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DOTTORATO DI RICERCA

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"Molecular genetic approaches to the study of early sex determination in the Mediterranean fruit fly *Ceratitis capitata*".

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Introduction

1.1 Insect Pests Control

Insects represent the most abundant group of organisms on earth, comprising about 800,000 described species. A small number of these species cause devastating crop losses or transmit disease to crops, animals and humans. So the insect pests continue to pose a major threat to agriculture.

Of more than 2000 serious insect pest species, 90% remain for which effective natural enemies have not been found. Pest control is at least as old as agricultural production, as there has always been a need to keep crops free from pests. In order to maximize food production, it is advantageous to protect crops from competing species of plants, as well as from carnivores competing with humans. Pest control interventions today are increasingly being implemented within the concept of Integrated Pest Management, known as “IPM”. IPM relies on a combination of practices to reduce damage by insects and related pests. As usually practiced, IPM can also include judicious use of chemical pesticides applied only after scouting reveals pests at economically damaging threshold levels. It also includes evaluation of the temporal distribution of the pest to determine the periods when the pest is most susceptible to preventive, rather than remedial interventions. For now, the Sterile Insect Technique (SIT) is the new strategy which improves specificity in the insect pest control and reduces any detrimental effects on the environment.

1.2 Fruit Flies

Fruit flies (*Insecta*; *Neoptera; Diptera; Brachycera; Muscomorpha; Tephritidae)* are the most agriculturally important family of flies. About 70 species of fruit flies are considered important agricultural pests, and many others are minor or potential pests (White and Elson-Harris, 1992). These pest species cause heavy losses annually because

of the phytophagous behaviour of their larvae, whereas other species are beneficial biological control agents of weeds. Fruits are the most important crops attacked, including citrus, mango, apples, and many others. Actually the fly's life cycle damages the crop. The female fly lays hundreds of eggs inside the fruit that is still healthy. Within few days, they hatch into hungry larvae, that gobble the pulp and destroy the crop. The larvae feed for 1-2 weeks in fruit and develop into pupae after larvae exit the fruit to pupate in the soil.

After 1-2 weeks the transformation from larva to adult is complete. Around 2 weeks later, adult flies emerge to mate and resume the cycle. Economic effects of pest species include not only direct loss by the larval activity and fruit damage, but also the cost of constructing and maintaining fruit treatment such as low oxygen and anoxia treatment to eradicate infestations in the fruit; and the loss in terms of exportations. In fact, to prevent the spread of the fruit fly species, in many countries that are free of that pest, the import of most commercial fruit from affected countries is severely restricted by quarantine laws.

In fact, among these pest species of fruit flies, the *Tephritidae* family is the dipteran group including most of the agricultural pest species, to which belong the genera *Ceratitis, Bactrocera, Rhagoletis* and *Anastrepha. Ceratitis* species are mostly restricted to Africa, except for the Mediterranean fruit fly (*Ceratitis capitata*), also known as Medfly, which has spread to many tropical and subtropical parts of the world. *Ceratitis capitata* is the most notorious pest species in the genus, and it is one of the most polyphagous and widespread species of *Tephritidae*. The genus *Bactrocera*, about 40 species, is local to Africa, the Mediterranean region, Australia, and the Pacific. One of theses species, the oriental fruit fly, *Bactrocera dorsalis* (Hendel), is a very destructive pest of fruit in asiatic and other areas where it occurs. *Rhagoletis* includes 17 species of which were listed as pests. The most serious are the apple maggot (*R. pomonella*), the European and eastern cherry fruit flies (*R. cerasi* and *cingulata*), the blueberry maggot (*R. mendax*), the walnut husk fly (*R. completa*), *R. striatella*, a pest of husk tomato, and *R. tomatis*, a pest of tomato (White & Elson-Harris, 1992).

1.3 Sterile Insect Technique

In recent years, molecular mechanisms regulating sex determination of species such as *Ceratitis capitata,* have received special attention due their potential use in SIT (Sterile Insect Technique) programs for the control and eradication of insect pests (Robinson *et al*., 1999; Saccone *et al*., 2002). SIT involves mass production of the target pest, sterilization by irradiation and sustained release over entire regions of large numbers of sterilized insects, which reduce the native population through infertile matings. Ideally, sterile insects competitively mate with the target population, and the subsequent reduction in the number of feral population is proportional to the number of sterile insects released.

The idea that populations of economically important insect species might be controlled, managed or eradicated through genetic manipulation was conceived by an American entomologist Dr. Edward F. Knipling in the late 1930s. A similar concept was published independently by the Soviet geneticist Serebrovsky (1940). The best example of a success of SIT is the New World screwworm (*Cochliomyia hominivorax*), which over the last fifty years has been eradicated from the U.S., Mexico and recently also from all of the central America and most of Panama (Wyss, 2000). The screwworm prey on warm-blooded animals, including humans, but especially cattle herds. In the 1950s, it was projected at about 200 million dollar annual losses to meat and dairy supplies in America , because the larvae of screwworm could attack open wound and eat into animal flesh, the flies could kill the cattle within 10 days of infection. Knipling and his colleague Bushland tried to find a best and the most efficient way to eliminate the entire screwworm population. Bushland researched chemical treatment of screwworm-infested wound 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). 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. SIT was advanced and promoted by the International Atomic Energy

Agency (IAEA) and the Food and Agricultural Organization of the United Nations (FAO).

SIT is the first method involving insect genetics for population control, and it is amongst the ecofriendly pest control methods. Unlike some other biologically-based methods, it is species specific and does not release exotic agents into new environment neither introduce new genetic variability into existing populations as the release organisms are sterile (Hendrichs, 2002). In SIT program, the item 'sexually sterile ' dose not indicate that the individuals do not produce any gametes but refers to the transmission of dominant lethal mutations, caused by X or gamma rays treatmens at pupal stage, that kill their progeny. It increases in effectiveness with decreasing density of the target pest (i.e. is inversely density-dependent), making it more useful in biosecurity applications in the early or final stages of eradication (Wimmer, 2005).

The application of SIT against medfly focused initially on the concept of eradication, following the successful example of the screwworm. In 1977, the first large SIT program against medfly was initiated in Southern Mexico, with the construction of a 500 million sterile fly mass rearing facility in Tapachula. The aim of the so called Moscamed program was to prevent the spread of medfly, which had become established in Central America, into Mexico and the U.S.A.

A potential problem with SIT is that it relies on the release of large numbers of sterile insects, but in some cases the adult females may themselves be unwanted or even hazardous. Mass rearing facilities initially produce equal numbers of two sexes, but generally try to separate and discard females before release. Possible reasons for such separation are to avoid assortative mating; to avoid any increase in the size of feral population during a genetic control procedure; to eliminate females which cause damage by the ovipositor to the fruitcrops or which may be disease vectors, as in the case of mosquitoes (Barlett and Staten, 1996). For this reason if the females can be removed from the production and release procedures then considerable economic advantages would accrue (Robinson, 1983). A variety of classical genetic approaches have been used to try to achieve this with several genetic sexing strains being developed and used in operational SIT programs (Bailey *et al*., 1980; Robinson *et al*., 1999). Such

methods are known as Genetic Sexing Mechanism (GSMs) or Genetic Sexing Strains (GSSs). All the genetic sexing strains used in operational programs involved the use of a chromosomal translocation to link the wild type allele of selectable/visible mutation to the male determining Y chromosome. In these systems, females are homozygous for the selectable mutation and males heterozygous. Current medfly genetic sexing strains (GSSs) contain two components: the Y-autosome translocation and the *temperature sensitive lethal* mutation (*tsl*, Franz *et al*., 1994). The *tsl* is used to eliminate the females by raising the temperature during egg incubation. The *tsl* mutation was recovered in a *white pupae* (*wp*, Rossler and Rosenthal, 1990) strain; both mutations are closely linked on the right arm of chromosome 5. A corresponding autosomal segment bearing wild type *wp* and *tsl* alleles (wp^+ and tsl^+) has been translocated on to the Y chromosome, conferring both brown colour to pupae and heat shock resistance to XY. Hence XX embryos are killed after a thermal shock to a 34°C temperature during late embryogenesis, 24-48 hours after eggs ovoposition. On the contrary because of the presence of wp^+ pupae marker and of the tsl^+ allele, males survive emerging from brown pupae. This sexing system is made possible by the close linkage of these two markers. However recombination does occur between wp^+ and tsl^+ resulting in a breakdown of the sexing procedure (Robinson A.S., 2002). Even in *C. capitata* the recombination in males is essentially absent, this is not the case when genetic sexing strains are reared in very large number (Robinson *et al*., 1999). Transgenesis can offer novel solutions to develop potentially more stable transgenic sexing strains (TSS). Sex separation methods based on female-specific expression of a conditional dominant lethal gene or the phenotypic transformation of females into males seem to be promising alternatives to the classical GSSs (Saccone *et al*.,2002; Horn and Wimmer, 2003).

1.4 *Ceratitis capitata* as pest

Ceratitis capitata, the Mediterranean fruit fly, or medfly, has capable of causing extensive damage to a wide range of fruit. Now the Medfly is seem to be the one of the world's most destructive fruit pests because of its global distribution, its wide range of hosts, its rapid dispersion through human transport, and its tolerance of colder climates

than most species of tropical fruit flies (Figure 1.1).

The Mediterranean fruit fly is native to tropical west Africa, but has spread to other parts of the world including America, Southern Europe, Australia, and the New World tropics. It has been recorded infesting over 300 cultivated and wild fruits. The host list includes apple, apricot, avocado, bell pepper, carambola, coffee, dates, fig, grape, grapefruit, guava, lemon, lime, loquat, lychee, mango, nectarine, orange, papaya, peach, pear, persimmon, plum, pomegranate, pummelo, quince, sapote, tangerine, tomato, and walnut.

The medfly adults can be easily recognized, *wild type* females has long pigmented bristles on the femur pointing towards the coxa of the foreleg and the ovipositor , *wild type* male exhibits two spatulated bristles on the head, a row of non-pigmented bristles on the ventral part of the femur towards the coxa of the foreleg, short pigmented bristled grouped on the dorsal part of the femur close to the coxa of the foreleg and male genitalia (Figure 1.2). The damage to crops caused by Mediterranean fruit flies result from 1) female lay the eggs inside the fruit and soft tissues of vegetative parts of certain plants, 2) the larvae feed in fruit , and 3) decomposition of plant tissue by invading secondary microorganisms (Figure 1.3).

The most damaging of crops is the larval feeding in fruit. The healthy young fruits become twisted and usually drop. Mature fruits that attacked by larvae may develop a water soaked appearance. The bacteria and fungi can enter the fruit by the larval tunnels, then cause the fruit to rot. These larva also invade young seedlings, succulent tap roots, and stems and buds of host plants (Figure 1.4).

1.5 Sex Determination in *Drosophila melanogaster*

The great genetic knowledge gained in the model system *Drosophila melanogaster* on the genetic control of sex determination offered the opportunity to start two decades ago in *Ceratitis* an evolutionary study (for a recent review see Saccone *et al*., 2010). The tephritid flies are quite different to the *Drosophila* group. The *Drosophila* flies are not agricultural pests and are mostly only a nuisance where fruit and vegetables are stored.

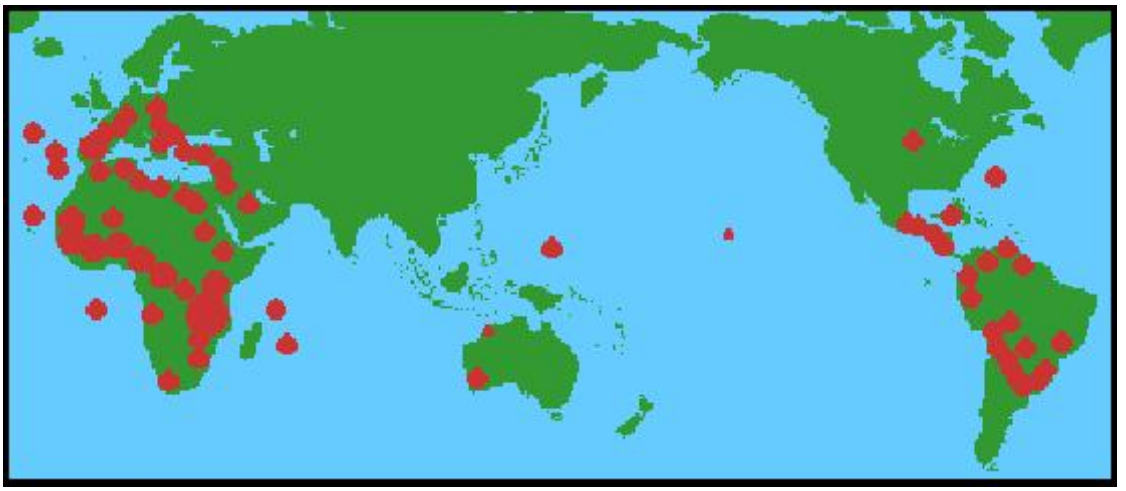


Figure 1.1 - World-wide distribution map of *Ceratitis capitata* Pest.

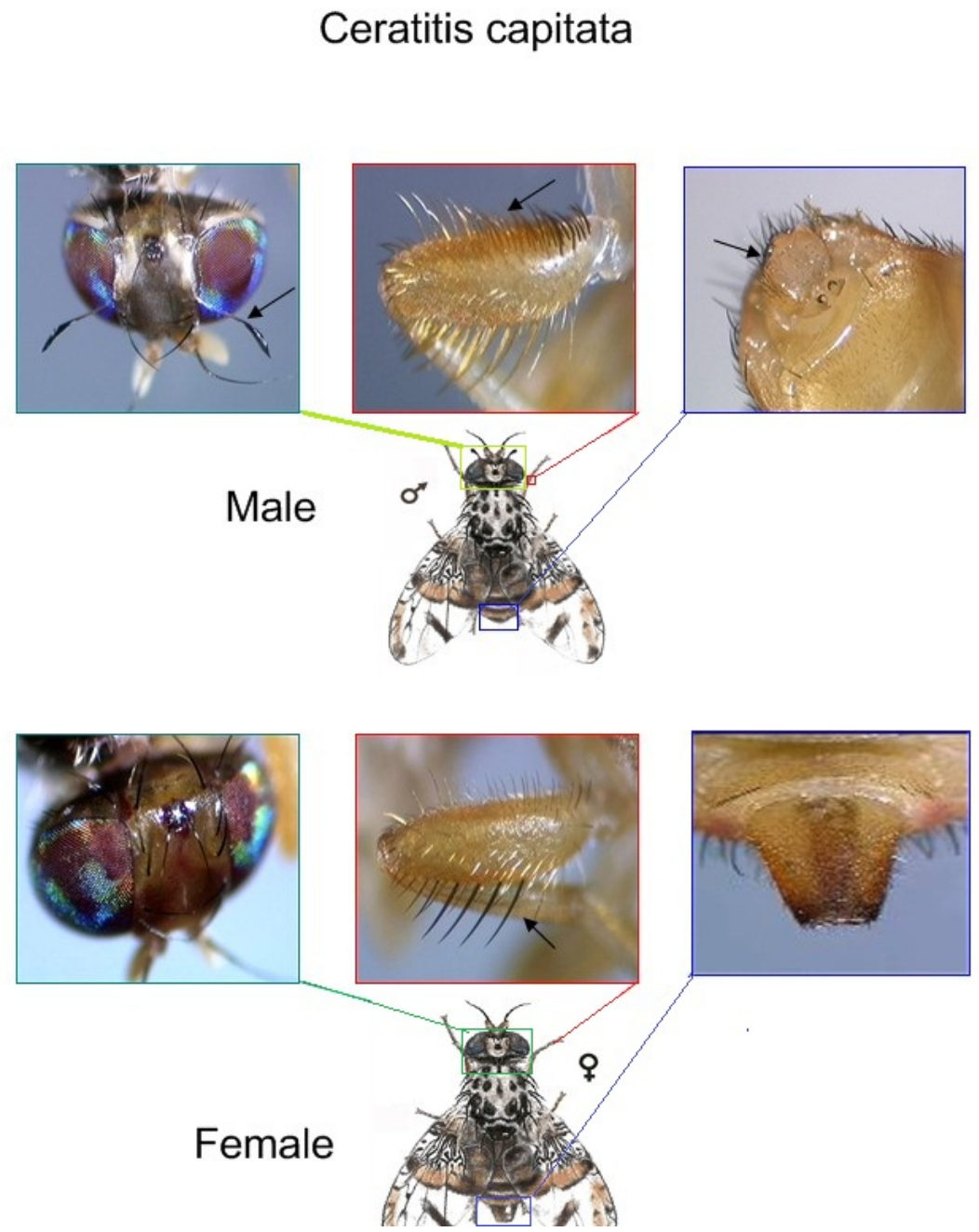


Figure 1.2 - Sexual dimorphism in *C.capitata* is manifested in morphological differences between the sexes.*wild type* females has long pigmented bristles on the femur pointing towards the coxa of the foreleg and the ovipositor , *wild type* male exhibits two spatulated bristles on the head, a row of non-pigmented bristles on the ventral part of the femur towards the coxa of the foreleg, short pigmented bristled grouped on the dorsal part of the femur close to the coxa of the foreleg and male genitalia.

Figure 1.3 - Damages to crops by *Ceratitis capitata.*

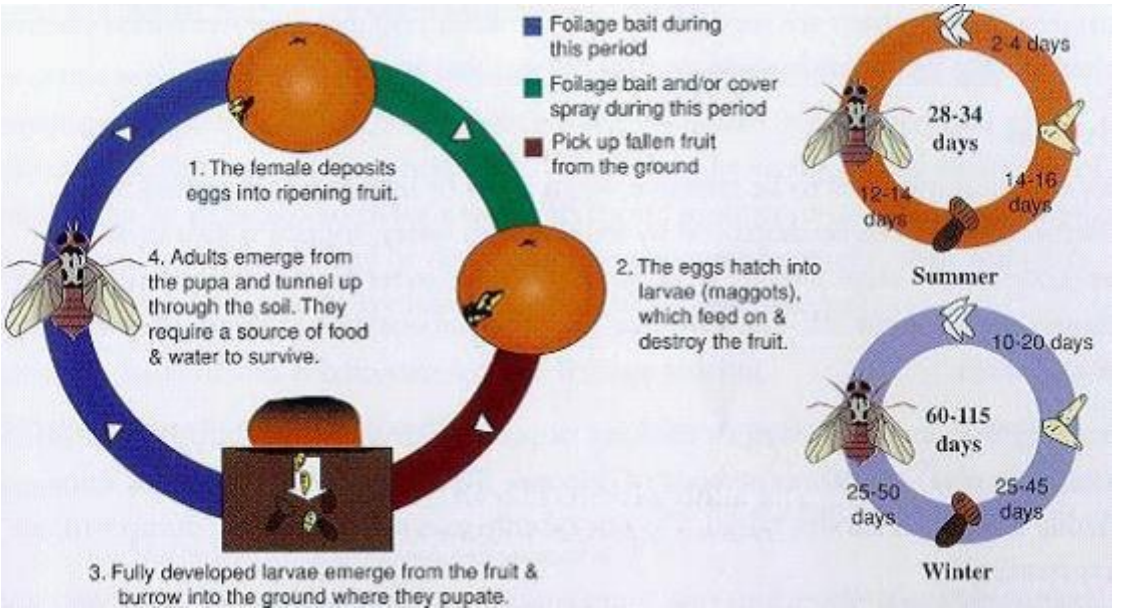


Figure 1.4 - Life cycle of medfly.

Drosophila sex determination provides the best-understood example of a regulatory pathway based on a cascade of alternative splicing events controlling and controlled by key regulatory genes: *Sex-lethal* (*Sxl*), *transformer* (*tra*), *transformer -2* (*tra-2*), *doublesex* (*dsx*) and *fruitless* (*fru*). *Sxl*, *tra*, *tra-2*, *dsx* and *fru* that interact according to a hierarchical organization (*Sxl>tra+tra-2>dsx/fru*) (Cline1988; Burtis and Baker, 1989; Inoue *et al*., 1990). The recent identification and evidence in *Drosophila* point to the number of X chromosomes rather than the X:A ratio as the primary signal (Erickson and Quintero, 2007). According to this point, the male or female dose of X chromosomes is defined by the number of X-linked signaling elements (XSE) in the zygote (1X or 2X), which function when in double dose in XX early embryos to transcriptionally activate the *Sex-lethal* gene (*Sxl*) (Figure 1.5).

The *Sxl* gene is the switch gene in response to the transient primary single (XSE) that can determine the choice between the male and female development in *Drosophila.* The *Sxl* gene starts to be active very early (2 hours from oviposition) only in XX embryonic cells and it produces female-specific transcripts coding for a *Sxl* RNA binding protein essential to maintain the female-specific splicing mode of *Sxl* pre-mRNA itself. Indeed in its own transcript, Sxl protein represses inclusion of default male-specific stop containing exon that aborts translation, thus initiating the positive feedback loop that maintains functional *Sxl* expression in females. After few hours (at 4 hours from oviposition) the gene starts to be transcribed constitutively using a different promoter. However only in XX embryos *Sxl* continues to be active, because the early SXL protein started the positive feedback loop and continues to promote male exon skipping; in XY embryos Sxl produces longer male-specific transcripts because of the absence of early SXL protein.

Sxl protein also regulates the choice between two alternative 3'splice sites in the *transformer* (*tra*) pre-mRNA , by binding specifically to a short cis regulatory element nearby to the non-sex-specific site, and preventing its use (Sosnowski *et al*., 1989). This cis element is also present nearby the *Sxl* male-specific exon and its used for the positive autoregulation.. In female SXL promotes also the female specific-splicing of its downstream target, the pre-mRNA of *transformer* (*tra*), so that the full-length TRA

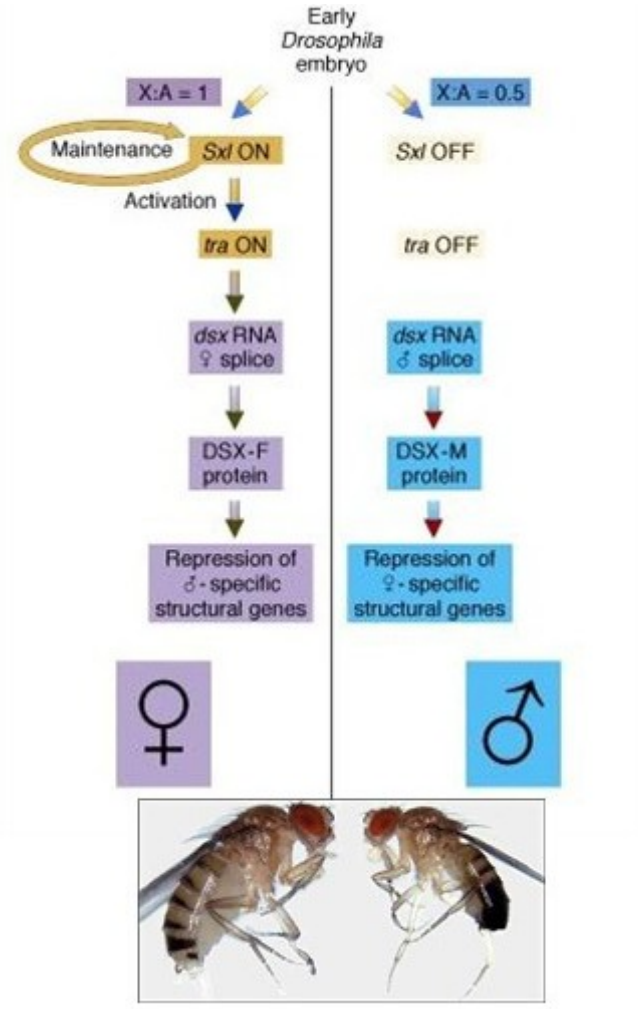


Figure 1.5 - Sex determination cascade in *Drosophila melanogaster.*

protein is produced only in females (Sosnowski *et al*., 1989; Inoue *et al*., 1990; Valcarcel *et al*., 1993). In males, the *Sxl* gene is "off", therefore *tra* male-specific splicing is governed by a default mechanism resulting in mRNA encoding for small non-functional TRA peptides (Butler *et al*., 1986; Boggs *et al*., 1987). The *doublesex* (*dsx*) and *fruitless* (*fru*), downstream regulatory components of the cascade, are controlled by TRA in the females and by default splicing in males. The protein isoforms produced by *dsx* and *fru* are responsible for the development of sex-specific somatic traits and behavioral traits. TRA is able to direct the female-specific splicing of *dsx* and *fru* pre-mRNAs (Baker and Ridge, 1980; McKeown *et al*., 1988; Hoshijima *et al*., 1991; Heinrichs *et al*., 1998). Tra can associate with Tra-2, via its arginine-serine (RS) domains, forming part of protein complex that binds to target *dsx* and *fru* pre-mRNA repeat elements (Inoue *et al*., 1992). The *dsx* and *fru* splicing regulation require not only the product of non-sex-specific *tra-2* gene but also other general splicing factors (Amrein *et al*., 1988; Burtis and Baker, 1989; Inoue *et al*., 1992). The sex-specific *dsx* transcripts code for two Dsx isoforms acting as transcriptional regulators of terminal genes responsible for the sexual dimorphism. DSX^M represses expression of female-specific genes and activates expression of male-specific genes, leading to male differentiation. DSX^F has the opposite effect, leading to female differentiation (Jursnich and Burtis, 1993). The fru gene encodes only in males for male-specific FRU isoforms which are required for the development of male courtship behaviour (Salvemini *et al*., 2010).

1.6 Sex Determination in *Ceratitis capitata*

The isolation of *Sxl*, *tra* and *dsx* homologous genes in the distantly related dipteran species such as *Ceratitis capitata* led to discovery of the partial conservation of the *Drosophila* regulatory *tra>dsx* module despite 120 Myr of phylogenetic distance between the two species (Saccone *et al*., 1998; Pane *et al*., 2002; Saccone *et al*., 2010). In particular, the *Ceratitis capitata* homolog of *Sxl* is not a "switch" gene as in *Drosophila*, it is expressed in both XX and XY embryos, irrespective of whether the

male-determining Y, which bears the M factor, is present or absent, and this observation is inconsistent with a main *CcSxl* sex-determining function (Saccone *et al*.,1998; Saccone *et al*., 2002). However, *Dmtra* is a subordinate target of *Sex-lethal* (*Sxl*) in *Drosophila*, but *Cctra* plays an essential role in sex determination of *Ceratitis capitata* by initiating an autoregulatory mechanism in XX embryos which provides continuos *tra* female-specific function and acts as cellular memory maintaining the female pathway (Figure 1.6).

The *tra* gene (*Cctra*) of *C. capitata*, as in *Drosophila*, is a key intermediate regulatory gene for femaleness through its regulation of the *dsx* and *fru* genes and has a novel additional master function begin able to positive autoregulate and maintain female sex determined state during all fly life (Pane *et al*., 2002; Salvemini *et al*., 2009). *Cctra* is apparently dispensable for maleness in *Ceratitis capitata*. In contrast in *Drosophila*, the presence of the Y chromosome is necessary for male fertility, but not for male development (Hardy *et al*., 1981). But, RNAi-treated female XX embryos of *Ceratitis capitata* can develop into fertile males, which indicate that transient repression of *Cctra* by RNAi is sufficient to implement fully normal male development. The cases of complete sexual transformation of genetic *Ceratitis capitata* females (XX) into fertile males by RNAi prove that the Y chromosome, except for the dominant male determiner M, does not supply apparently any other contribution to both somatic and germline male development, as suggested by previous Y-chromosome deletion analysis (Willhoeft and Franz, 1996). *Cctra-2* is also conserved in medfly and exerts a novel key function on *Cctra* autoregulation. *Cctra-2*, as in *Drosophila*, is necessary for promoting *Ccdsx* and *Ccfru* pre-mRNAs female-specific splicing and that unlike in *Drosophila*, *Cctra-2*, as *Cctra* too, appears to be necessary for establishing female sex determination in early XX embryos and possibly for maintaining the positive feedback regulation of *Cctra* during development (Salvemini *et al*., 2009). In XX embryos, maternal *Cctra* and Cctra-2 mRNAs provide full-length *Cc*TRA and CcTRA-2 proteins that initiate a positive feedback regulation. These proteins promote a female-specific splicing of the zygotically transcribed *Cctra* pre-mRNA so that new full-length *Cc*TRA protein can be produced. The newly synthesized protein controls the maitenance of *Cctra*

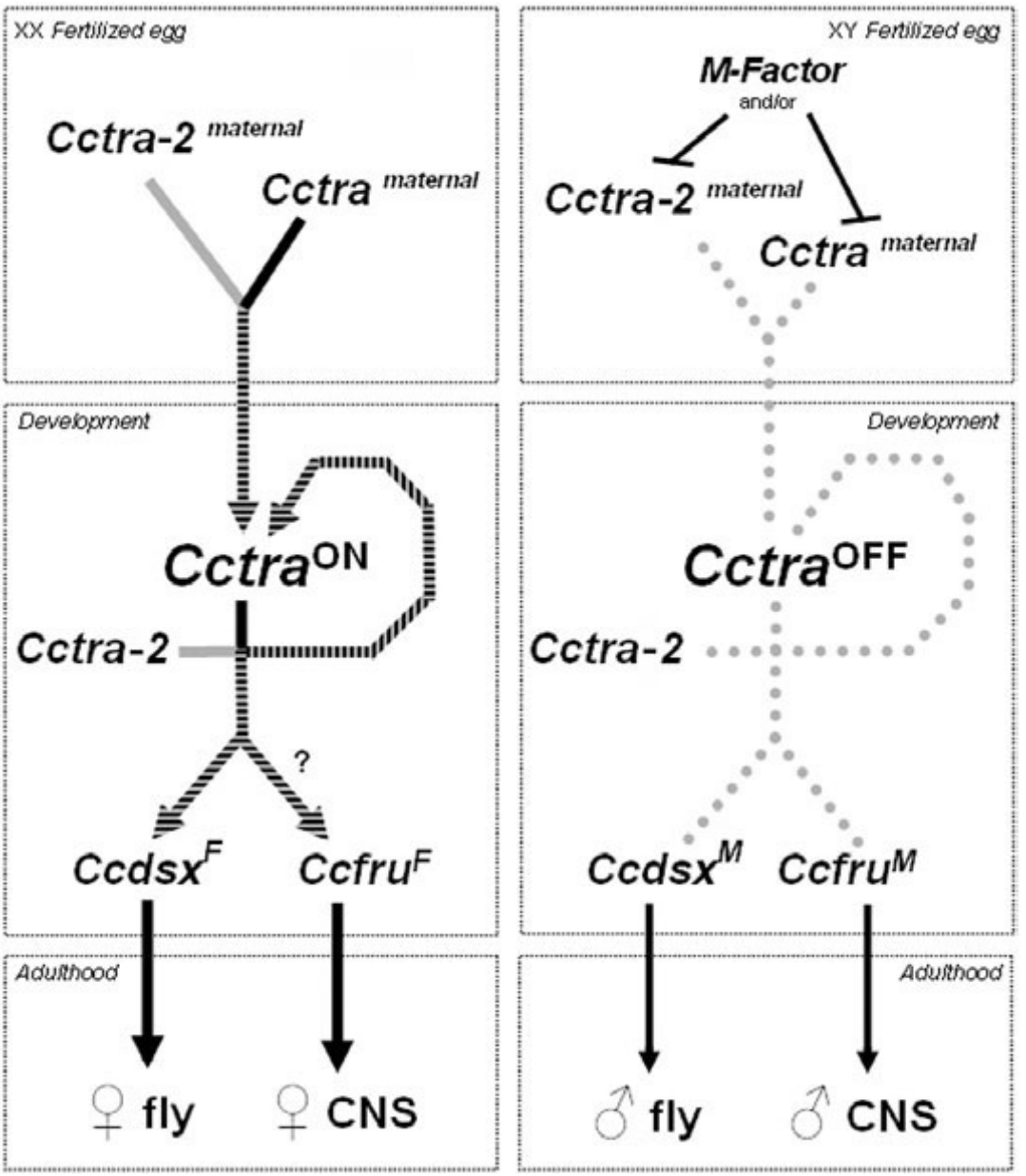


Figure 1.6 - Sex determination cascade of *Ceratitis capitata*.

autoregulation and the female-specific splicing of *Ccdsx* pre-mRNA. The *Cc*DSX^F protein is produced promoting, most probably as in *Drosophila*, female development and repressing male development. In XY embryos, the M factor which presents on the Y chromosome would prevent the autoregulation of *Cctra*. So the non functional TRA protein is produced, embryos develop into males.

The medfly *dsx* gene, *Ccdsx*, produced sex-specific transcripts by alternative splicing as in *Drosophila*, suggesting its functional conservation as a sexual differentiation regulator (Saccone *et al*., 1996; Saccone *et al*., 2008). The pre-mRNA of *Ccdsx* is also alternatively spliced giving rise to sex-specific products that show a remarkable structural conservation when compared with the corresponding male and female products in *Drosophila* (Saccone *et al*., 2008). The putative TRA/TRA-2-binding sites are found by sequence analysis of *Ccdsx*, close to the regulate splice site and within the untranslated female-specific exon, as in *Drosophila*. The splicing of *Ccdsx* is hence apparently conserved and under the control of TRA and TRA-2 (Saccone *et al*., 2002).

1.7 Sex Determination homologous genes in other species

The molecular comparative study between *Drosophila* and *Ceratitis* is very useful not only to understand evolution of sex determining genes and pathways but also to develop a sexing strategy to improve the S.I.T., and also to obtain novel information on the basic molecular genetics in this insect pest. The presence of putative TRA-TRA2 binding sites in *M.* d*omestica*, *A. gambie* and *A. aegypti dsx* genes suggests that a tra and tra-2 homologues are likely to exist in all these species. Autoregulating versions of the *Ceratitis transformer* gene, have been isolated later on also in other dipteran species. Homologues of *tra* and *tra-2* have been reported not only in *Drosophila* species (O'Neil and Belot, 1992) and *Ceratitis capitata* (Pane *et al*., 2002; Salvemini *et al*., 2009), but also in *Musca domestica* (Burghardt *et al*., 2009) and *Lucilia cuprina* (Concha and Scott, 2009). Niu and his colleagues (2005) have identified *Bombyx mori tra-2* gene (*Bmtra2*) cDNA by blasting the EST database of *B. mori*. Six types of *Bmtra-2* cDNA clones were identified; all isoforms of *Bm* TRA-2 protein showed striking structural

similarity to *Drosophila* TRA-2 proteins.

The Australian sheep blowfly *Lucila cuprina*, is an economically important pest insect belonging to the Caliptratae subsection of dipterans and thus closely related to the housefly *M. dometica* (Beck *et al*., 1985; Heath and Bishop, 2006). Homologues of *Sxl* gene have been isolated from species in which the male sex is determined by a dominant male determiner, such as *C. capitata* (Saccone *et al*., 1998), *Megaselia scalaris* (Sievert *et al*., 2000), *Musca domestica* (Meise *et al*., 1998), *Chrysomya rufifacies* (Muller-Holtkamp, 1995), *B. tryoni* and *Lucila cuprina*. The *Sxl* gene has been also characterized in the lepidopteran *Bombyx mori* (Niimi *et al*., 2006). In all these species the *Sxl* homologues, although they encode highly conserved protein, display a splice pattern identical in females and males and do not appear control sex determination. Interestingly, in both *M. scalaris* male and female, *sxl* appears to be expressed only in the germline and not in the soma (Siecert, Kuhn and Traut, 1997; Sievert *et al*., 2000).

Homologues of *dsx* gene have been isolated from *C. capitata* (Saccone *et al*., 2002), *A. obliqua* (Ruiz *et al*., 2005), *B. tryoni* (Shearman and Frommer, 1998), *M. scalaris* (Kuhn *et al*., 2000), *Anopheles gambie* (Scali *et al*., 2005), *Aedes aegypti* (Mauro *et al*., 2005), *Bombyx mori* (Ohbayashi *et al*., 2001), *Apis mellifera* (Cho *et al*., 2007) and very recently in haplo-diploid wasp *Nasonia vitripennis* (Oliveira *et al*., 2009). In all these species male- and female-specific mRNAs are produced by the *dsx* homologues. They encode for male-specific Dsx^M and female-specific Dsx^F proteins, which are highly conserved. These homologues show a similar gene structure and, with the exception of *Bombyx mori*, putative dsxRE in the 3' untranslated region of the female-specific exons which suggest that the same sex-specific control of *dsx* expression occurs in these species as in *D. melanogaster*.

Dsx gene of *Nasonia vitripennis* has been identified by Oliveira and his colleagues (2009), but they did not known which gene regulate alternative splicing of *dsx* in *Nasonia*. And recently Verhulst and his colleagues have identified the *Nasonia vitripennis transformer* (*Nvtra*) regulates the female-specific splicing in the sex determination. The maternal input of *Nvtra* messenger RNA, in combination with

specific zygotic *Nvtra* transcription, in which *Nvtra* autoregulates female-specific splicing, is essential for female.

Sex determination in the honeybee *Apis mellifera* is controlled by the *complementary sex determination* (*csd*) locus; Beye and colleagues reported the cloning of *csd* locus of *Apis mellifera* (Beye *et al*., 2003), it codes for an SR protein, and different alleles have very different aminoacidic sequences. Injection of *csd*-dsRNA into developing eggs caused genetic females to develop as male larvae and, interestingly, common region of different alleles shows similarity (29%) with *Cc* TRA N terminal region.

The housefly (*Musca domestica*) is another excellent model system to study sex determination in dipteran species. In the sex determination of housefly, the M factor which is located on the Y chromosome is the dominant male-determining factor (Hiroyoshi, 1964); the female determiner F plays as the master switch in the housefly pathway, like *Sex-lethal* (*Sxl*) in *Drosophila*. The *transformer* of *Musca* (*Mdtra*) expresses functional products only in female by alternative processing which corresponds to female determiner F. The homolog of *doublesex* of *Musca*, *Mddsx*, acts as a important effector in the pathway downstream of F (Hediger *et al*., 2004). It can produce a set of sex-specific protein isoforms that functionally correspond to the *dsx* variants in *Drosophila*. Similarly to the model proposed by Pane et al., (2002), in *Musca* XX embryos, *Mdtra* is activated by maternal $Mdtra^{mat}$ and $Mdtra2^{mat}$ products; then *Mdtra* maintains its productive as (ON) mode of expression throughout development by a positive feedback loop (Hediger et al., 2010). *Mdtra*, together with *Mdtra2*, sets its direct downstream target *Mddsx* into the female mode of expression, which leads to overt female differentiation. In a standard M-containing zygote, the activation of *Mdtra* is prevented by the paternally transmitted M. Then *Mdtra* is "OFF" and *Mddsx* is set by default into the male mode of expression and male development ensues.

Gabrieli *et al*., (2010) recently used a PCR-based sexing method for *Ceratitis*, which takes advantage of a putative LTR retrotransposon MITE insertion on the medfly Y chromosome and analysed the transcriptomes of individual early male and female embryos by RT-PCR. They found that the heterogeneity of the *Cctra* mRNA population

during the “splicing-resetting” phase (5-8 h after oviposition) is indicative of a threshold dependent activity of the CcTRA protein. So the maternally inherited *Cctra* transcripts in the female embryos are insufficient to produce enough active protein to promote *Cctra* female-specific splicing. The slow rate of development and the inefficiency of the splicing mechanism in the pre-cellular blastoderm facilitates the male-determining factor (M) activity, which probably acts by inhibiting CcTRA protein activity.

1.8 Suppression Subtractive Hybridization (SSH)

Suppression subtractive hybridization (SHH) is a well established molecular subtraction method (Lukyanov *et al*., 1994; Diachenko *et al*., 1996). SSH is a powerful technique for studying the biological processes and allowing comparison of two mRNA populations and isolation of fraction enriched in differentially distributed molecules. This method is used to identify genes with differential expression pattern in different samples (tissues, sexes, etc.), in particular genes involved in the regulation of basic biological processes and compare the genes that either are over-expressed or exclusively expressed in one population compared with another. The SSH technique has many potential applications in molecular genetics and positional cloning studies, including the identification of disease-related, developmental, tissue-specific, and other differentially expressed genes.

SSH is a method that requires only one round of subtractive hybridization , it eliminates any intermediate steps for physical separation of single-stranded (ss) and double-stranded (ds) cDNAs and can obtain greater than a 1000-fold enrichment for differentially expressed cDNAs (Figure 1.7). It based on the suppression PCR by inverted terminal repeats (ITR). The long inverted terminal repeats when attached to DNA fragments can selectively suppress amplification of undesirable sequences in PCR procedures.

Two types of tissues or cell populations being compared of cDNA are needed to synthesized that is the first step to prepare for the SSH. The cDNA population in which specific transcripts are to be found is called tester cDNA, and the reference cDNA

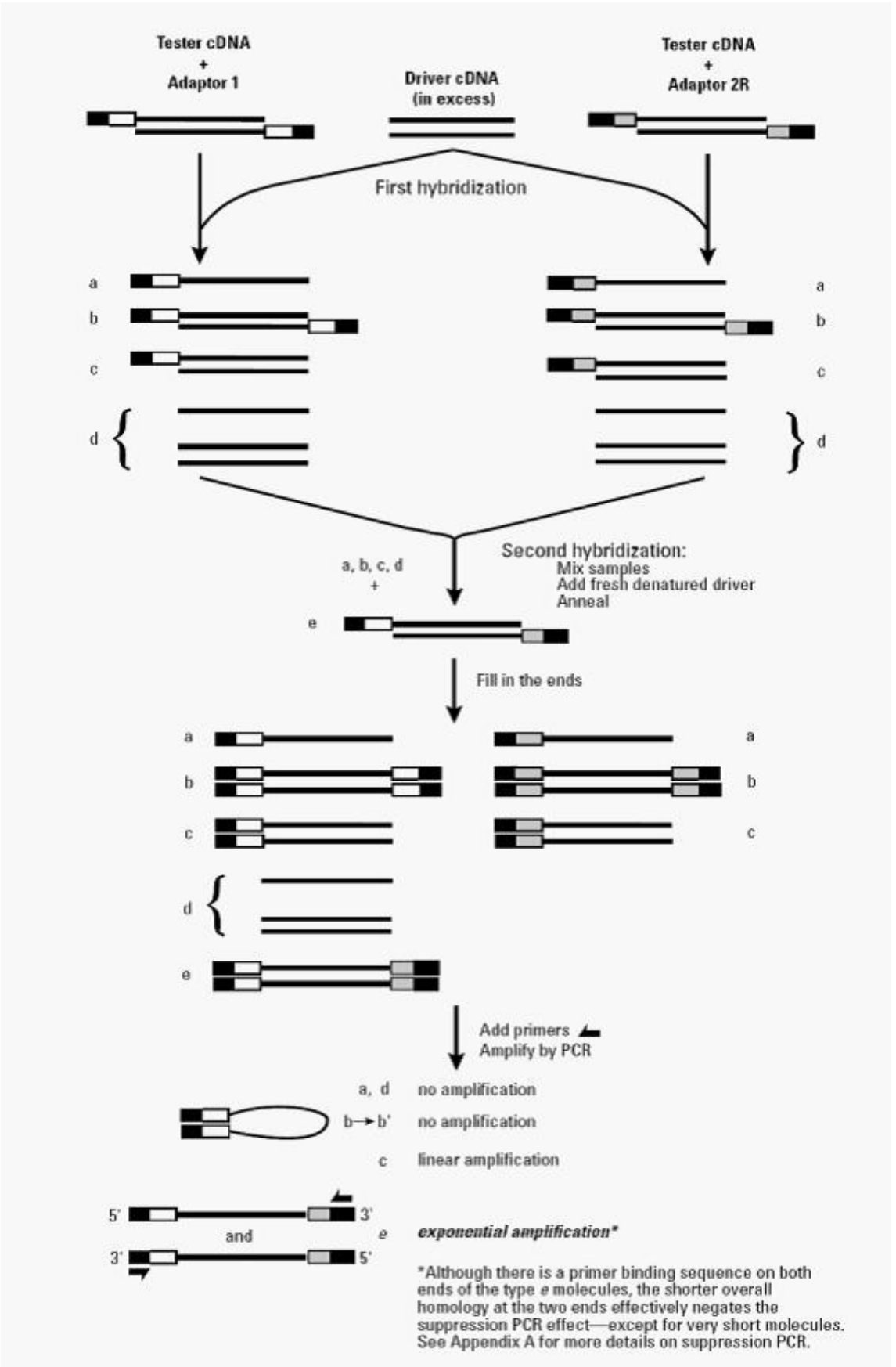


Figure 1.7 - Schematic representation of the SSH method.

population is called driver cDNA. Then the tester and driver cDNAs are digested with a four-base-cutting restriction enzyme and the tester cDNA is divided into two portions, and each is ligated to a different ds adaptor (adaptor 1 and 2R). Adaptor 1 and 2R do not contain a phosphate group of their ends and attach to the 5' ends of the cDNAs. The adaptor 1 and 2R have two parts (Figure 1.7): the outer part is represented by a solid box (for example:it is a T7 promoter) and the inner parts are represented by a clear box of adaptor 1 and the shade box of adaptor 2R (for example: inner part may has the sites of *Not I* ,*Sma I*, *Xma I*,*Eag I* or *Rsa I*). After two round of hybridization, the first PCR use the primer 1 which is correspond to the outer part of the Adaptor 1 and 2R; the second PCR amplification use the Nested PCR primer 1 that is correspond to the inner part of adaptor 1 and the Nested PCR primer 2R which is correspond to the inner part of adaptor 2R.

In the first hybridization, an excess of driver cDNA is added to each sample of tester cDNA. After annealing, four types of molecules generated in each sample are amplificated (Figure 1.7). Type a molecules are single-stranded (ss) tester molecules with adaptor, include equal concentrations of high- and low-abundance sequences because reannealing is faster for more abundant molecules due to the second-order kinetics of hybridization. Type b molecules are double-stranded (ds) tester molecules with inverted repeats. Type c molecules are double-stranded (ds) tester-driver molecules with only one type of adaptor. Type d molecules are ss- or ds-driver molecules without adaptor.

During the second hybridization, the two primary hybridization samples are mixed together in the presence fresh denatured driver. Type a cDNAs from each tester sample are now able to associate and form a new type e hybrids. The novel type e hybrids are amplificated that are double-stranded tester molecules with different ss ends that correspond to Adaptor1 and Adaptor2R. Freshly denatured driver cDNA is added to enrich fraction e further for differentially expressed sequences. The entire population of molecules is then subjected to two rounds of PCR to amplify selectively the differentially expressed sequences.

Prior to the first cycle of primary PCR, the adaptor ends are filled in, creating the

complementary primer binding sites needed for amplification. Type a and d molecules lack primer annealing sites and cannot be amplified. Type b molecules form a panhandle-like structure that suppresses amplification. Type c molecules have only one primer annealing site and can only be amplified linearly. Only type e molecules, which have two different primer annealing sites, can be amplified exponentially. These differentially expressed sequences are greatly enriched in the final subtracted cDNA pool. Then a secondary PCR is performed using nested primers to reduce any background PCR products and enrich for differentially expressed sequences. The secondary PCR product is ready for the further analysis, including cloning, screening, etc.

Results and Discussion

Summary of the research project

The Y chromosome of *Drosophila* has several unusual features that together have made the molecular identification of its genes difficult. In addition to ribosomal DNA and a few other multiple copy genes, it is known to contain six single-copy genes essential for male fertility (*kl-1*, *kl-2*, *kl-3*, *kl-5*, *ks-1*, and *ks-2*) (Carvalho *et al*., 2000). In *Drosophila* X0 males are completely normal (except for the sterility), so the Y chromosome seems to have an unusual functional specialization, apparently containing only genes directly involved with male fertility. In contrast with the usual eukaryotic chromosomes, the *Drosophila* Y contains a coherent set of genes, being an assemblage of male-related genes collected during evolution from the whole genome. The molecular identification of the Y-linked genes is revealing the underlying logic of the process of gene recruitment (Clark *et al*., 2001).

My Ph.D. experimental work was focused on the search and genetic study of Y-linked putative genes of *Ceratitis capitata*. In particular, I have identified a Y-linked gene, *yt1*, which is actively transcribed from very early developmental stages of XY individuals. Then I attempted to identify and subsequently clone by suppressive subtractive hybridization technique novel Y-derived early transcripts and putative early male-specific genes, in the aim of cloning also the Male determining factor of *Ceratitis capitata*.

Expression analysis of Y-linked early transcripts

Genomic DNA fragments that are derived from the Y chromosome of *Ceratitis capitata*, have been previously isolated using a differential hybridization approach (Anleitner and Haymer, 1992). In the GenBank database, these authors published DNA sequences of 4 genomic partially overlapping clones (named 5Kb, pM21, pM11 and pY114), all containing a repetitive element which seems to be Y-specific. We have used the longest of these sequences (5kb; Accession Number: AF115330.1 - 5,642 bp long) to

search by Blast algorithm for homologous hits in NCBI databases. Interestingly, when using BlastX on *Drosophila* genome database, we have found that a *Ceratitis* DNA sequence is related to a gene (CG13340) located on the *Drosophila* chromosome 2R, which encodes a leucyl aminopeptidase (Lap) (Figure 2.1). This *Drosophila* gene lacks of genetic mutations but it is known to be expressed in adult testis, suggesting a putative role in the control of *Drosophila* male fertility. Furthermore *Dmlap* has three paralogs in *Drosophila* genome encoding proteins of aminopeptidase family. The *C. capitata lap* (*Cclap*) ORF is interrupted in its genomic sequence by multiple stop codons, suggesting that corresponds to a pseudogene but on the other side it could be possible that this genomic region corresponds to an intron (Figure 2.2). Furthermore considering that *Cclap* is strictly linked to a repetitive Y-specific sequence, it is thinkable that other Y-linked *Cclap* copies are present in other regions. However, we decided to investigate if this Y-linked gene or pseudogene is transcribed. We designed two specific primers on the most conserved regions of the BlastX alignment with *Drosophila* genome (named Y2+ and Y2-) to amplify the Y-linked gene/pseudogene in RT-PCR experiments on total RNA extracted adult male and female flies, as well as, from early embryonic samples and various developmental stages (Figure 2.2). In figure 2.3A is shown the PCR amplification with Y2+/Y2- primer pair on cDNA of *Ceratitis* adult males and females. We got only in males a prominent band of expected size (0.3 kb) and two low abundance bands of 0.6 kb and 0.8 kb, probably due to aspecific primer annealing. The cloning and the sequencing of the 0.3 kb amplification product confirm us that it is the expected 5 kb derived product. We next performed similar analysis on different developmental stages from unfertilized eggs (X0) to pupae. Embryonic RNAs, at 30 min, 3 and 24 hours, were purified from mixed XY/XX embryos and from XX only embryos, produced as described in next paragraph. Results are shown in Figure 2.3B. No amplification signals were detected in unfertilized X0 eggs, in XX larvae or XX pupal stages. At embryonic stages the 0.3 kb expected band was detected in XY/XX embryonal samples from 30 min to 24h where a strong amplification was observed, probably corresponding to a very high expression level. This signal is absent in XX-only embryos, indicating that is its male-specific and Y-linked nature.

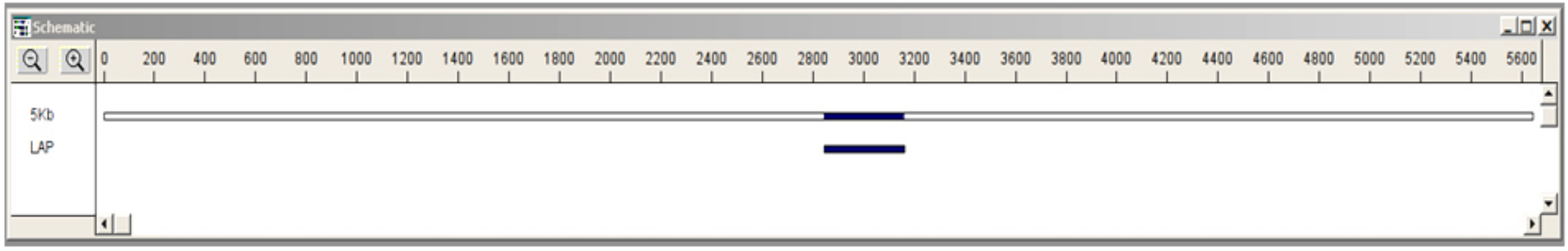


Figure 2.1 - Localization of the *Cclap*-encoding sequence in the Y-specific 5Kb element of *Ceratitis capitata*.

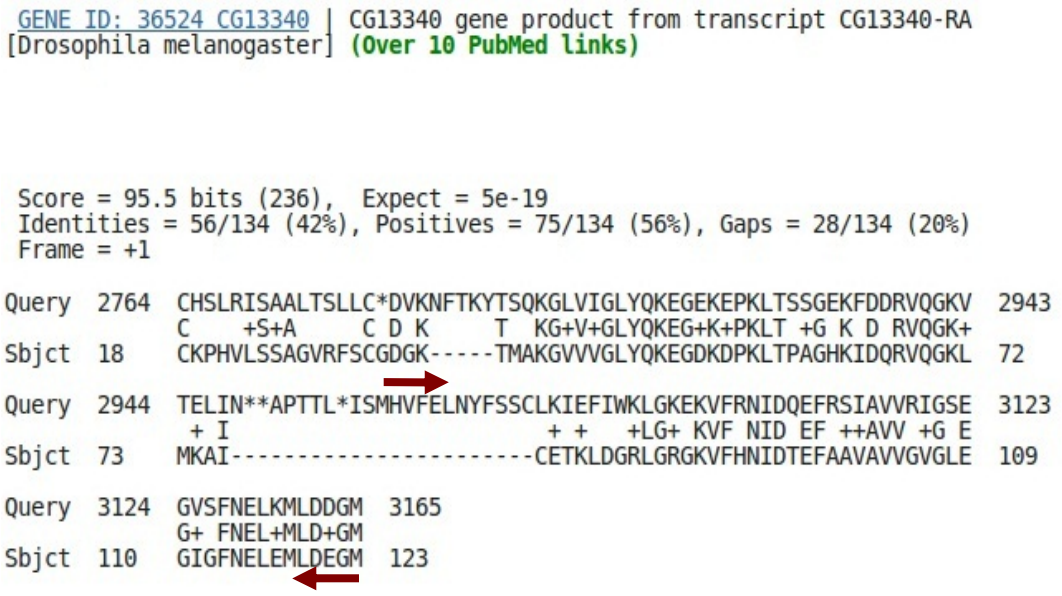


Figure 2.2 - A BlastX alignment of *Ceratitis capitata* 5kb element with the *Drosophila* genome database. The red arrows represent the positions of specific primers (Y2+/Y2-) designed and used in the RT-PCR experiments. Asterisks indicate stop codon position.

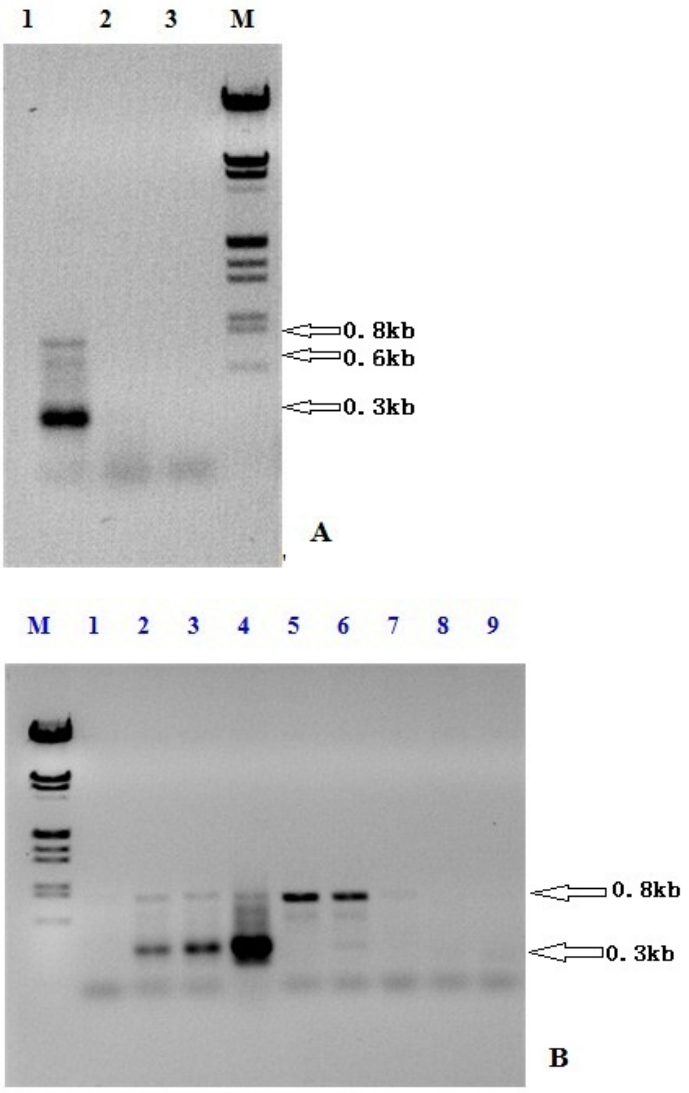


Figure 2.3 – Analysis of the transcriptional activity of the Y-linked *Cclap*. (A) RT-PCR with on RNAs from males (Lane 1), females (Lane 2) and without template (Lane 3) as negative control.(B) RT-PCR on RNA from unfertilized eggs (Lane 1), from mixed XX/XY individuals (Lane 2-4 and 8-9) and from XX-only individuals (5-7) of different developmental stages. Embryos 30 h from OP (OviPosition)(Lanes 2 and 5), 3h from OP (Lanes 3 and 6), 24 h from OP (Lanes 4 and 7). The expected 0.3 kb cDNA product is present in XX/XY individuals and absent in unfertilized eggs and in XX-only embryos. The unexpected 0.8kb cDNA product is only present in 30 min and 3h from OP XX embryos, but also weakly visible in XX/XY embryos (Lanes 2-4). Positive control with rpP1 gene primers are not shown.

In XX-only embryos a strong 0.8 kb band was amplified at 30 min and 3 h stages. The cloning and the sequencing of this cDNA product revealed that it is not derived from Cclap and hence has originated by an aspecific primers annealing event.

These data suggested that a very early *Cclap* male-specific transcriptional activity is detectable in *Ceratitis capitata* embryos and hence we decided to perform a bioinformatical analysis of the entire 5Kb sequence to identify putative promoter(s) of the *Cclap* gene/pseudogene. We used three different prediction software available at:

http://www.fruitfly.org/seq_tools/promoter.html
http://www-bimas.cit.nih.gov/molbio/proscan/
http://www.cbs.dtu.dk/services/Promoter/

The analyses revealed the presence of three putative promoters at region +1000, +1400 and +2700 of the 5 Kb sequence (Figure 2.4), compatible with the position of the *Cclap* encoding sequence. The further molecular characterization and *in vivo* validation of these putative promoters could be very useful for developing sensitive transgenic marker systems and/or conditional sex-specific expression systems useful for generating transgenic sexing strains that could increase the performance of the Sterile Insect Technique.

SSH cDNA library construction

In previous studies we have established a putative window of action for the Male Determining Factor (MDF) of *Ceratitis capitata* at 8-10h from egg laying (Pane and collegues, 2004). At this stage, XY embryos and XX embryos display indeed a differential splicing pattern of *Cctransformer* gene (*Cctra*) which is a target of MDF. These data were recently confirmed by independent experiments of another group (Gabrieli *et al*., 2010). At this early stage we can imagine that also the *CcLap* transcripts are actively transcribed, seeing its active expression at both 30 min and 24h from oviposition.

Hence we approached the problem of identifying early male-specific expressed

Promoter predictions for 5Kb element:

DDGP Promoter Scan Output:

Start	End	Score	Promoter Sequence
160	210	0.97	tgcgttaaagtatataaccggcactccggaactttgacgactttcagtac
307	357	0.93	ttaataaacttttaaaacaggcaaaacagacgcagtcgacacgggaaatc
965	1015	0.93	gcgttcacaatatataaaatctcaagcacatagctcatctacggaatctg
1126	1176	0.89	aagaatatcataaataaccgaggatgctttcatttcaagagggggagtagt
1431	1481	0.85	gattctagtttataaggctagcggacagtaccttacaccggagcacgccg
1908	1958	0.96	ccaacggcataaatataatggaccactctaatgtgcgaacaaaaaaaaac
2059	2109	0.87	aatatatgtatatatattcctatttattgcaacttctattattcgacaat
2589	2639	1.00	aaatatataaataaaaaaaggcgatagtaaaattagattaatcaaacatt
3633	3683	0.89	atagattctgtatgagtggtgcatcactcagccgacgcgcatgatttgt
3692	3742	0.82	acacatttgtatatttaacgcatatatgcaagtgggtacatatataagt
3926	3976	0.89	cgtatcctcatatatgtctggcctcggcaagattattgaaatatatgaat
4188	4238	0.92	cgttttcctatataaaaatttccataaaagacgatataataaatttgatta
5536	5586	0.95	aagtgtggttctaaaaaggaccgtttctattcttgtgtttaataagtatc

BIMAS Promoter Scan Output:

Proscan: Version 1.7
Processed Sequence: 5642 Base Pairs

Promoter region predicted on forward strand in 767 to 1017
Promoter Score: 61.95 (Promoter Cutoff = 53.000000)
TATA found at 974, Est.TSS = 1014

Significant Signals:

Name	TFD #	Strand	Location	Weight
Oct-factors	S01029	+	757	2.754000
IgHC.2	S00814	-	774	2.754000
Sp1	S00801	+	802	2.755000
CCF	S01964		806	2.284000
Sp1	S00781	-	807	2.772000
CREB	S00489	-	826	1.147000
TFIID	S01540	+	975	1.971000
TFIID	S00007	+	977	2.618000
TFIID	S00615	+	977	2.920000

Promoter region predicted on forward strand in 1213 to 1463
Promoter Score: 56.66 (Promoter Cutoff = 53.000000)
TATA found at 1438, Est.TSS = 1463

Significant Signals:

Name	TFD #	Strand	Location	Weight
SIF	S02021	-	1232	1.161000
NFI	S00342	+	1232	2.450000
Sp1	S00301	+	1339	2.755000
CCF	S01964		1403	2.284000
Sp1	S00781	-	1404	2.772000

Promoter region predicted on forward strand in 2347 to 2597
Promoter Score: 56.20 (Promoter Cutoff = 53.000000)
TATA found at 2581, Est.TSS = 2611

Significant Signals:

Name	TFD #	Strand	Location	Weight
AP-1	S00475	+	2519	6.606000
TFIID	S01540	+	2532	1.971000
TFIID	S00007	+	2534	2.618000
TFIID	S00515	+	2534	2.920000

Promoter 2.0 Promoter Scan Output:

Sequence, 5641 nucleotides

Position	Score	Likelihood
2700	1.262	Highly likely prediction
4400	1.095	Highly likely prediction

Figure 2.4 - Search for putative promoters in 5kb Y-specific element with 3 different prediction software tools. The arrows show the position of the putative promoters identified. The DBGP, BIMAS and Promoter 2.0 promoter scan outputs are shown: the highest scores identified simultaneously with the first 2 software were chosen as best candidate promoters, highlighted in yellow, green and pink.

transcripts of *Ceratitis capitata*, including hopefully transcripts corresponding to the Male Determining Factor, by applying a suppressive subtractive hybridization (SSH) technique (Diatchenko *et al*., 1996; Gurskaya *et al*., 1996) on two different RNA samples extracted respectively from mixed XY/XX and XX-only embryos populations. This technique has been used with success to isolate differentially expressed genes for example in different social insect castes (Donnell and Strand, 2006), in specific insect tissues (Wolfner *et al*., 1997), as well as, in other eukaryotic systems (Beilinson *et al*., 2005; Bree *et al*., 2005). The advantage of this method is that it allows also the isolation of ESTs expressed a very low levels in a specific tissue.

The first step of the experimental procedure was to obtain two different populations of embryos of *Ceratitis capitata* to be used in subtraction experiments: the first one, composed of XY and XX embryos, and hence containing also Y-chromosome derived transcripts, and the second one, made of XX embryos, without them.

In our laboratory a transgenic strain of *Ceratitis capitata* was developed to produce XX only progeny (Saccone *et al*., 2007). In this strain the action of a transgene (inserted by a PiggyBac vector, Pane *et al*., unpub. res.), able to produce dsRNA molecule of *Cctra* gene during ovogenesis, prevents the early production of maternal and zygotic CcTRA protein in female XX embryos (Saccone *et al*., 2007). The transient lack of this early protein cause the lack of the establishment of the *Cctra* autoregulatory positive loop in XX embryos and the development of XX pseudomales. The transgenic individuals are identified through the presence of a fluorescent marker, the dsRed. The progeny of a cross between a transgenic female (bearing one copy of the transgene) and a non transgenic male is then composed of XY normal males (50% transgenic, 50 non transgenic) and XX pseudomales (also 50% transgenic, 50 non transgenic), which are all fertile. Hence by crossing individually these males (either transgenic or not; the transgene acts through the mother, not the father) with non transgenic females, it is possible to identify those cages having an XX male, being the progeny composed then of all XX females. With this peculiar method we crossed non-transgenic XX pseudo-males with XX non transgenic females and we obtained only XX embryos that developed as female only progeny.

For the subtraction procedure are required RNA polyA+ amount of at least 2 μg for each of the two samples. To obtain this quantity of RNA polyA+ we have set up two crosses for medium scale production of *Ceratitis* embryos with XX only karyotype (from which to extract the XX-only RNA, named driver RNA) and embryos with mixed karyotypes XY/XX (from which to extract the mixed XY/XX RNA, named tester RNA). The two crosses were set up in cages of size 60x60x70 cm to contain about 2400 flies each: 800 XX pseudo-male and 1600 XX wild-type females with in the "driver" cage and 800 XY wild- type males and 1600 XX wild-type females with in the "tester" cage. We collected about 1 ml of embryos from each cross. Embryos collection was carried out through two hours long intervals and the collected embryos were left to develop until they reach the stage of 8-10 hours after ovideposition. Then we extracted total RNA with cesium chloride density gradient protocol and we purified by affinity chromatography molecules of RNA polyA+. Figure 2.5 shows an electrophoresis of total RNA samples extracted by ultracentrifugation and respective RNA polyA+ obtained after chromatography.

Utilizing the PCR-Select Subtractive Hybridization Kit (Clontech, Palo Alto, CA) we produced two subtracted libraries. The first one, named **forward subtracted library**, is constituted of XY-XX 8-10h old *Ceratitis capitata* embryos cDNAs population subtracted with XX-only 8-10h old *Ceratitis capitata* embryos cDNAs population. This SSH library should contain early male-specific expressed transcripts and hopefully MDG transcripts. The second library, named **reverse subtracted library** and required as a control for the successive differential screening procedure, was produced by subtracting the XX-only 8-10 old *Ceratitis capitata* embryos cDNAs population with the XY-XX 8-10h old *Ceratitis capitata* embryos cDNAs population.

The validity of a SSH library can be confirmed by providing that subtraction had indeed taken place. We evaluated our forward subtracted library by comparing the abundance of two housekeeping genes, *rpP1* (Gagou *et al*., 1999) and *rpS21* (Verras *et al*., 2004) in the subtracted respect to non-subtracted cDNA by PCR amplifications. *rpP1* transcripts were detected in the non-subtracted cDNA after 18 cycles of amplification and *rpS21* transcripts were detected after 28 cycles (Fig. 2.6). In

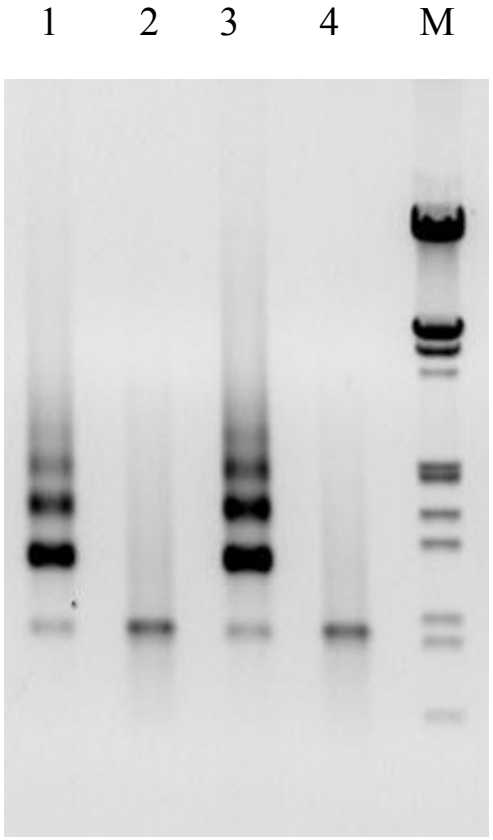


Figure 2.5 – Gel electrophoresis of total RNA (Lanes 1 and 3) and poly (A+) RNA (Lanes 2 and 4), from XX/XY (Lanes 1 and 2) and XX-only embryos (Lanes 3 and 4) 8-10h from OP .

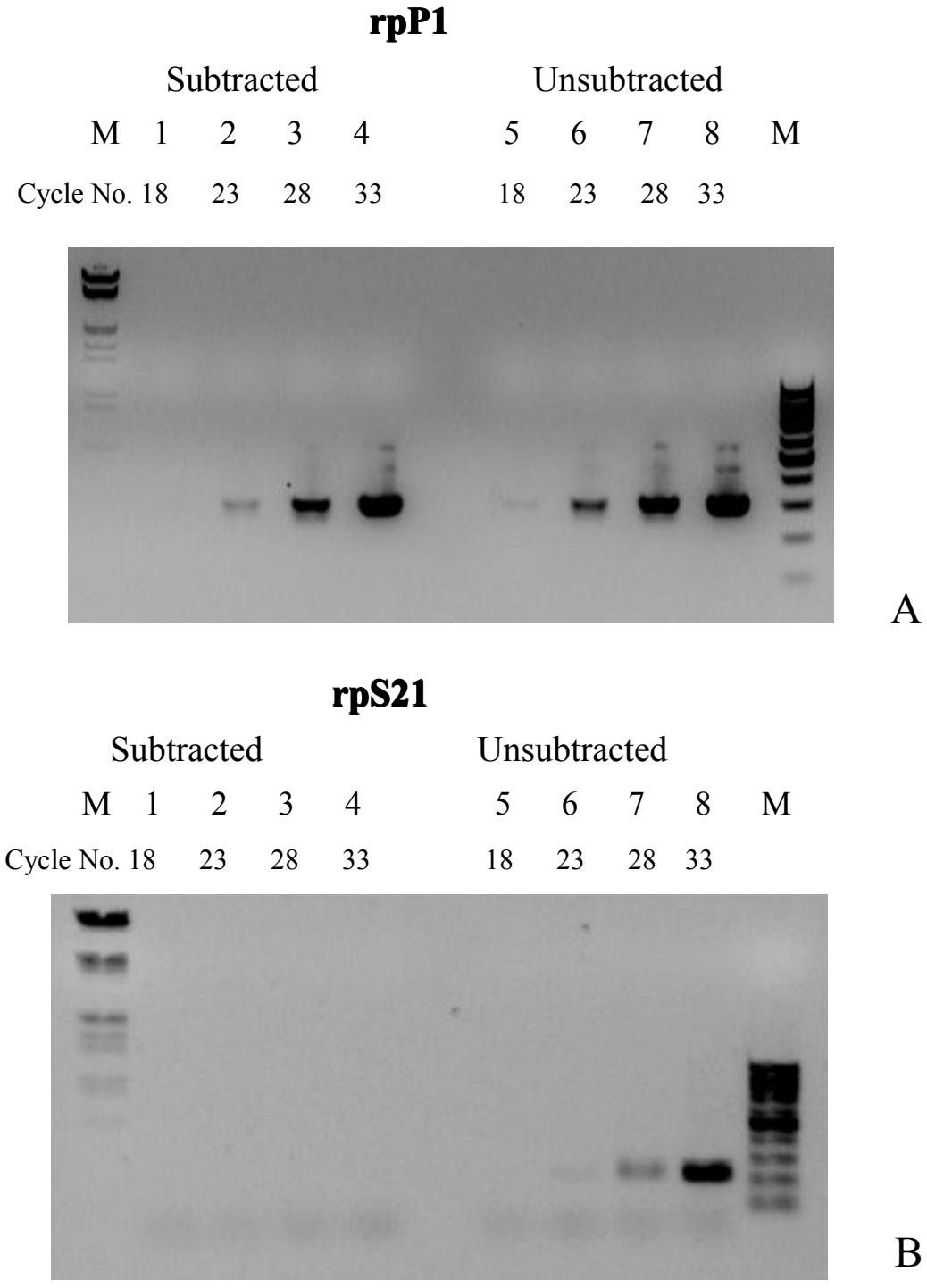


Figure 2.6 – RT-PCR on two constitutively expressed genes to control the efficiency of the subtraction procedure. (A) The primers rpP1+/rpP1- are used for RT-PCR on 8-10h from OP: Lanes1-4 subtracted XX/XY embryos. Lane 5-6 are unsubtracted cDNA of 8-10H old XX/XY embryos.Lanes 1 and 5: 18 cycles; Lanes 2 and 6: 23 cycles; Lanes 3 and 7: 28 cycles; Lanes 4 and 8: 33 cycles.(B) PCR is performed on the subtracted (Lanes 1-4) and unsubtracted (Lanes 5-8) cDNA of 8-10H old XX/XY embryos with primers rpS21+/rpS21-. Lanes 3 and 7: 28 cycles; Lanes 4 and 8: 33cycles.

subtracted cDNA *rpP1* transcripts were detected after 23 cycles of amplification while *rpS21* transcripts were not detected at all. This marked reduction in the abundance of both housekeeping genes in the SSH subtracted cDNA indicates that subtraction had indeed correctly taken place.

Mirror Orientated Selection

One of the major drawbacks of subtraction methods is the isolation of false positive clones. These background clones are generated from non-specific annealing of PCR primers or non-ligated adaptors (type-I background) and from redundant cDNA molecules that evade elimination by hybridization (type-II background). In order to reduce the number of background clones we applied also Mirror Oriented Selection (MOS) procedure (Rebrikov *et al*., 2000). MOS utilizes the principle that background molecules have only one orientation of the present adaptor sequence, whereas truly differentially expressed molecules have many progenitors with adaptor sequences present in both orientations. The result is achieved by removal of one adaptor by restriction digestion, heat denaturation and re-annealing of the resulting molecules. In this case only hybrid molecules with the remaining adaptor at the opposite ends are amplified. MOS procedure hence eliminates background molecules reducing the complexity of the remaining cDNA mixture. We confirmed the validity of MOS procedure using the male-specific *CcLap* transcript as positive control. As evidenced by expression analysis at embryonic stages the *CcLap* 0.3 Kb amplification product is amplifiable only in Y chromosome-containing samples, from early stage of development (30 minutes). For this reason this transcript should be enriched during the molecular subtraction procedure in the XY/XX minus XX sample. In SSH subtracted cDNA this transcript is surprisingly not detectable at all by PCR amplification, most probably due to the high complexity of the subtracted cDNA mixture due to abundant background molecules. In addition SSH method has a better efficiency for genes differentially expressed at low levels then for those having high expression such as *Cclap*. After the MOS procedure instead *CcLap* trascripts are detected after 33 cycles of amplification (Figure 2.7). These finding suggest us that MOS background reduction

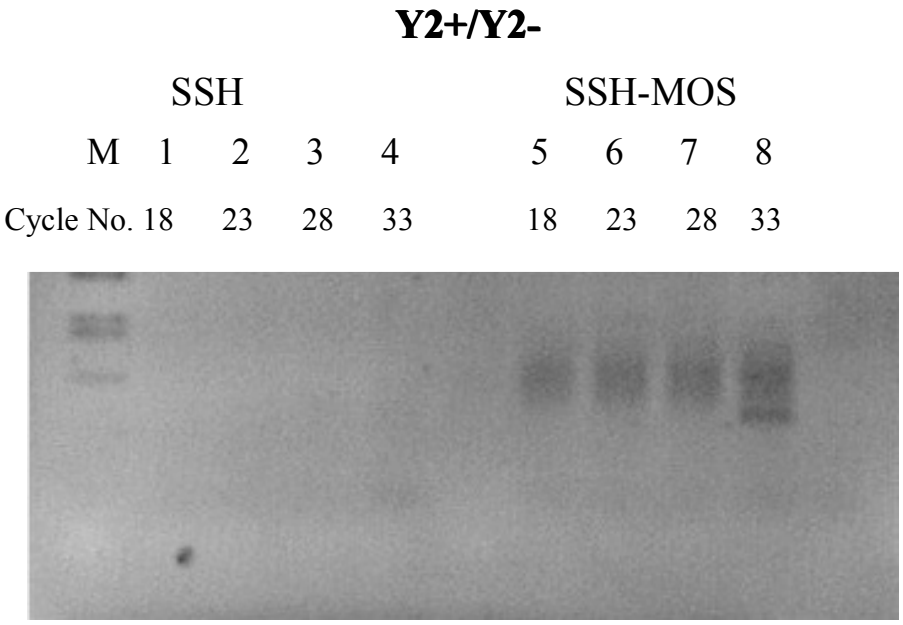


Figure 2.7 – The male-specific *Cclap* mRNA is amplified following MOS procedure. RT-PCR is performed on the SSH (Lanes 1-4) and SSH-MOS cDNA of 8-10h XX/XY embryos with *Cclap*-specific Y2+/2- primers.

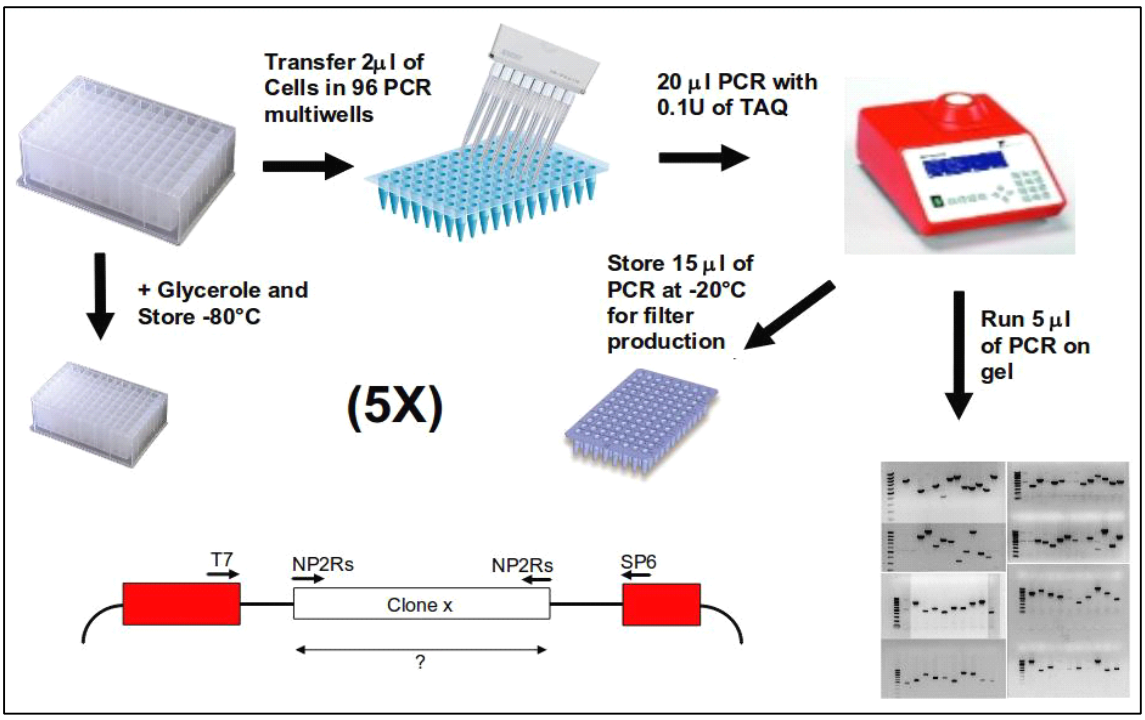
had indeed correctly taken place and that male-specific transcripts are present in the subtracted library.

The **forward subtracted SSH-MOS** cDNAs were directly cloned into a T/A cloning vector (pDrive Vector – Qiagen) and electroporated into ultra competent *E. coli* cells, resulting in the **forward subtracted library**. With homolog procedure we produced also the second library, named **reverse subtracted library**.

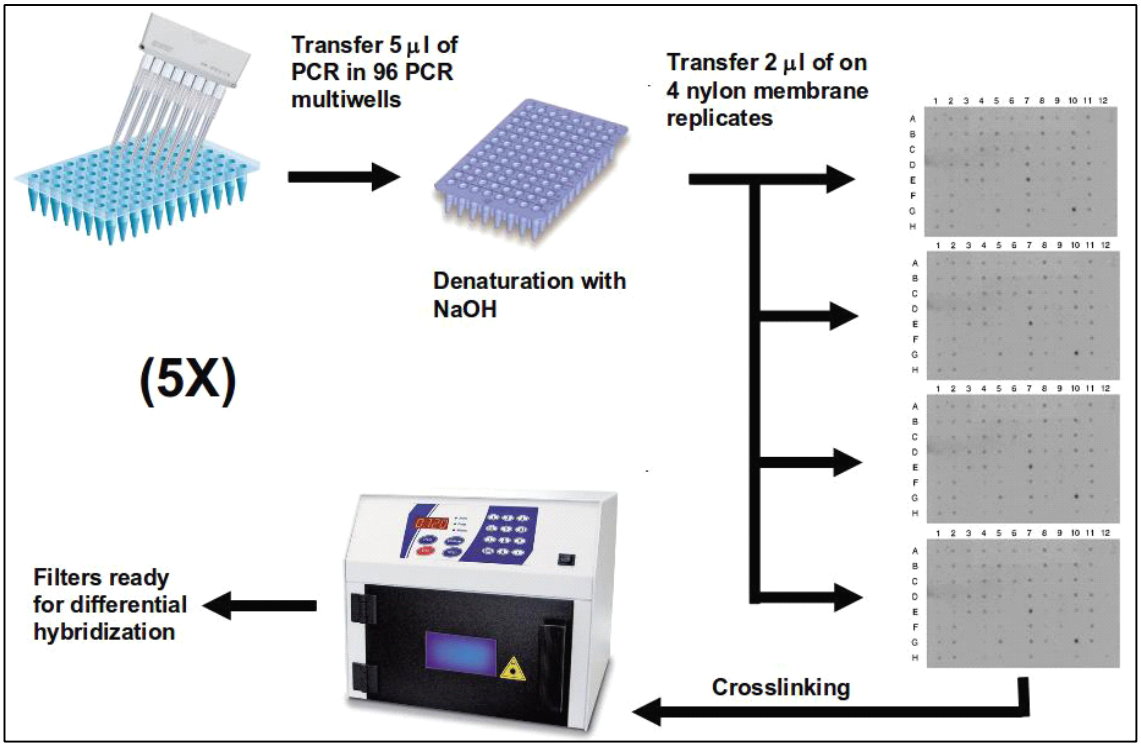
We estimated the forward subtracted library size by plating an aliquot of the transformed cells on 10 LB-agarose plates. We get a medium colony number of 643 for each plate and a relative library size of 2,5 x 10^4 clones.

Differential screening.

Differential screening of the subtracted cDNA library was performed by cDNA dot blots of PCR products obtained with NP2Rs primer amplification of 480 forward subtracted randomly chosen clones (NP2Rs primer is utilized during the MOS procedure and is present at both sides of all the subtracted clones). In figure 2.8A-B is presented a graphical overview of the differential screening procedure. 480 forward subtracted plated colonies were picked out and incubated in five Eppendorf 1.2 ml 96-well plates containing LB medium and ampicillin (named Plate A to E). The bacterial cultures were analyzed individually by PCR amplification of plasmid inserts that were analysed on agarose gel and then blotted on nylon filter replicates (4 for each plate) (Figures 2.9-2.11). The electrophoresis analysis revealed that 38 clones out of 480 were empty cloning vector and 32 clones out of the remaining 442 clones contain two or more cloned products, which were excluded from the analysis. This led to a total number of 410 single cloned subtracted cDNA fragments. It's important to analyze further by differential hybridization only those clones having a single fragment derived from a specific mRNA. Indeed the presence of two independent cDNA fragments, derived from two different mRNAs in the same plasmid, will hide a possible differential hybridization of one of them. Considering that 10-30% of the clones are expected to be truly positives, most probably only one of the two fragments would be differentially expressed in *vivo*. The other cDNA fragment (false positive) would be expressed



A



B

Figure 2.8 – An overview of the differential screening procedure.(A) The PCR is performed with NP2Rs primer on samples from bacteria cultures.(B) The cDNA PCR products are transferred on and crosslinked to nylon filters.

Plate A SSH-MOS XYXX-XX 8-10h

Plate B SSH-MOS XYXX-XX 8-10h

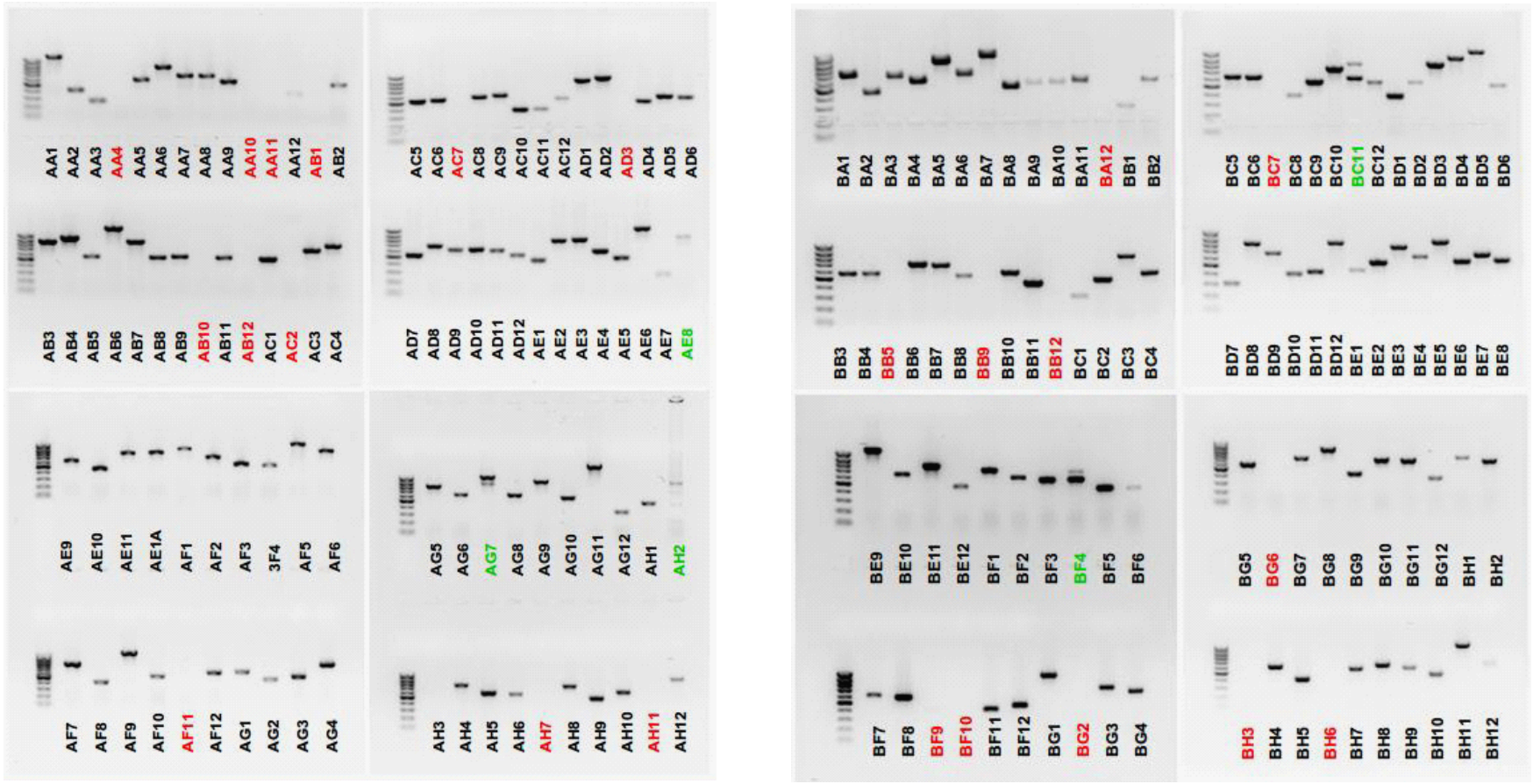


Figure 2.9 – Gel electrophoresis of PCR products obtained with NP2Rs primers amplification of cDNA clones from bacterial sample of plates A and B. The positive amplifications of cDNA products were blotted on filters for hybridization. The negative amplifications indicated in red and those having more than one fragment were excluded from further analysis.

Plate C SSH-MOS XYXX-XX 8-10h

Plate D SSH-MOS XYXX-XX 8-10h

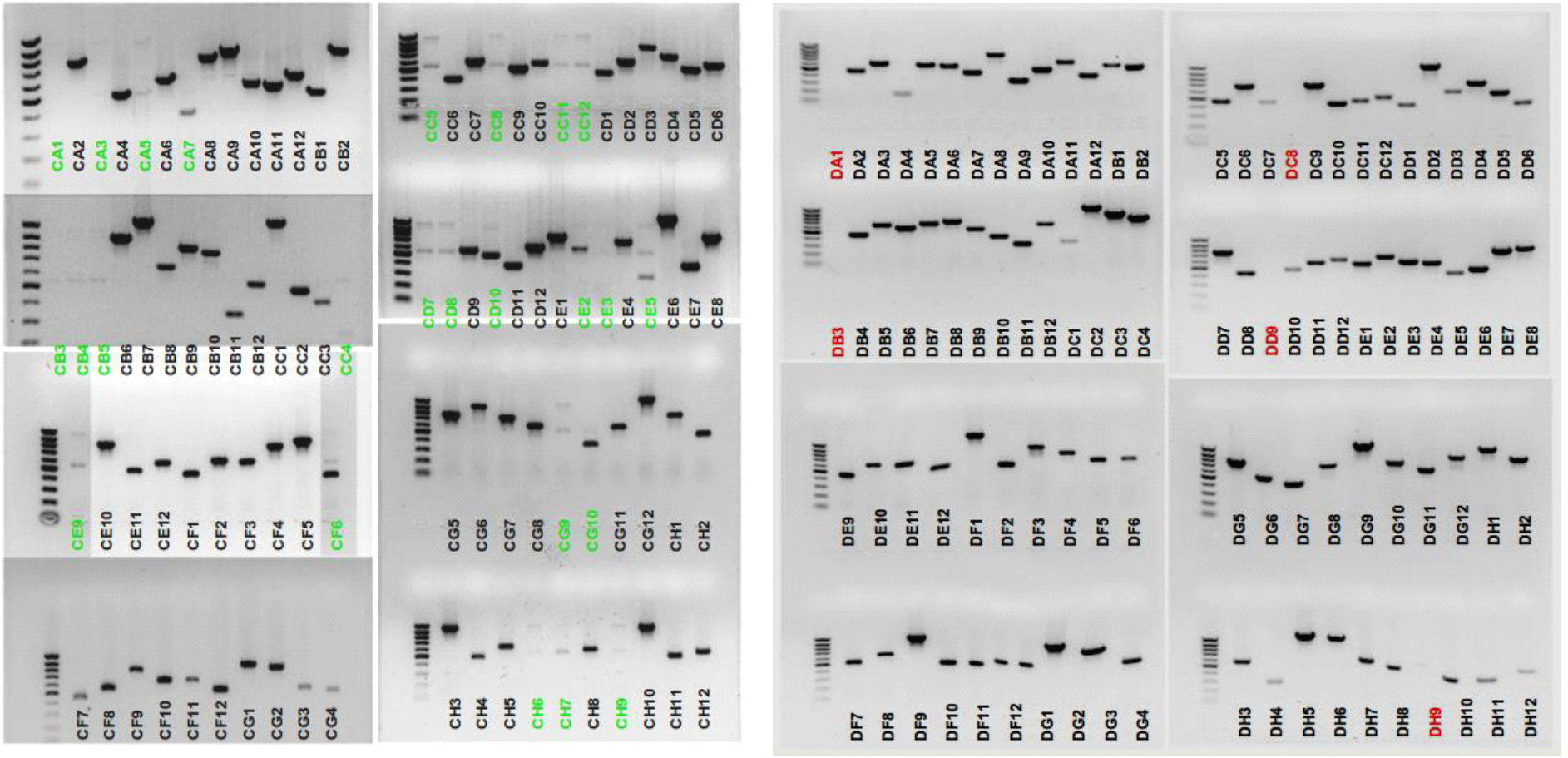


Figure 2.10 – Gel electrophoresis of PCR products obtained with NP2Rs primers amplification of cDNA clones from bacterial sample of plates C and D. The positive amplifications of cDNA products were blotted on filters for hybridization. The negative amplifications indicated in red and those having more than one fragment were excluded from further analysis.

Plate E SSH-MOS XYXX-XX 8-10h

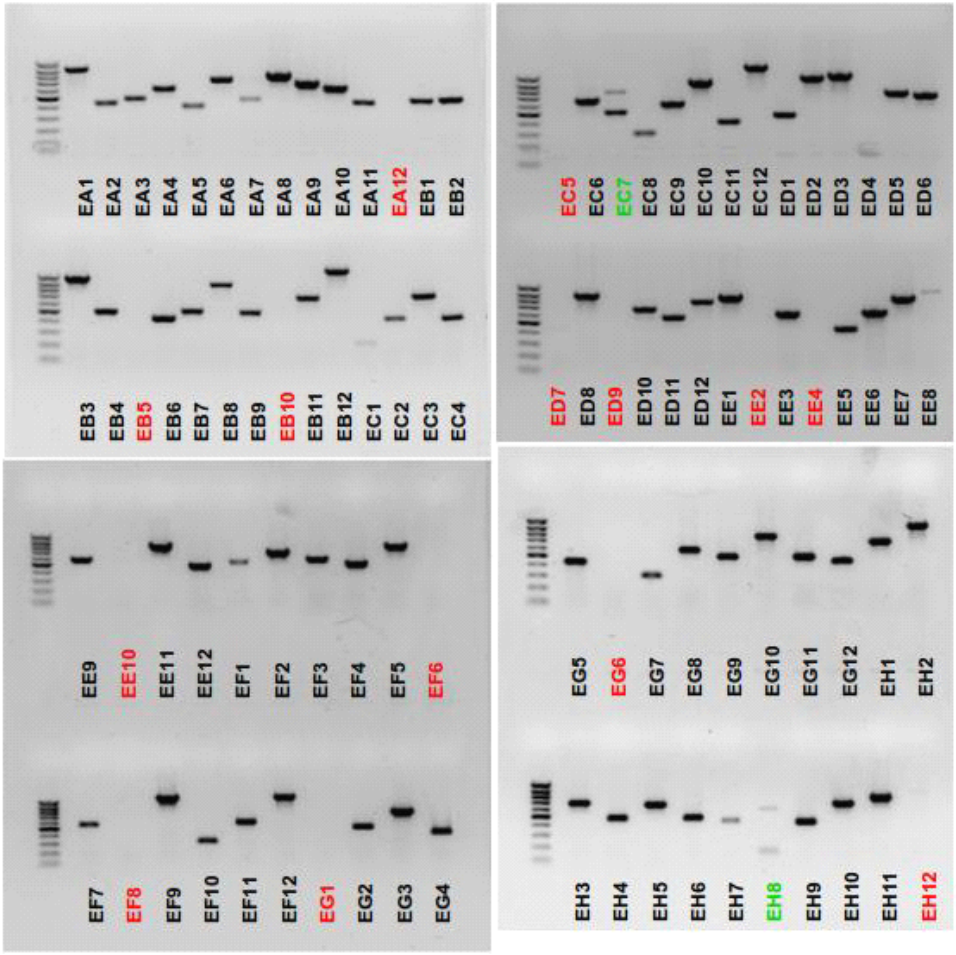


Figure 2.11 – Gel electrophoresis of PCR products obtained with NP2Rs primers amplification of cDNA clones from bacterial sample of plate E. The positive amplifications of cDNA products were blotted on filters for hybridization. The negative amplifications indicated in red and those having more than one fragment were excluded from further analysis.

similarly in both tester and driver and hence would hybridize with both probes on dot spotted filters, hiding the presence of a second positive cDNA fragment.

4 identical filters were produced for each 96-well plate by arraying 2μl of each PCR product on nylon filter. Each filter replicate was hybridized with one of the four following probes: 1) forward-subtracted tester probe (XY/XX 8-10h cDNA minus XX 8-10h cDNA), which identifies differentially expressed clones plus false positives. 2) reverse-subtracted tester probe (XX 8-10h cDNA minus XY/XX 8-10h cDNA, which identifies only false positives which hybridize also with the first probe, 3) unsubtracted-tester probe (XY/XX 8-10h cDNA) which identifies differentially expressed clones plus false positives both highly expressed, 4) unsubtracted-driver probe (XX 8-10h cDNA), which identifies only false positives highly expressed,

Hence the clones that hybridize only with the forward-subtracted tester probe (XX/XY) can correspond to differentially expressed cDNA clones. The clones that hybridize with the forward-subtracted (XX/XY) and unsubtracted tester probes (XX/XY), but not with the reverse-subtracted (XX minus XX/XY) or unsubtracted driver probes (XX), usually correspond to differentially expressed genes, namely real positive clones. Those clones having no detectable hybridization signals with any probe could represent differentially expressed transcripts or false positives having a very low abundance. Finally, those hybridizing equally with both subtracted probes and unsubtracted probes, were the most highly expressed clones, including either real positive clones or false positive. The results of the differential screening are shown in Figures. 2.12-2.14. In our experiment, from 410 clones, 25 of them were identified as hybridizing with the forward-subtracted tester probe (XX/XY minus XX). A further clone was identified as hybridizing also with unsubtracted tester probe (XX/XY). All 26 clones either failed to hybridize or weakly hybridized with the reverse-subtracted (XX minus XX/XY) and driver (XX) probes.

The 26 clones were sequenced with T7 and SP6 plasmid primers and aligned via Macaw software. The sequence and length of AE5 and BE6 clones are respectively identical to those of DB4 and BE8 clones. Hence we have isolated 24 different clones and we analyzed them by computational analysis. A BLASTx analysis revealed that 18

Plate A filter replicates are hybridized with four probes

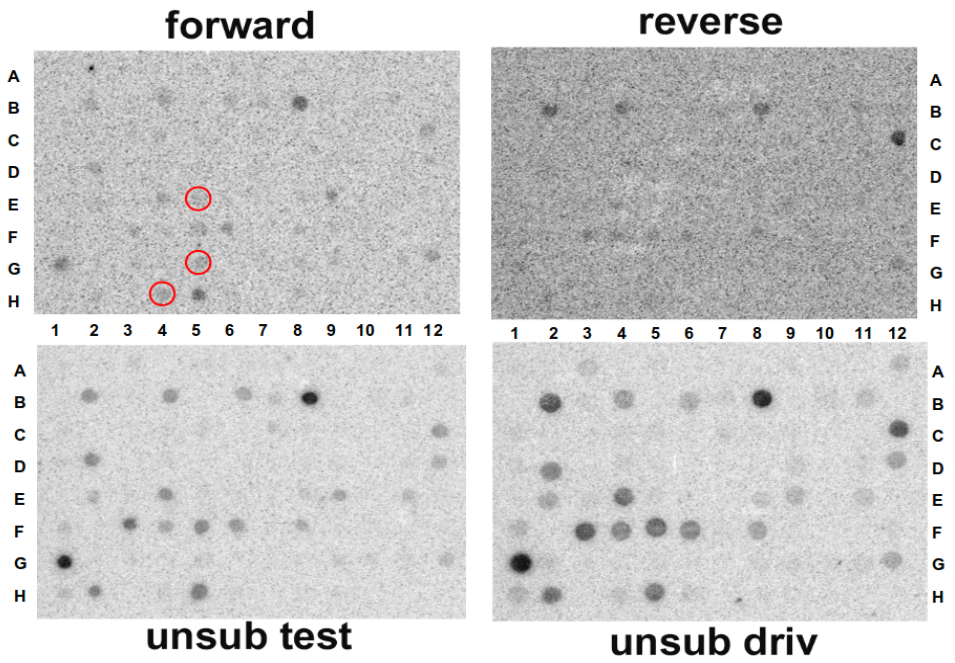


Plate B filter replicates are hybridized with four probes

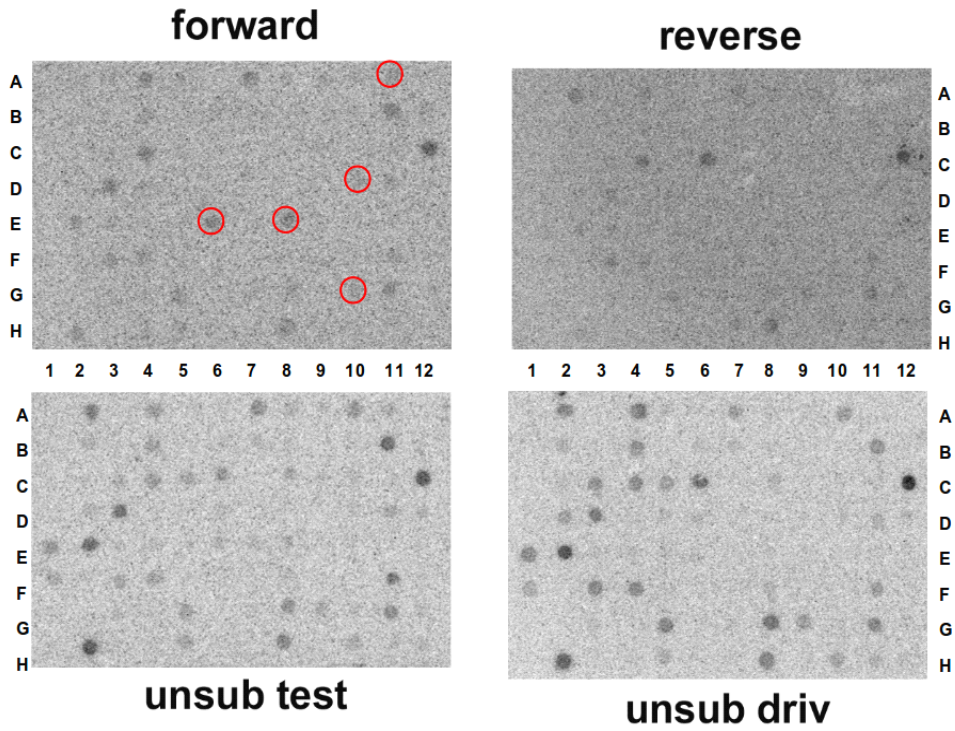


Figure 2.12 – 4 blot replicates of Plate A and B in total 8 filters, individually hybridized with 4 different probes (forward, reverse, unsubtracted tester and unsubtracted drive).

Plate C filter replicates are hybridized with four probes.

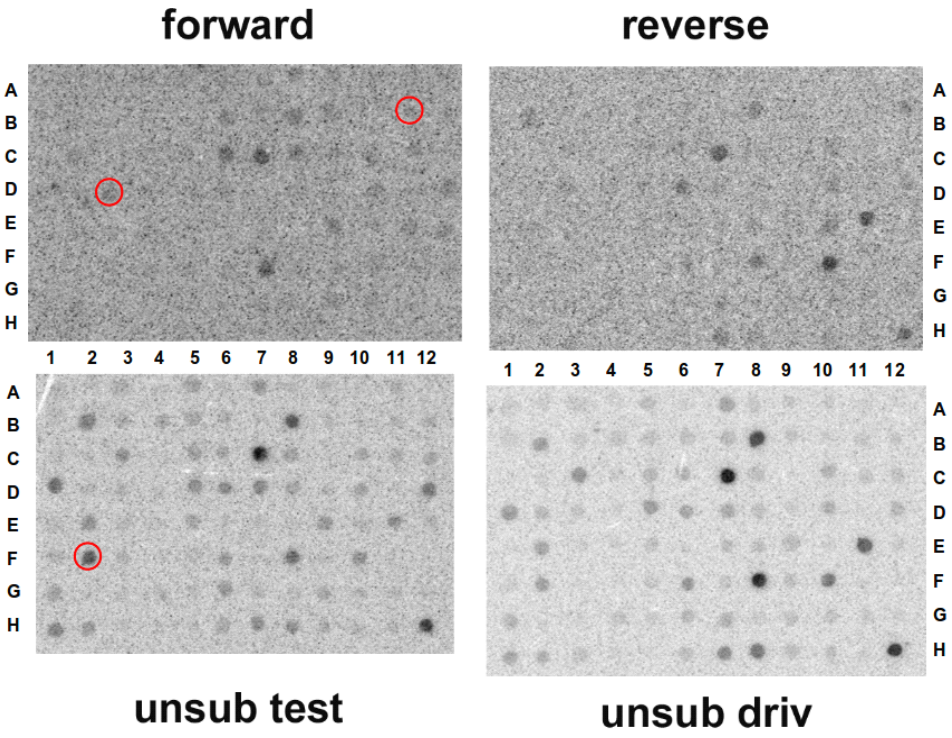


Plate D filter replicates are hybridized with four probes

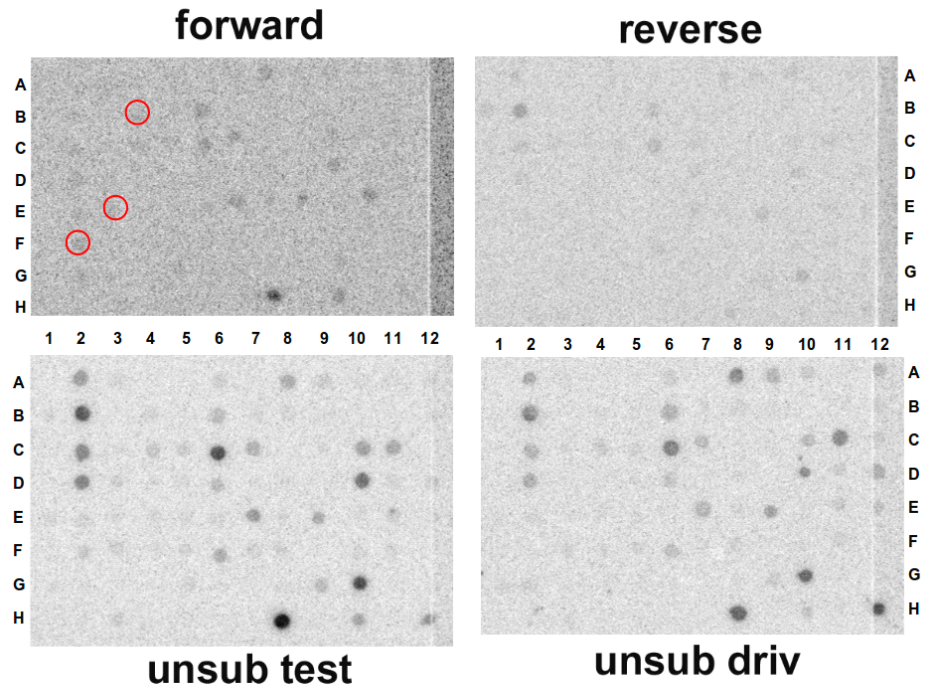


Figure 2.13 – 4 blot replicates of Plate C and D in total 8 filters, individually hybridized with 4 different probes (forward, reverse, unsubtracted tester and unsubtracted drive).

Plate E filter replicates are hybridized with four probes

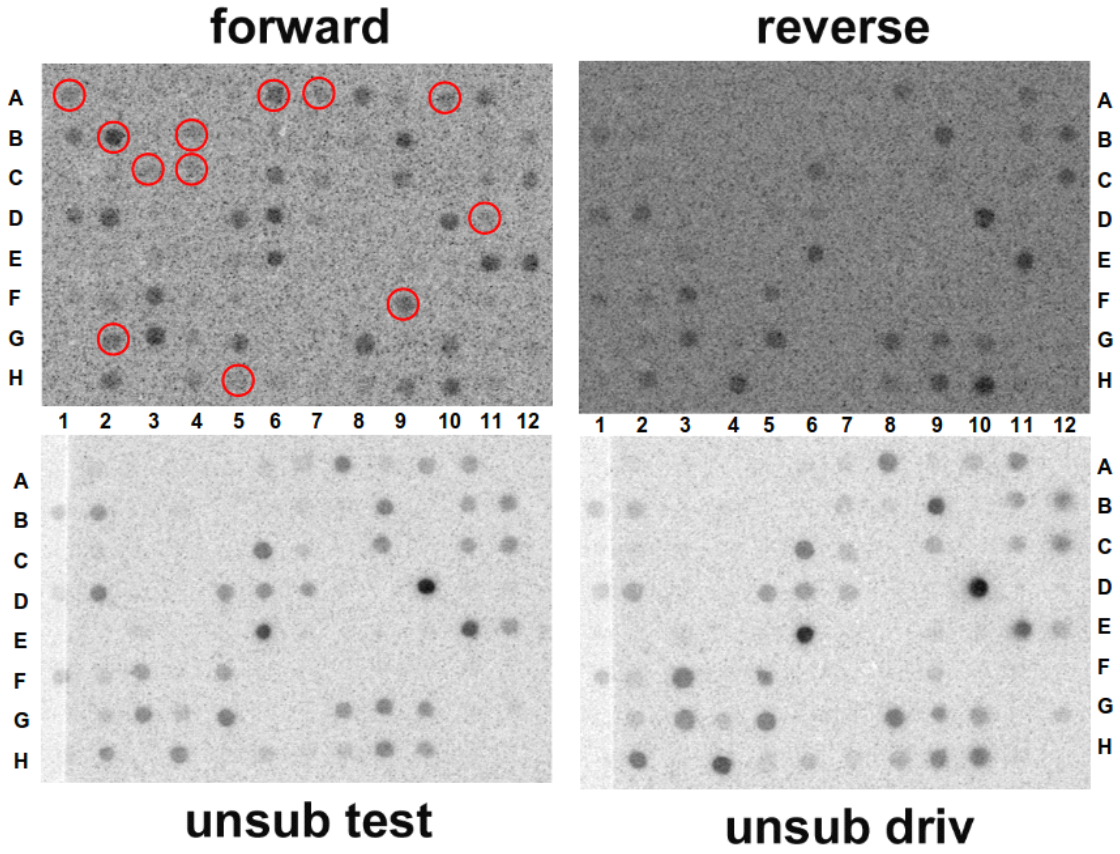


Figure 2.14 – 4 blot replicates of Plate E in total 4 filters, individually hybridized with 4 different probes (forward, reverse, unsubtracted tester and unsubtracted drive).

transcripts encode for putative protein domains with significant homology with known proteins of varying functions. The remaining 6 cDNA clones seem to encode either unknown or low conserved proteins or to correspond to untranslated regions of the transcripts. The results of these analyses are reported in Table 1.

Real Time PCR Validation

To validate the differential screening results and confirm the differential expression of the 24 clones, we use quantitative real time PCR analysis. The Ambion RetroScript Kit was used to prepare cDNA from the same XY/XX RNA polyA+ and XX-only polyA+ samples used for the SSH-MOS procedure. cDNA were diluted 1:5 and 1 μl was used in each reaction for Real-Time PCR using SYBR Green PCR Mastermix (Applied Biosystem). *rpP1* specific primers were used for normalization step. As reported in figure 2.15, 11 out of 26 clones are male-biased, with expression levels ranging from 1,47 to 11,58 fold (Tab. 2).

We performed on these 11 differentially expressed clones, a Blast2Go analysis (Götz *et al*., 2008), which automatically finds similarity between sequences (either nucleotidic or aminoacidic), extracts the Gene Ontology (GO) terms associated to each of the obtained hits and returns an evaluated GO annotation on putative biological function (F), molecular process (P) and cellular component (C) for the query sequence(s). The results are reported in Tab. 3. We have also performed a Blastx analysis in Flybase, a *Drosophila* genome database (data not shown). Unfortunately, the clone BA11, showing the most –male biased expression (11 times more expressed in males), as also DE3 and EC4 clones have apparently no significant homology with DNA or proteins. These 3 clones however could correspond to untranslated regions of the corresponding transcripts.

EG2 clone encodes an aminoacidic sequence which has significant homology with a blastoderm-specific protein of *Drosophila*, whose molecular function is unknown (data not shown). Clone AE5 (2 times more expressed in males) and clone EA1 (4 times more expressed in males) encode aminoacidic sequences having possibly related to functions such as spermatogenesis and in *Drosophila* are both related to Twin of m4

Clone	Size (bp)	Encoded protein fragment length (amino acids)	Best BlastX database match – Gene Name	*E*-value	Best BlastN *C. capitata* ESTs database match*	*E*-value	Predicted protein/domains	Putative function and involvement in biological processes
AE5	427	100	GA18719 [D. pseudoobscura]	2e-15	gb|FG075523.1|	0.0	Twin of m4	Sensory organ precursor cell fate determination; sensory organ development; establishment of planar polarity; Notch signaling pathway; cell fate specification
AG5	813	271	GL22350 [D. persimilis]	1e-64	gb|FG080302.1|	0.0	eIF3-S10, PCI (Proteasome, COP9, Initiation factor 3) domain	Translation initiation
AH4	717	105	AAB17945.1 [C. capitata]	3e-40	gb|FG086741.1|	1e-150	Mariner transposase	Transposable element integration
BA11	541	---	---	---	gb|FG073114.1|	0.0	?	?
BD10	391	98	ADD20011.1 [G. morsitans] – Serpine1	1e-18	gb|FG070497.1|	0.0	Vasa intronic gene, Hyaluronan/mRNA-binding domain	Remodeling of chromatin and regulation of transcription, regulation of mRNA stability
BE6	554	62	GH23158 [D. grimshawi]	1e-04	gb|FG076670.1|	0.0	?	?
BG10	442	101	GI23731 [D. mojavensis]	1e-16	---	---	Distal Antenna	DNA binding, antennal development
CB11	305	66	TA01224p [D. melanogaster]	2e-30	gb|FG075026.1|	6e-96	Ribosomal protein S6	Control of cell growth and proliferation through selective translation of particular classes of mRNA
CD3	776	258	XP_001661254 [Ae. aegypti]	1e-115	gb|FG089286.1|	0.0	Trifunctional protein	Acetyltransferase, thiolase
CF2	573	191	GJ14499 [D. virilis]	83-101	gb|FG086241.1|	0.0	RNA helicase DEAD-box / Rm62	Pre mRNA splicing, nucleocytoplasmic transport, translation, RNA decay
DE3	572	---	---	---	gb|FG078089.1|	0.0	?	?
DF2	407	34	GE16302 [D. yakuba]	3e-7	gb|FG080796.1|	0.0	TROVE domain	RNA binding of the RNA components of three RNPs: telomerase RNA, Y RNA and vault RNA
EA1	881	136	GE22037 [D. yakuba]	8e-44	gb|FG068332.1|	0.0	Ocho, Enhancer of split m4	?
EA6	397	113	GK25636 [D. willistoni]	3e-41	gb|FG089028.1|	0.0	Innexin 2	Gap junction channel activity
EA7	743	148	Phum_PHUM596260 [P. humanus corporis]	5e-10	---	---	Spatzle protein	Activation of the Toll signaling pathway in the embryonic development and innate immune defense of Drosophila
EA10	436	---	---	---	gb|FG072069.1|	4e-104	---	?
EB2	454	73	GK15407 [D. willistoni]	6e-34	gb|FG071243.1|	0.0	Profilin	Actin binding
EB4	485	---	---	---	gb|FG086874.1|	5e-59	?	?
EC3	680	117	RE41765p [D. melanogaster]	5e-47	gb|FG079610.1|	0.0	DUF758 domain	This is a family of eukaryotic proteins with unknown function, which are induced by tumour necrosis factor
EC4	393	---	---	---	gb|FG074936.1|	0.0	?	?
ED11	540	179	ADD18300.1 [G. morsitans] - ATP-dependent RNA helicase	2e-79	---	---	RNA helicase DEAD-box / Rm62	Pre mRNA splicing, nucleocytoplasmic transport, translation, RNA decay
EF9	460	59	GJ24617 [D. virilis]	1e-16	---	---	Hemomucin	Immune response
EG2	441	145	GH13539 [D. grimshawi]	2e-19	gb|FG069128.1|	2e-17	Blastoderm Specific Gene	Transcription factor
EH5	634	212	GI13274 [D. mojavensis]	1e-23	---	---	Ribosome Biogenesis protein	Ribosome Biogenesis

Table 1 - Similarity sequence analyses of 24 clones by BlastN and BlastX algorithms on NCBI diptera databases.

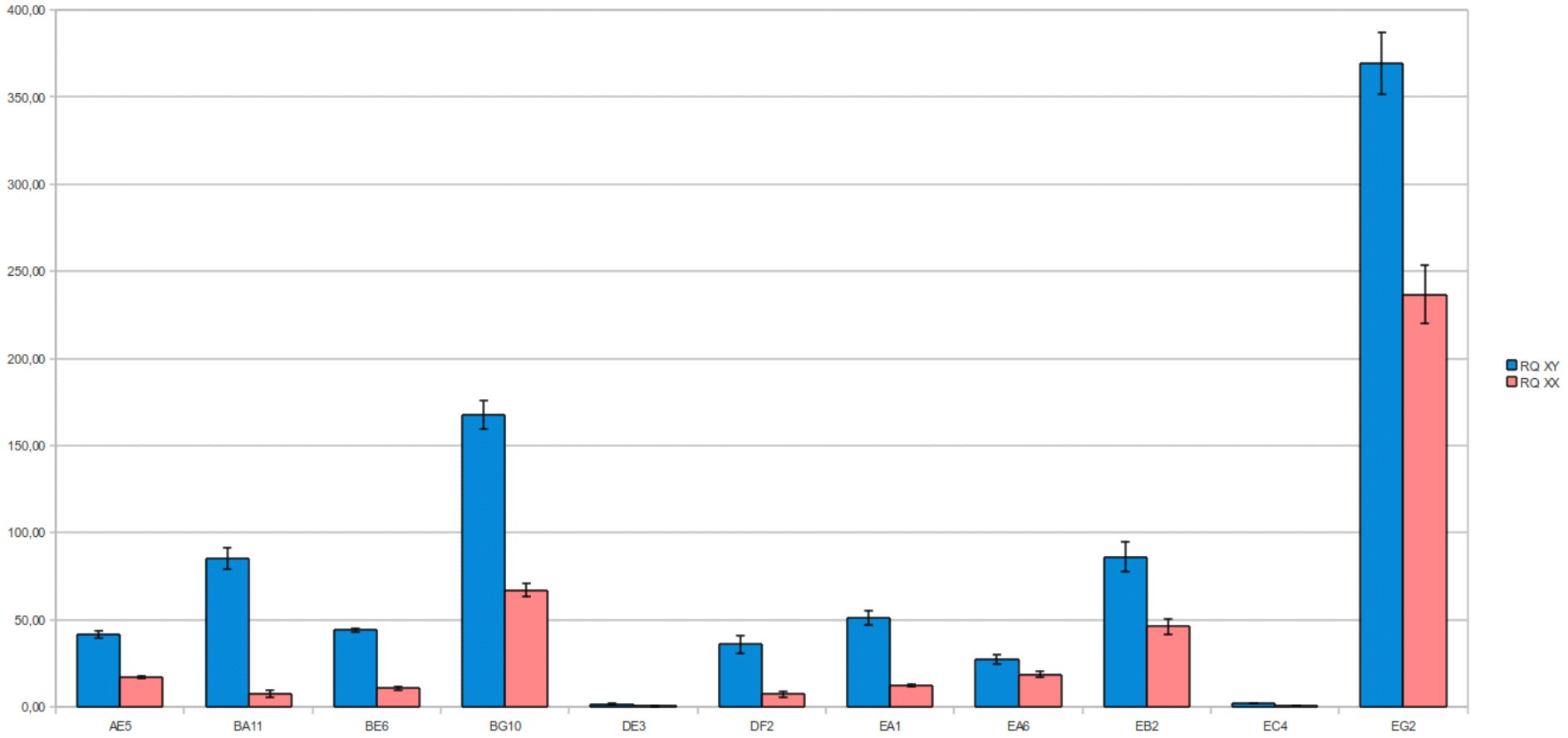


Figure 2.15 - Validation of the male biased expression of 11 cDNA clones by the Real Time PCR.

Name	RQ XY vs XX	SD	T-test p Value
AE5	2,41	0,15	0,00
AG5	0,53	0,16	0,04
AH4	0,15	0,26	0,01
BA11	11,58	3,10	0,00
BD10	0,34	0,13	0,01
BE6	4,06	0,30	0,00
BG10	2,50	0,10	0,00
CB11	0,25	0,02	0,00
CD3	0,23	0,18	0,01
CF2	0,31	0,12	0,00
DE3	3,32	0,77	0,03
DF2	5,18	2,03	0,01
EA1	4,09	0,46	0,00
EA6	1,47	0,27	0,01
EA7	0,66	0,11	0,01
EA10	1,17	0,48	0,77
EB2	1,89	0,33	0,01
EB4	1,20	0,18	0,08
EC3	0,57	0,16	0,02
EC4	3,52	0,41	0,01
ED11	0,53	0,02	0,00
EF9	0,74	0,19	0,08
EG2	1,56	0,09	0,00
EH5	0,20	0,22	0,00

Table 2 – Relative quantization (RQ) in XY embryos versus XX embryos of the 24 SSH-MOS subtracted clones. Clones shaded in light gray are more expressed in male embryos respect to female embryos.

nr	sequence name	seq description	length	#h...	min. eValue	sim mean	#GOs	GO IDs
1	AE5	twin of m4	427	10	2,1E-15	72.0%	19	P:nuclear division; P:central nervous system development; P:embryonic cleavage; F:SUMO binding; P:embryonic development ending in birth or egg hatching; C:microtubule; C:tubulin complex; P:sperm aster formation; P:pronuclear migration; P:Notch signaling pathway; P:mitotic spindle organization; P:female pronucleus assembly; P:peripheral nervous system development; P:sensory organ precursor cell fate determination; F:structural constituent of cytoskeleton; P:cell fate specification; C:cytoplasm; P:establishment of planar polarity; F:GTP binding
2	BA11	---NA---	541	0	-	-	0	
3	BE6	GH23158 [Drosophila grimshawi]	554	1	1,4E-4	59.0%	0	
4	BG10	distal antenna	447	19	1,1E-16	68.68421...	7	P:antennal development; P:regulation of transcription; F:protein binding; F:transcription factor activity; P:segment specification; P:compound eye development; C:nucleus
5	DE3	---NA---	572	0	-	-	0	
6	DF2	---NA---	407	0	-	-	0	
7	EA1	enhancer of split malpha	881	20	8,2E-44	66.65%	2	P:Notch signaling pathway; P:sensory organ development
8	EA6	innexin 2	397	20	3,0E-41	91.95%	6	C:gap junction; P:morphogenesis of embryonic epithelium; C:integral to membrane; P:olfactory behavior; F:gap junction channel activity; P:foregut morphogenesis
9	EB2	profilin	454	20	5,9E-34	92.25%	30	P:positive regulation of cell growth; P:determination of adult lifespan; P:male germ-line stem cell division; P:vitellogenesis; F:phosphatidylinositol-4,5-bisphosphate binding; P:insulin receptor signaling pathway; P:regulation of tube length, open tracheal system; P:positive regulation of multicellular organism growth; C:actin cytoskeleton; P:larval central nervous system remodeling; P:cell morphogenesis; P:maintenance of protein location in cell; P:actin filament polymerization; C:cytoplasm; P:positive regulation of organ growth; F:actin binding; P:dorsal closure; P:cytokinesis, actomyosin contractile ring assembly; P:brain development; P:primary spermatocyte growth; P:germ-line stem-cell niche homeostasis; P:response to starvation; P:growth of a germarium-derived egg chamber; F:insulin-like growth factor receptor binding; P:histoblast morphogenesis; P:ovarian nurse cell to oocyte transport; P:karyosome formation; P:metabolic process; P:pole plasm oskar mRNA localization; F:insulin receptor binding
10	EC4	---NA---	393	0	-	-	0	
11	EG2	blastoderm-specific gene isoform c	441	16	2,5E-19	60.5%	3	

F:molecular_function; P:biological_process; C:cellular_component

Table 3 - Analysis of 11 differentially expressed clones by Blast2Go tool.

and Ocho proteins, whose molecular functions are unknown but either for genetic mutation or for similarity they are known to be involved in biological processes such as sensory organ precursor cell fate determination, sensory organ development, establishment of planar polarity, Notch signaling pathway and cell fate specification.

Clone BG10 encodes an aminoacidic sequence having similarity to transcription factors such as the *Drosophila* DAN protein. The *dan* gene, *distal antenna*, encodes a protein which has a transcription factor activity and protein binding and seems to be involved in the biological processes such as segment specification and compound eye development. EB2 encodes an aminoacidic sequence showing homology to Profilin, an actin binding protein and in *Drosophila* to the *Chickadee*, a Profilin-related protein, encoded by a gene showing various mutant alleles with mutant phenotypes interestingly in the female and male germ lines and in the nervous system.

Conclusions

We have identified and analysed the expression of the first Y-linked gene in *Ceratitis capitata*, *Cclap*, which appears to be transcribed very early during embryogenesis (starting from the very first stages). Putative promoters of *Cclap* have been identified which will be investigated for their use as early drivers of transgene expression during embryogenesis. Further functional RNAi analysis of *Cclap* will clarify its possible function during very early embryogenesis.

We used a *Ceratitis* transgenic line which can produce male only progeny by an *in vivo* maternal RNAi specific for the *Cctra* master gene to obtain XX non transgenic males. These males have been crossed to non transgenic XX females to produce XX-only embryos. These method to produce female only progeny was very useful to approach the problem of identifying male-specific or male-biased genes in *Ceratitis capitata* through a PCR-based molecular subtractive technique.

We have produced a subtracted cDNA library from XX/XY embryos of 8-10h from oviposition, in which we have found by differential hybridization out of 410 dot spotted cDNA clones, 26 putative differentially expressed cDNAs out of which 11 cDNAs were real positive clones, showing a male-biased expression confirmed by real time PCR. The overall efficiency of our SSH-MOS in leading to isolate cDNA clones having putative differential expression is 6,3% (26/410), which is 1/3 of the one obtained by Rebrikov et al., (2000). The observed efficiency of the MOS in reducing the false positive with the respect of the real ones, is about 42% (11/26), a value which is half of the one observed by Rebrikov. We observed indeed that 11 out of 26 clones showed by real time PCR a significant differential expression, Considering the different complexity and the different expected number of differentially expressed genes in the Rebrikov and our studies, we think that the method worked very good in our case and that interesting genes having differential expression in males have been identified.

We apparently failed to identify by the SSH strategy novel Y-linked genes, considering that none of the clones showed an expression exclusively in the XX/XY sample versus XX one, such as for example the *Cclap* gene. If the Y-linked M factor

corresponds to a non polyadenilated mRNA such as microRNAs, then a novel SSH dedicated to this type of RNAs will be necessary to approach this new challenge. However it still possible that one of the 11 isolated clones correspond to a Y-linked gene, such as M, which however could have for example also a copy on an autosomal localization. In this case mRNAs would be present in both sexes but biased in the male one, because of the Y-linked extra copy, which could have evolved a male determining or a male-specific function. These 11 *Ceratitis capitata* genes showing a male-biased expression will be in future investigated also in their *in vivo* functions during embryogenesis by applying transient RNAi on XX-only and on XX/XY mixed embryos. Their functional study will contribute to a better understanding of the genetic and molecular differences underlying the first stages of embryogenesis when male sex determination takes place in *Ceratitis capitata*. This knowledge will be possibly useful not only to understand evolution of sex determination in different dipteran species, but also to develop novel strategies of biological control for this so relevant agricultural pest insect.

Acknowledgment

I wish to sincerely thank all the people that have helped me during this three years of Ph.D. experimental thesis.

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Materials and Methods

Fly Strains

The medfly were reared in standard laboratory conditions at 25°C, 70% relative humidity and 12:12 h light-dark regimen. 800 males mated 1600 females were maintained in every sex cages (60cm×60cm×70cm).After 3-4 days, eggs were collected in water dishes for 8-10H and 23-25H.

RNA isolation

The total RNA was extracted from embryos, using the standard guanidinium isothiocyanate procedure (T. Maniatis, E. F. Fritsch and J. Sambrook, 1982). The 1ml embryos are mixed with guanidinium isothiocyanate solution at the ratio of 1:7 (1 ml embryos : 7 ml solution) and centrifuge at 10,000×g for 10 min at 4°C.The remove the supernatant in a new tube with a sterile pipette. The supernatant fraction is highly enriched for the denatured RNases and must be removed carefully to avoid bringing the floating film into contact with the RNA pellet. The supernatant is mixed with 4 mL 5 M CsCl and centrifuge at 31,000×g for 16 h at 18°C, then discard the supernatant fraction, the RNA pellet is at the bottom of the tube. Dry the pellet at the room temperature and dissolve the pellet in 500-1000 µl DEPC-treated 1 mM EDTA (pH 7.5) solution (5 to 50 µl is recommended). The concentration and purity of the RNA concentration were determined spectrophotometrically by measuring the absorbance at 260 and 280 nm, and the integrity of the RNA was assessed using denaturing agarose gel electrophoresis.

Reverse Transcription PCR

RT-PCR was performed using RNA from embryos with Advantage® RT-for-PCR Kit (Clontech). 1 µl of RNA from each sample was treated with 1 µl of Dnase I (2 U/µl,

Ambion) to remove contaminating DNA following the manufacturer's instruction, and then reverse transcribed using 1 μl of oligo (dT), incubate the sample at 70°C for 2 min. Then the sample is mixed with 5× 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 1 h and 94°C for 5 min. The primer RpP1(ribosomal protein P1) was used as the positive control (RpP1+:5'-TTGCGTTTACGTTGCTCTCG-3';RpP1-:5'-AATCGAAGAGACCGAAACCC-3'). 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. RT-PCR expression analysis was performed with the following primers: Y2+(5'-AAGGACTTGTGATTGGATTG-3'), Y2-(5'-ATGCCGTCGTCCAACATC-3') that located in Y chromosome of *C. capitata*. Cycling conditions were denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min, with a final 10 min extension at 72°C.

Poly A+ RNA isolation

The poly A+ RNA was isolated using the Oligotex mRNA Mini Kit (QIAGEN). The 200 μg total RNA is diluted in 250 μl RNase-free water, 200 μl OBB (binding buffer) and 15 μl Oligotex suspension, mix gently. Incubate the sample for 3 min at 70°C in a water bath, then remove the sample and place it at 20 to 30°C for 10 min. Centrifuge the sample at maximum speed for 2 min and carefully remove the supernatant by pipetting. The mRNA pellet which is at the bottom of tube is diluted in 400 μl OW2 (wash buffer) and remove the sample onto spin column. After centrifuge the sample at maximum speed for 1 min, transfer the spin column to a new microcentrifuge tube, and add 400 μl OW2, centrifuge at maximum speed for 1 min and discard the flow-through. Transfer the spin column to a new microcentrifuge tube, add 100 μl hot (70°C) OEB (elution buffer) and centrifuge for 1 min at maximum speed.

Suppression subtractive hybridization, mirror orientation selection and differential screening of subtracted library

2,2 µg of poly (A) RNA from each sample was used for reverse transcription to perform the cDNA suppression subtractive hybridization using the Clontech PCR-select cDNA Subtraction Kit according to the manufacturer's protocols. cDNA from the XX/XY embryos was used as a tester, which the sample from the XX embryos was used as a driver in forward subtraction (and the cDNA from XX embryos was used as a tester, the sample from the XX/XY embryos was used as a driver in reverse subtraction). Mirror orientation selection was implemented as described by Rebrikov (Rebrikov *et.al*., 2000) with some modifications. For each direction, two tester populations were created separately by ligating suppression adapters 1 and 2R to the blunt-ended *Rsa*I-digested cDNA synthesis products.

(Adaptor1:5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3')

(Adaptor2R:5'-TAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT-3')

The two tester populations were mixed with driver excess (driver cDNA had no adaptors) in two separate tubes, denatured, and allowed to renature. After the hybridization, the two samples were mixed and hybridized together. 1 µl cDNA was taken for 24 µl first PCR with 1.0 µl primer 1(5'-CTAATACGACTCACTATAGGGC-3') by the following temperature program: 2 min at 72°C for initial extension of 3'-ends, followed by 27 cycles with 30 s at 94°C, 30 s at 66°C, and 1.5 min at 72°C. 3 µl of the first PCR products was diluted in 27 µl sterile water and then 1 µl of diluted sample was taken for the second PCR with 1.0 µl nested primer 1 (5'-TCGAGCGGCCGCCCGGGCAGGT-3'; *Xma*I restriction site underlined) and 1.0µl nested primer 2R (5'-AGCGTGGTCGCGGCCGAGGT-3') by the following 12 cycles with 10 s at 94°C, 30 s at 68°C, and 1.5 min at 72°C. PCR products were extracted by phenol/chloroform solution and ethanol precipitated. The pellet was dissolved in NTE

buffer (10 mM NaCl, 10 mM Tris-HCl, 0.1 mM EDTA). To remove nested primer 1 adaptors, 5 µl of the cDNA samples was mixed with 2 µl of 10× *Xma*I restriction buffer, 12 µl H_2O and 1 µl *Xma*I (10U/µl). The reaction was incubated at 37°C for 1 h. The enzyme was then inactivated by adding 2 µl of 200mM EDTA and incubated at 70°C for 10 min. One microliter of *Xma*I-digested cDNA was mixed with 1 µl of 4× hybridization buffer (2 M NaCl, 200 mM HEPES pH 8.3, 0.8 mM EDTA) and 2 µl of H_2O and incubated in a thermal cycle at 98°C for 1.5 min and then at 68°C for 4 h. After hybridization the sample was mixed with 200 µl of dilution buffer (50 mM NaCl, 20 mM HEPES pH 8.3, 0.2 mM EDTA) and heated at 70°C for 5 min. One microliter of diluted cDNA was taken for subsequent PCR in a total volume of 20 µl. The PCR mixture containted 50× Advantage cDNA Polymerase Mix (Clontech), 10× PCR reaction buffer, 10 mM dNTPs and 0.6µM adapter-specific primer NP2Rs (5'-GGTCGCGGCCGAGGT-3'). The primer NP2Rs is shorter than NP2R, was designed to reduce the strong suppression PCR effect that occurs for short DNA fragments. The PCR mixture was incubated in a thermal cycle at 72°C for 2 min and followed the 23 cycles with 7 s at 94°C, 20 s at 62°C and 2 min at 72°C. 1.5 µl of PCR products was cloned into pGEM-T Easy Vector (Promega) and transformed in *E.coli* cells (Promega). After blue/white selection on LB Ampicillin/IPTG/X-Gal plates, 480 white colonies were picked and arrayed in 100µl of LB-Ampicillin medium in a standard 96-well plates overnight at 37°C. 2 µl of each bacterial culture were mixed with Master Mix (10× PCR reacion buffer, primer NP2Rs, dNTP mix, 50× PCR enzyme mix, H_2O) in the total volume of 20 µl. The PCR mixture was incubated in a thermal cycle at 94°C for 30 s and followed the 23 cycles with 10 s at 95°C and 3 min at 68°C. After PCR, electrophorese 5 µl from each reaction on agarose gel to observe how much PCR products were corresponded to the cDNA insert. Then 5 µl of PCR product is combined with 5 µl of 0.6 N NaOH in 96-well plats (NaOH would denature the DNA for hybridization) and transfer 1 µl of each mixture to a nylon membrane, neutralize the blots for 2-4 min in 0.5 M Tris-HCl (pH 7.5) and wash in H_2O, cross-link the DNA to the membrane using a UV linking device. Forward and reverse subtracted hybridization probes were prepared from SSH-MOS secondary PCR products digested with *Xma*I to

remove and degrade adaptors. 3 µl (20-100 ng) of each probe was labeled with [α-^{32}P]dATP. Colony lifts were prehybridized with hybridization solution (50 µl of 20× SSC and 50 µl of blocking solution) for 1 h at 72°C, and then hybridized overnight at 72°C with labeled probes. Membranes were then washed four times with low-stringency solution (2 × SSC,0.5 % SDS), twice with high-stringency solution (0.2 × SSC, 0.5 % SDS), each for 20 min at 68°C, and then exposed to BioMax MR film (Kodak) overnight. Clones representing mRNAs that are truly differentially expressed hybridize only with the forward-subtracted probe. Plasmid DNA from positive clones was isolated using the Wizard® Plus SV Minipreps DNA Purification System (Promega). The DNA of each sample is sequenced with the Big Dye® Terminator v1.1 sequencing Kit (Applied Biosystem) using the primer T7 and SP6 (0.8pmol/µl) and then analyzed by BLAST.

Real Time PCR

The Ambion RetroScript Kit was used to prepare cDNA from 0,5 µg of the same XY/XX RNA polyA+ and XX-only polyA+ samples used for the SSH-MOS procedure. cDNA were diluted 1:5 and 1 µl was used in each reaction for Real-Time PCR using SYBR Green PCR Mastermix (Applied Biosystem) and Applied 7500 Real-Time PCR System. *Ceratitis capitata rpP1* gene was was selected as endogenous control (Acc. Numb.: Y11907.1). Primers of 24 target genes and *rpP1* gene were designed by using Primer Express 3.0 Software (Applied Byosistem) and are reported here:

AE5+: 5'-GCGAGCGTTTTCTGCAACA-3'
AE5-: 5'-GCCTTTCCTAACACGCGAATA-3'
AG5+: 5'-TTGCGCGCTCAGATGGTATA-3'
AG5-: 5'-TTCTAGTCGACGCGCTTCTTC-3'
AH4+: 5'-AACGACGAAACGCAGTTGATT-3'
AH4-: 5'-AGGAAATGGTTCGCGAAATTT-3'
BA11+: 5'-TCGTATGCACTTACGATCTTC-3'

BA11-: 5'-AAACAGCTCAGAACTCTTGAC-3'

BE6+: 5'-GGCACTTCATCGAGACTCTTCA-3'

BE6-: 5'-AAGCAGTGCAGCGCCTAAAG-3'

BG10+: 5'-ATCAGCTACGCAAGCGACAA-3'

BG10-: 5'-CGAGGATTGCTACATTTTTCTAACC-3'

CB11+: 5'-CGTTACCACCGGCAATACG-3'

CB11-: 5'-TCTTTTCCGGCGACTGGAT-3'

CD3+: 5'-TGTGTGTGACCGTAGCGCATA-3'

CD3-: 5'-ACATTGCGCCCAAATTTCTT-3'

CF2+: 5'-ATGAGGCTGATCGTATGTTGGAT-3'

CF2-: 5'-TGTAATTGCCGAGAAAATCTTCTG-3'

DE3+: 5'-AAGACGGTTTTCTCGCTTGCT-3'

DE3-: 5'-GCTCATCATCAGTCTCCTCTGTTC-3'

DF2+: 5'-TGCTGCAGCGTCGTTTCTT-3'

DF2-: 5'-TAATATTGTTGATACACATGCGTGAAG-3'

EA1+: 5'-GCGCCCAACGATCCATAAAG-3'

EA1-: 5'-ACGCATCGTCTGCAAACGA-3'

EA6+: 5'-GGCCAAATCCCGATATCACTT-3'

EA6-: 5'-TTCATTGGTTTATCGTATGGCTGTT-3'

EA7+: 5'-GGCAGACCATCAGCAATGC-3'

EA7-: 5'-TTCGATGCACACGATTTCATC-3'

EA10+: 5'-TACTTCATTCTTCCTCATCATC-3'

EA10-: 5'-TTTTCGGCTGCTACACCAAC-3'

EB2+: 5'-CTGCTATTAACTTCGCCAACTCTTC-3'

EB2-: 5'-CGAAAATGAGCTGGCAAGATT-3'

EB4+: 5'-AGCAACCAAATAAAGAAGCATCCA-3'

EB4-: 5'-TTCGCTCGTAAAATCGGAATG-3'

EC3+: 5'-GACTTTAGGGCGGCATGTGA-3'

EC3-: 5'-TAGTGCCGAGGAACTGAAGGA-3'

EC4+: 5'-AGGTGGTTGTTGCAACTGTTTCT-3'

EC4-: 5'-GCGTCTTCAAGCCATCATCA-3'

ED11+: 5'-TCGCTCTAAGTGGACGGGATA-3'

ED11-: 5'-GTGTCGGTGCCAAAATCAGA-3'

EF9+: 5'-AGCGCCCTCATACCTGACAT-3'

EF9-: 5'-AGATCTGCAGGCGCTGTTTC-3'

EG2+: 5'-CGTCCCCCGACTACTAATGG-3'

EG2-: 5'-TGCTTGTTTTTGTTGAAGCTTATCC-3'

EH5+: 5'-TGCTATACACAAAGCGAGAGCAA-3'

EH5-: 5'-AACACTGTTCCATTTTGTCTCTTATATC-3'

RPP1 qPCR+: 5'-GGCTTTGGAAGGTATCAACGTT-3'

RPP1 qPCR-: 5'-TCTGGTTCTTCCTCCTTCTTCTTCT-3'

All the real-time PCR reactions were performed in triplicates on 1:5, 1:25, 1:125, 1:625 and 1:3125 dilution of starting 1:5 diluted cDNAs. Nontemplate control (NTC) reactions were performed in triplicates for each pair of primers, and two biological repeats were performed. Real-time PCR were carried out in a final volume of 50 µl, including 100 nM of each primer, and 1 µl of a cDNA dilution. PCR reactions were performed in 96-well optical reaction plates (Applied Biosystem). The reactions were heated for 10 min to 95°C followed by 50 cycles of denaturation for 30 sec at 95°C and annealing-extension for 45 sec at 60°C. For each pair of primers, the PCR efficiency was calculated using different template dilutions and the equation $(1+e) = 10^{(-1/slope)}$. Only primer pairs with an efficiency between 0.85 and 1.15 were considered valuable. At the end of the amplification experiment, a melting curve was realized between 55°C to 95°C by steps of 0.5°C, to ensure that the signal corresponded to a single PCR product.

The relative gene expression levels of XY embryos versus XX embryos were represented by relative quantification (RQ) values, which were calculated with the $\Delta\Delta^{Ct}$ method. RQ values of XY-only embryos were calculated as follow: XY RQ = XY/XX RQ -[(XX RQ)/2].

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