 kit (Ambion). The synthetic RNA was precipitated with ethanol and resuspended in injection buffer (Rubin and Spradling, 1982). Embryos were collected 30 minutes after egg laying, hand dechorionated and microinjected with 0.250 $\mu\text{g}/\mu\text{l}$ synthetic RNA. Injected embryos were allowed to develop at room temperature.

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