HOW TO PRODUCE MALE-ONLY PROGENY IN PEST INSECTS FOR SIT: A BIOTECH APPROACH.

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Ai miei genitori.
Perche senza di voi
nulla di ciò che ho fatto
sarebbe stato possibile.
Perchè credete in me
anche quando io non ci riesco.

A Giovanni Chiarelli.
Perchè non ho potuto dirti grazie.

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RIASSUNTO

Gli Insecta sono il gruppo di animali terrestri con la più grande biodiversità e probabilmente biomassa, pertanto molto interessanti per comprendere i fenomeni biologici ed evolutivi che plasmano la vita sul nostro pianeta.
Numerosi sono gli insetti di rilevanza medica, in modo particolare gli ematofagi, come le zanzare, che sono spesso vettori di patogeni dell'uomo. Tra questi, particolare interesse è stato rivolto allo studio del maggiore vettore della febbre dengue, *Aedes aegypti*, che da ormai decenni viene studiato sia da un punto di vista biologico, per la comprensione di meccanismi biologici di base, sia biotecnologico, per la messa a punto di nuove strategie di controllo. Ma varie sono anche le specie che costano ogni anno diversi milioni di dollari in termini di perdite di raccolto per numerose specie vegetali. Ne è un esempio *Ceratitis capitata*, anche nota come Medfly, mosca della frutta particolarmente diffusa nelle aree del Mediterraneo: il danno alla frutta è dovuto alla deposizione delle uova poiché le femmine posseggono un ovopositore con cui iniettano all'interno del frutto centinaia di embrioni che, una volta sviluppatisi in larve, cresceranno nutrendosi della polpa del frutto. Il danno provocato al frutto successivamente favorisce anche infezioni batteriche e fungine.
Negli ultimi decenni, è cresciuto fortemente l'interesse nel trovare tecniche di lotta alla Medfly, ed in generale agli insetti di interesse agri-economico e bio-sanitario, alternative ai pesticidi, che si sono rivelati potenzialmente dannosi per la salute umana oltre che non sempre efficaci.
Particolarmente interessante ed efficace è la SIT (*Sterile Insect Technique*), proposta già nel 1955 da Knipling ed applicata da decenni contro varie specie di insetti; la tecnica si basa sull'allevamento su vasta scala e rilascio nelle aree infestate di individui della specie che si intende combattere, sterilizzati mediante l'esposizione ai raggi gamma, i quali competendo con gli individui selvatici per l'accoppiamento riducono il potenziale riproduttivo della popolazione infestante. Le potenzialità di tale strategia possono essere estese superando alcuni dei principali limiti fino ad oggi riscontrati (Dyck et al., 2005). Si è infatti osservato che in genere l'immissione di mosche sterili di entrambi i sessi nelle aree di interesse comporta una riduzione della potenziale efficacia del sistema poiché i maschi sterili tendono ad accoppiarsi con le femmine sterili invece che con le selvatiche (che è l'accoppiamento desiderato che provoca la soppressione della riproduzione). Inoltre le femmine, sebbene sterilizzate, eseguono l'ovoposizione (di embrioni portatori di cromosomi con mutazioni letali) e danneggiano quindi la frutta che tende poi a marcire a causa delle successive infezioni. In quest'ottica quindi, il rilascio di femmine sterili non solo non è produttivo, ma addirittura deleterio sia per l'efficacia della SIT sia per il danno alla frutta (McInnis et al., 1994). Al fine quindi di migliorare i risultati applicativi della SIT nella lotta a *Ceratitis capitata*, è stato opportuno ottenere un sistema di separazione dei sessi, o "sexing", che permettesse di selezionare i soli maschi.
La SIT è già stata impiegata con successo nella lotta alla Medfly in diversi paesi, come anche contro varie altre specie di Tephritidae (nel Mediterraneo è stata applicata ad esempio in Israele, in Spagna ed in Libia). Esistono varie bio-fabbriche anche in Europa, in paese quali la Spagna (Valencia), ed il Portogallo (Madeira in cui si effettua una produzione di massa di decine di milioni di maschi a settimana) che vengono impiegati in campo nell'ambito della SIT (Neil et al., 2010).
La tecnica ad oggi più usata nelle bio-fabbriche per ottenere una progenie

esclusivamente maschile di *Ceratitis* si basa su mutazioni genetiche e su riarrangiamenti cromosomici indotti mediante esposizione ai raggi X (Franz , 2005). Il ceppo più usato permette di uccidere selettivamente gli embrioni femminili durante la loro embriogenesi, grazie ad uno shock al caldo, al quale resistono invece i maschi grazie ad una traslocazione autosomica sul cromosoma Y che contiene una versione selvatica di un gene conferente questa resistenza.
E' però da tener presente che tali riarrangiamenti cromosomici determinano una instabilità cromosomica che si verifica in condizioni di allevamento in massa e deve essere tenuta sotto controllo. Per ovviare a tali problemi, l'approccio sviluppato negli ultimi due decenni è stato basato sulla genetica molecolare e l'uso di insetti transgenici, sia per ottenere un sistema alternativo di produzione di soli individui maschi sia per indurre una sterilità per via genetica o per sviluppare altre strategie di soppressione veicolando, tramite il rilascio di insetti transgenici fertili nelle popolazioni selvatiche, dei transgeni che provochino letalità in un solo sesso e determinino uno squilibrio tra i due sessi.
Questo approccio di tipo biotecnologico è possibile grazie all'uso di sistemi di trasformazione genica stabile, mediata da elementi trasponibili, opportunamente modificati e capaci di integrarsi nel genoma ospite. La prima specie non Drosophilidae ad essere stata trasformata geneticamente è la *Ceratitis* ed a questo primo necessario passo biotecnologico ha contributo il laboratorio nel quale ho svolto la mia tesi (Zwiebel et al., 1995).
Alcuni dei più recenti sviluppi biotecnologici volti al miglioramento della SIT per la lotta a *C. capitata*, in modo particolare nell'ambito del "sexing", sono basati sulla possibilità di intervenire sui meccanismi genetici della determinazione del sesso.
Il segnale primario nella determinazione del sesso in *C. capitata* é costituito da un fattore di mascolinizzazione M, definito "male determining factor", non ancora determinato molecolarmente, localizzato sul cromosoma Y (Willhoeft and Franz, 1996; Saccone et al., 2011). Mentre in *Drosophila melanogaster* la determinazione del sesso dipende da un segnale primario cromosomico che é diverso in maschi (XY/AA) ed in femmine (XX/AA) e che si traduce nell'attivazione o nella repressione del gene chiave *Sex-lethal*, in *C. capitata* a monte della cascata genica, dove agirebbe il segnale primario M, non è conservato *CcSxl*, che non presenta infatti un'espressione sesso-specifica (la proteina CcSXL viene prodotta in entrambi i sessi già durante gli stadi embrionali in maniera indipendente dalla presenza del cromosoma Y). Gene chiave nella determinazione del sesso in *Ceratitis* è *transformer* (*Cctra*) (Pane et al., 2002) che è infatti trascritto già a stadi embrionali e persiste per tutto lo sviluppo, e che agisce con un sistema di regolazione basato su splicing alternativo di tipo "exon-skipping".
E' stata dimostrata la presenza nel pre-mRNA di *Cctra* di sequenze altamente conservate corrispondenti agli elementi regolativi (RE) dello splicing, specifici per il legame delle proteine TRA e TRA-2 (t*ra-2* è un gene con funzioni ausiliari di *tra*). Da ciò l'ipotesi di un modello di autoregolazione del gene *transformer* in *Ceratitis*, in base al quale la proteina CcTRA, funzionale solo in femmine, si legherebbe insieme con la proteina CcTRA-2 alle sequenze *CctraRE* generando un trascritto femmina-specifico mentre nei maschi, in assenza di una proteina TRA funzionale, viene generato un trascritto maschio-specifico. CcTRA e CcTRA-2 sono coinvolti anche nella regolazione dello splicing dei geni "a valle" quale ad esempio *Ccdoublesex* (*Ccdsx)*(Saccone et al., 2008), coinvolto nel controllo del dimorfismo sessuale, e

Ccfruitless (*Ccfru*) (Salvemini et al., 2009), che è invece fondamentale nel dirigere il comportamento sessuale maschile.Recentemente diversi studi hanno proposto proprio *Cctra* come potenziale elemento chiave nel miglioramento della SIT. Un approccio particolarmente promettente è basato sull'osservazione che iniezioni di dsRNA con sequenza specifica per regioni esoniche di *Cctra* in embrioni di *C. capitata* portano, mediante il meccanismo regolativo dell'RNAi, ad un silenziamento post-trascrizionale transiente di *Cctra* (Pane et al., 2002). Essendo *Cctra* richiesto per la determinazione del sesso femminile negli embrioni XX, se viene artificialmente spento si produce una progenie composta da maschi XY (in cui il dsRNA non ha effetto alcuno perché *Cctra* è normalmente spento), e da individui XX revertiti in maschi per effetto del silenziamento post-trascrizionale di *Cctra*. Circa il 5% della progenie totale è composta da individui XX che presentano caratteristiche intersessuali. Un simile risultato si ottiene con l'impiego di dsRNA per *Cctra-2* (Salvemini et al., 2009). Interessante è il fatto che i maschi XX sono in realtà fertili quando accoppiati con femmine del ceppo selvatico allevato in laboratorio. L'uso dell'RNAi contro il gene *transformer*, come metodo per produrre progenie maschile, è stato oggetto di brevetto internazionale da parte della Università Federico II di Napoli (Saccone, G. et al., PCT WO 02/070686). E' stato inoltra prodotto un vettore di trasformazione che porta un transgene in grado di produrre dsRNA specifico per il gene *Cctra* sotto controllo del promotore inducibile Dmsp70. In una delle linee transgeniche ottenute, l'unica in cui è stato osservato l'effetto fenotipico atteso, l'RNAi mediato dal transgene agisce per via materna ed è quindi possibile ottenere progenie di soli maschi (XX mascolinizzate ed XY) solo quando si incrociano femmine transgeniche per maschi (De Simone, Ph.D. thesis; Saccone et al., unpub. res.). Ad esempio dall'incrocio di femmine emizigoti (cioè portatrici di un singolo vettore) con maschi non transgenici, la progenie di maschi che si osserva è composta dal 50% di maschi transgenici (sia XX che XY) e 50% di maschi non transgenici (sia XX che XY). Ciò sta a significare che l'effetto materno del dsRNA contro il gene *Cctra* si esercita anche in quelle uova prodotte dalla madre che non portano il transgene. Il fatto che il transgene in questo ceppo produca dsRNA che agisce per via materna ha fatto immaginare che l'espressione del costrutto risenta del cosiddetto effetto di posizione, un fenomeno ben studiato nel noto sistema modello *Drosophila*. Nel caso specifico il transgene verrebbe attivato durante l'oogenesi, grazie all'azione di un enhancer di un gene ad espressione ovarica.
Parte del mio lavoro di dottorato è stato quindi incentrato sullo studio di tale linea transgenica al fine di caratterizzare il sito di inserzione del transgene nel genoma, e di approfondire i meccanismi di azione dell'RNAi per effetto materno.
Tramite inverse-PCR all'estremità 3' dell'elemento trasponibile, è stata sequenziata una regione genomica di 96 bp a partire dalla quale, mediante una ricerca nel database delle sequenze prodotte per il progetto genoma di *C. capitata*, disponibili al sito NCBI, nella banca dati SRA, è stata ottenuta una sequenza di 544 nucleotidi. Un successivo "walking" *in silico*, ottenuto mediante allineamento delle sequenze omologhe via via identificate,ha permesso di ricostruire una regione di 1.7 Kb (1200 Kb a valle e 0,5 Kb a monte rispetto al sito di inserzione) che mostra una significativa similarità con una elemento trasponibile di *Drosophila* (Hoppel-like transposon) (Reiss et al., 2003). Tramite RT-PCR è stato osservato che entrambe le regioni (a valle ed a monte del sito di inserzione) sono trascritte in femmine sia wild-type che transgeniche, sia nella regione superiore del corpo che negli addomi, contenenti gli

ovari.
Per verificare la competitività dei maschi XX della linea transgenica rispetto agli XY, ho condotto insieme con il Dott. G. Saccone (Dip. Biologia) ed il Dr. G. Franz esperimenti di “mating” sia su larga scala, in serre in accordo con le linee guida della FAO-IAEA (FAO/IAEA/USDA Product Quality Control and Shipping Procedures for Sterile Mass-Reared Tephritid Fruit Flies) sia su piccola scala, mantenendo sempre un rapporto di 1:1:1 per maschi XX:maschi XY:femmine. Entrambi i test hanno rivelato che le femmine non mostrano avere una preferenza nell'accoppiamento verso i maschi XY rispetto agli XX, che risultano quindi essere competitivi nell'accoppiamento con i maschi selvatici. Mediante dissezioni degli ovari è stata inoltre osservata la presenza di spermatozoi nelle spermateche e nelle camere di fecondazione delle femmine accoppiatesi con i maschi transgenici, a dimostrazione che questi trasferiscono efficientemente gli spermatozoi negli organi di raccolta degli spermatozoi delle femmine.
Inoltre per verificare una possibile influenza del contesto genetico materno rispetto all'efficienza dell'RNAi per effetto materno, la linea transgenica è stata incrociata con un altro ceppo di laboratorio originario del Sud Africa (SA): maschi transgenici omozigoti sono stati incrociati con femmine SA. Le femmine emizigoti (A-) derivanti dalla F1 sono state successivamente incrociate con maschi SA. Sorprendentemente la F2 è risultata essere composta da circa il 50% di maschi, mentre il restante 50% era costituito da intersessi, maschi malformati e femmine, rispetto ad un atteso 95% di maschi e 5% di intersessi. Tale risultato avalla l'ipotesi che il contesto genomico materno possa influenzare l'efficienza del fenomeno dell'RNAi per effetto materno.
Al fine di comprendere come il fenomeno dell'RNAi nella linea transgenica Cc5.3 agisca sul prodotto del gene endogeno *Cctra* durante l'embriogenesi, ed a che stadio di sviluppo si inizi ad osservare un cambiamento nello splicing di *Cctra* e *Ccdsx* in senso maschile negli embrioni XX, il corrispondente pattern di splicing è stato analizzato mediante RT-PCR su embrioni ed uova non fecondate sia di femmine selvatiche che di femmine transgeniche. Le uova non fecondate e gli embrioni sono stati raccolti a 2-3h, 7-8h, 23-24h e 47-48h dalla ovodeposizione. Negli embrioni derivati da femmine selvatiche la comparsa dell'isoforma maschio-specifica di *Cctra* avviene a 6h dalla ovodeposizione (Salvemini e Saccone , dati non pubblicati; Gabrieli et al., 2012), mentre nei campioni esaminati, fino a 7-8h sono presenti solo i trascritti femmina-specifici; ciò induce a ipotizzare che l'azione delle molecole di dsRNA contro *Cctra* accumulatesi nella cellula uovo durante l'oogenesi possano causare un ritardo nella comparsa dei trascritti maschio-specifici. Una possibile spiegazione di tale fenomeno può essere legata al fatto che, data la loro scarsa abbondanza, i trascritti maschio-specifici neosintetizzati risentano maggiormente dell'azione dell'RNAi fino a quando la riduzione dei livelli relativi di mRNA di *Cctra* di origine materna porterà al collasso il feedback positivo di automantenimento dell'isoforma femmina specifica. Tramite real-time PCR su uova non fecondate ed embrioni a 0-6h dalla ovodeposizione, derivanti da femmine transgeniche e selvatiche, si è inoltre osservato che i livelli di espressione di *Cctra* risultano essere minori nei campioni derivanti da femmine transgeniche rispetto alle femmine selvatiche, e ciò è attribuibile all'azione materna della RNAi.
Il pattern di splicing di *Cctra* e *Ccdsx* è stato inoltre analizzato tramite RT-PCR su maschi selvatici, e maschi XX e XY eterozigoti per il transgene: in circa la metà dei campioni analizzati, sia XX che XY, si è osservato un pattern di splicing di *Cctra* e

Ccdsx alterato, data la coesistenza di trascritti sia maschio-specifici che femmina-specifici. Poichè tutti i maschi analizzati si presentavano fenotipicamente normali, si è ipotizzato che tale alterazione avvenisse dopo un normale sviluppo embrionale e larvale. Per confermare tale ipotesi è stato effettuato il medesimo esperimento su larve di maschi di III instar (maschi selvatici e maschi XX e XY eterozigoti per il transgene, tutti precedentemente cariotipizzati). In tutti i campioni analizzati, fatta eccezione per un maschio transgenico XX ed un maschio transgenico XY) il pattern di splicing di *Cctra* e *Ccdsx* risulta normale.
Una seconda parte del mio lavoro di ricerca è stato volto alla ricerca di nuovi geni potenzialmente implicati nella cascata della determinazione del sesso, che possano in futuro essere impiegati nelle strategie di "sexing".
Le proteine TRA e TRA-2 sono proteine SR, contenenti domini RS. Le proteine SR sono regolate mediante fosforilazione ad opera di differenti chinasi, quali quelle appartenti alla famiglia LAMMER/CLK (Rabinow 2012). In particolare, in *Drosophila melanogaster*, l'isoforma da 69kDa del gene *Darkner of Apricot* (DOA), espressa maggiormente in femmine, fosforila le proteine TRA e TRA-2 (Rabinow et al., Person. Comm.). Nell'ambito di una collaborazione con il Prof. Leonard Rabinow, sono stata ospite del "Centre de neurosciences" della Universitè Paris-Sud. Partendo dalle isoforme da 69kDa, 105kDa, 238kDa e 55kDa, di *D. melanogaster* si è risaliti alla struttura del gene ed alla sequenza del locus DOA.
Le quattro isoforme sono state poi analizzate mediante qPCR in embrioni misti raccolti a 0-48h dalla ovoposizione, larver di III instar maschi e femmine, e singoli adulti maschio e femmina. A differenza di quanto osservato in *Drosophila*, dove l'isoforma da 69kDa risulta essere espressa prevalentemente in femmine, l'isoforma 69kDa-like di *C. capitata* risulta essere espressa più in maschi che in femmine sia in adulto che nelle larve di III instar. Le isoforme 69kDa-like, 105kDa-like e 55kDa-like sono state analizzate mediante qPCR anche in embrioni a 8-10h e 23-25h dalla ovoposizione.
Ancora una volta l'isoforma 69kDa-like è risultata essere maggiormente espressa in maschi. Per valutare una possibile implicazione dell'isoforma 69kDa-like nella determinazione del sesso in *C. capitata*, è stato svolto uno studio di analisi funzionale mediante RNAi: è stato prodotto un dsRNA specifico per l'esone N1 esclusivo dell'isoforma 69kDa-like che è stato iniettato in embrioni allo stadio di preblastoderma. Tra gli adulti ottenuti, un maschio mostrava caratteristiche intersessuali (genitali maschili ma con setole femminili su una zampa e ed una sola antenna) ed una femmina presentava genitali malformati. Dall'analisi molecolare è emerso che la femmina malformata non presentava alterazioni del pattern di splicing di *Cctra* e *Ccdsx*, mentre il maschio con caratteristiche intersessuali presentava un pattern di *Ccdsx* alterato.
Dati i limiti delle tecniche di genetica inversa al momento disponibili quali l'RNAi (che non permette, ad esempio, di studiare la funzione di geni che vengono espressi in fasi tardive dello sviluppo), parte del mio lavoro di dottorato è stata volta alla verifica dell'efficienza in *C. capitata* ed *Ae. aegypti* di una nuova strategia di "gene destruction", basata sull'impiego dei TALENs (Transcriptional Activetor-Like Effector Nuclease). I TALENs nascono dalla fusione di un effettore di un transattivatore trascrizionale (TAL) con la endonucleasi FokI; ciò gli permette di riconoscere sequenze specifiche di DNA a livello delle quali la FokI indurrà un taglio a doppio filamento, la cui riparazione indurrà una mutazione per delezione o per inserzione

(Cermak et al., 2011).
In collaborazione con la company Oxitec (Milton,Oxford-UK), dove ho potuto lavorare per due mesi, è stato disegnato un TALEN specifico per la DsRed2. Il nuovo TALEN è stato prima testato in *Aedes aegypti*: sono stati disegnati due costrutti, ognuno contenente uno dei due domini del TALEN, che sono stati iniettati nel polo posteriore della linea transgenica di *Ae. aegypti* Ox3978D, che possiede come markers sia la DsRed che AmCyan, entrambi visibili a stadio larvale. Da 1894 embrioni iniettati sono stati ottenuti 25 adulti, che sono stati successivamente incrociati singolarmente con adulti selvatici ed è stata ottenuta una singola famiglia di positivi, in cui persisteva la AmCyan ma era assente la DsRed. Mediante PCR e sequenziamento è stata rilevata una delezione di 6 nt.
Con lo stesso approccio sono stati prodotti due vettori iniettati nella linea transgenica di *C. capita* Ox3647 che possiede i markers ZsGreen e DsRed (Dafa'alla Et al., 2006).Sono stati iniettati 1335 embrioni, ed ottenuti 77 adulti che sono stati incrociati singolarmente con adulti selvatici. I 4685 individui ottenuti sono stati esaminati, ma in nessun caso si è osservata la presenza della sola ZsGreen. Sono al momento in corso ulteriori studi per la produzione di costrutti diversi da testare in *Ceratitis*.
Un'altra parte del mio progetto di dottorato è stato volto invece allo studio del gene *fruitless* in *Aedes aegypti*: tale gene, espresso nel tessuto nervoso, è responsabile del corteggiamento, e quindi necessario nei maschi ai fini riproduttivi. Nel laboratorio in cui ho svolto il mio lavoro di dottorato è stato recentemente caratterizzato il gene *Aeafru* e sono state identificate due classi di trascritti: una prima classe codificante per un'isoforma espressa in maniera non sesso-specifica, ed una seconda classe che dà origine a trascritti maschio o femmina-specifici.
Mediante l'analisi del pattern di espressione di *Aeafru* durante gli stati di sviluppo, è emerso che i trascritti sesso-specifici vengono prodotti a partire dallo stadio larvale tardivo (larve di III instar) fino allo stadio adulto, mentre i trascritti non sesso-specifici sono presenti in *A. aegypti* già a stadi embrionali e continuano ad essere espressi durante tutto lo sviluppo fino a stadi adulti. I nostri risultati sono stati oggetto di pubblicazione (Salvemini et al., 2013).
Le conclusioni del mio lavoro sono quindi:

1) La linea 5.3 funziona con effetto materno probabilmente grazie all'azione di elementi regolativi che appartengono ad un trasposone endogeno relato ad Hoppel.
2) Questo sito genomico potrà in futuro essere oggetto di targeting mirato per introdurre un nuovo vettore ottimizzato che induca in modo condizionale l'effetto materno.
3) La strategia di mascolinizzazione delle femmine com metodo di sexing può essere una concreta alternativa alla letalità sesso-specifica; i maschi XX sono in gradi di accoppiarsi.
4) La disponibilita di un nuovo strumento di genetica inversa quale i TALENs, permetterà in futuro uno studio rapido ed efficiente dei geni implicati nei meccanismi riproduttivi, permettendo la messa a punto di nuove strategie di sexing molecolare.
5) La caratterizzarione del gene *fruitless* in *Aedes aegypt*i e la disponibilità di un nuovo strumento di genetica inversa quali i TALENs, permetteranno in futuro di valutare la possibilità di utilizzare il silenziamento del gene *fru* nell'ambito delle strategie di controllo di *A. aegypti*.

Summary

Insects represent the most abundant group of animals on earth, comprising about 800,000 described species, and approximately 10,000 of these species can be actually destructive for human activities. Pest control interventions, alternative to pesticides, are increasingly being implemented within the concept of Integrated Pest Management, involving the biological control to eradicate a pest from the area of interest. This concept has been recently extended also to those hemathophagous insects transmitting human diseases, such as for example mosquitoes.
The Sterile Insect Technique (SIT) is a species-specific and environmentally friendly method for insect biological control, based on release of sterilized insects within reasonable proximity of all native females to decrease the next progeny, due to the reduced mating between their fertile wild counterparts. Release of insects of both sexes is less effective than male-only release in reducing the wild population, while removing females from the production and release procedures, would produce considerable advantages in economic and effectiveness terms. Hence a genetic sexing system to produce male-only progeny, would improve the effectiveness of SIT.
My PhD project has been focused on novel biotechnological approaches to control insect species, such as the agricultural pest *Ceratitis capitata* (Diptera; Tephritidae) and the mosquito disease vector *Aedes aegypti* (Diptera; Culicidae). The first part of my Ph.D. experimental work has been focused on the molecular and functional studies of the *C. capitata* transgenic strain Cc5.3, which is a first prototype of a novel sexing system, based on masculinization of XX individuals. This strain can produce male-only progeny in specific crosses, by a maternal transgene-mediated RNAi against a key gene for female sex determination. The male only progeny obtained by this method could be used, in future, for release programs in SIT, after sterilization.
I asked some questions to which I partially answered during my PhD thesis: 1) are the XX males competitive in mating with wild type females? 2) is the masculinization observed at phenotypic level, also fully penetrant at molecular level? 3) which are the regulatory elements influencing the peculiar maternal effect in this transgenic strain?
Moreover, I have also investigated additional novel genes, such as Drosophila orthologues of *doa* and *fru* genes, potentially involved in sex determination and sexual differentiation of *C. capitata (doa gene, a kinase involved in splicing control)* and *Ae. aegypti (fru* gene, a transcriptional factor involved in courtship behaviour) which can be possible targets for novel transgene-mediated RNAi and hence the development of improved sexing strains. Interestingly, I have found similar to Drosophila, sex-biased isoforms of *Ceratitis* DOA encoding transcripts, which could be involved in controlling sex-specific alternative splicing of target genes. Furthermore I have contributed to clarify the molecular differences underlying the sex-specific splicing of the fru gene in *Aedes* with the respect of other dipteran species, proposing a model for sex determination in this mosquitoes species.
In addition to RNAi, novel reverse genetic tools are available. I've tested the TALENs' action in *Ae. aegypti* and *C. capitata* and more recently the CRISP-CAS9 method in Ceratitis.

1 INTRODUCTION

Insects represent the most abundant group of animals on earth, comprising about 800,000 described species,occupying about two thirds of animal's known species. Approximately 10,000 of these species can be actually destructive. Usually insects fed on plants, as well as crops and weeds, causing deterioration and loss of a large amount of food, estimated to be US $2,000 billion per year (Briceño & William, 2002). Moreover bloodfeeding mosquitoes transmit many of the world's more deadly diseases, as malaria (Kamareddine, 2012), and yellow and dengue fever (Lupi, 2011).
Pest control interventions, alternative to pesticides, are increasingly being implemented within the concept of Integrated Pest Management, that involves the integration of different strategy, including chemical and biological control to eradicate a pest from the area of interest (Rust & Su, 2012).
During the last few years a considerable expansion in the number of tools available to perform molecular and genetic studies on the genome of insects have occurred and this knowledge can be translated into concrete benefits for pest insects control strategies (Franz, & Robinson, 2011). In order to improve SIT programs and to develop new strategies of pest control, a good tool could be a biotechnological approach using transgenic strains based on the control of a key gene for sex determination (Gilles et al., 2013; Saccone et al., 2007; Saccone et al., 2011).

1.1 SIT

The Sterile Insect Technique (SIT) (Kipling, 1955) is the first method involving insect genetics for population control of pest species. It has been used extensively against pest insects, in area-wide control programs of economically and medically important species, as alternative to insecticides, promoted by the International Atomic Energy Agency (IAEA) and the Food and Agricultural Organization of the United Nations (FAO) (Dyck et al., 2005).
SIT is a species-specific and environmentally friendly method for insect biological control, based on release of sterilized (by X or gamma ray) insects within reasonable proximity of all native females to decrease the progeny, due to the reduced mating between their fertile wild counterparts and, because released insects are mobile and would actively seek mates, control of a pest can be effective on an area-wide basis, within an integrated pest management (IPM) program.
SIT has been already used successfully against several pest insects: it allowed the eradication of *Glossina austeni* from Zanzibar, *Cochliomyia hominivorax* from Libya and North and Central America, and *Bactrocera cucurbitae* and *B. tryoni*, respectively from Japan and Australia. Establishment of the Mediterranean fruit fly (*Ceratitis capitata*) in California and Florida, Mexico and Chile has been prevented by SIT (Neil et al., 2010).
SIT is moreover applied in population suppression programmes against several other insect species: there are at least 14 facilities around the world rearing *C. capitata* (including some european countries, such as Spain and Portugal), and another 11 rearing related species of fruit flies. Due to the possibility of preferential mating between sterile released insects, release of insects of both sexes is less effective

than male-only release in getting sterility into wild population (McInnis et al., 1994). Furthermore the sterile females can lay into the fruit non productive embryos causing mechanical damage to the fruit and consequent fungal and viral infections. For those insect species vectors of animal diseases, the blood-feeding females are responsible for transmitting infections. For this reason, if females could be removed from the production and release procedures, considerable advantages would occur in economic and effectiveness terms. Sex-separation can be achieved using Genetic Sexing Strains (GSS), involving the use of a chromosomal translocation linking the wild-type allele of a selectable mutation to the male determining chromosome (Fig.1).
First genetic sexing strain was employed in a small trial in 1972 against the Australian sheep blowfly (*Lucilia cuprina*) using a sex-linked pupal color mutation to separate sexes before emergence (Robinson, 2002).
Despite the benefits introduced with this female-killing (FK) system, mutation and chromosome translocations resulted in a high level of sterility and instability, because of the lost of the mutation and reversion of the translocation.
Genetic sexing strains have been developed for *C. capitata* based on reciprocal translocations between the male determining chromosome and an autosome carrying a selectable marker as *tsl*, (temperature sensitive lethal) mapped to the right arm of autosome 5 (Kerremans & Franz, 1994) and linked to *white pupae* (*wp*) mutation (Fig.1). Using males carrying a Y-linked translocation and heterozygous for the two selectable markers, and females with a normal karyotype and homozygous for the two mutations, females can be eliminated during embryogenesis by incubating eggs in a 34°C water bath, while males are not affected. The introduction of a filter rearing system (FRS) enables to solve problems based on strain stability during large-scale mass rearing removing any recombinant individuals that threaten the integrity of the strain from the colony (Fisher & Caceres, 2000).
GSS have been developed for 20 insect species (Robinson, 2002) but only *C. capitata* and *An. albimanus* GSS can be presently mass-reared to the levels required for area-wide programmes. Unfortunately a genetic strategy which is efficient in one species can be not applicable in other one. For this reason it is necessary for each species to start from different new points and sexing strains can be developed only *de novo* for each new species. In addition the use of radiation as sterilizing mean induces chromosomal damage not only in the germ cells, but also in the somatic ones, causing some deleterious effects that reduce the competitiveness of the released males. Hence a strategy to avoid radiation sterilization is also needed to achieve a more cost-effective and efficient implementation of the SIT in area-wide programs against major insect pests of agriculture and human health (Franz. et al., 2011). Furthermore it is desirable a genetic marker to distinguish released insects from wild insects, which could substitute the use of fluorescent dye powders which are unhealthy for the biofarm workers.
By eliminating radiation at pupal stage, insect competitiveness would be improved and the insects could be released at earlier stages of development, (Catteruccia et al., 2009).

1.2 GENETICALLY MODIFIED INSECTS TO CONTROL PEST INSECTS

The first genetically modified insect was obtained in 1982 using P element

transposition in *D. melanogaster* (Rubin and Spradling, 1982), opening the way to the use of this new technology to pest control. It has been however difficult to achieve similar results in other insect species, until 1995 when medfly, the first non Drosophilidae species was genetically modified (Ashburner, 1995); *Ceratitis* was transformed using the *Minos* transposable elemen, derived from *D. hydei* and the *Ceratitis white* gene marker (Loukeris et al., 1995; Zweibel et al., 1995).
Since then, genetic transformation has been achieved in many pest insect of human and agricultural interest (Diptera, Hymenoptera, Lepidoptera and Coleoptera), due to the discovery of other more effective transposable elements of class II (transposing via DNA intermediate), as *Hermes*, *mariner*, and *piggyBac*. Transformation is achieved by co-injection into early embryos of transposon-carrying bacterial plasmids together with plasmids producing an enzyme (transposase) that integrates the transposon into the chromosomal DNA (Franz & Robinson, 2011). Transposase catalyses vector integration and because of the ad hoc deletion of the transposase gene, vector integration is stable in germ-line chromosome. Genetic transformation could be used to introduce three types of required traits into insect strains to improve their use in the SIT: 1) a female-specific lethality system allowing efficient genetic sexing for male-only release; 2) a system able to cause lethality after transmission to progeny in order to replace the male sterilization by irradiation; 3) a marker systems to enable discrimination of released and naturally occurring insects (Schetelig et al., 2010), which should be dominant and hence visible in wild-type background. Fluorescent proteins, expressed under the control of a regulatory sequence, have been used for this last purpose in the vast majority of transgenesis work on pest insects, as the green fluorescent protein (GFP) isolated from the jellyfish *Aequorea victoria* (Prasher et al., 1992), its more soluble mutant enhanced-GFP (EGFP; Cormack et al., 1996), and the red fluorescent protein DsRed from the sea anemone *Discosoma* (Martz et al., 1999).
In order to avoid irradiation, the use of genetic systems,to induce sterility or death at given life stages have been proposed but all need conditional expression. The best-known of these are combined with the tetracycline-regulated systems (Gossen & Bujard, 1992; Gossen et al., 1994) based on the use of a synthetic tetracycline-controlled transactivator (constitutively produced under the control of a promoter of choice to provide tissue or cell-specificity) able to activate gene expression binding to a minimal promoter sequence of the human cytomegalovirus promoter *IE* (*Pcmv*) fused with *tet* operator sequences of *E. coli* (*tetO*).
In this prokaryotic derived system, presence of tetracycline prevent expression of the target gene under the control of *tetO,* because tetracycline induces tTA conformational change avoiding it to bind to the tetO site. In the absence of the antibiotic, tTA binds to tetO and activates the promoter, which initiates transcription of the target gene (Gossen & Bujard, 2002). Because the addition of tetracycline switches the system off, this system is also called “Tet-Off” system. To improve the stategy, a second inducible system have been developed: the reverse tetracycline-controlled transcriptional activator system (rtTA) or “Tet-On” system, where two DNA constructs are used: a transcription regulatory unit, which is a mutant Tet repressor fused to VP16 to form rtTA and the the same as in the tTA system responsive element *tetO* sequences linked to *Pcmv* driven target gene. In tet-ON system, in absence of tetracycline rtTA doesn't bind to *tetO* sequences and the target gene is not transcribed. However, in the presence of tetracycline, rtTA binds to *tetO* allowing

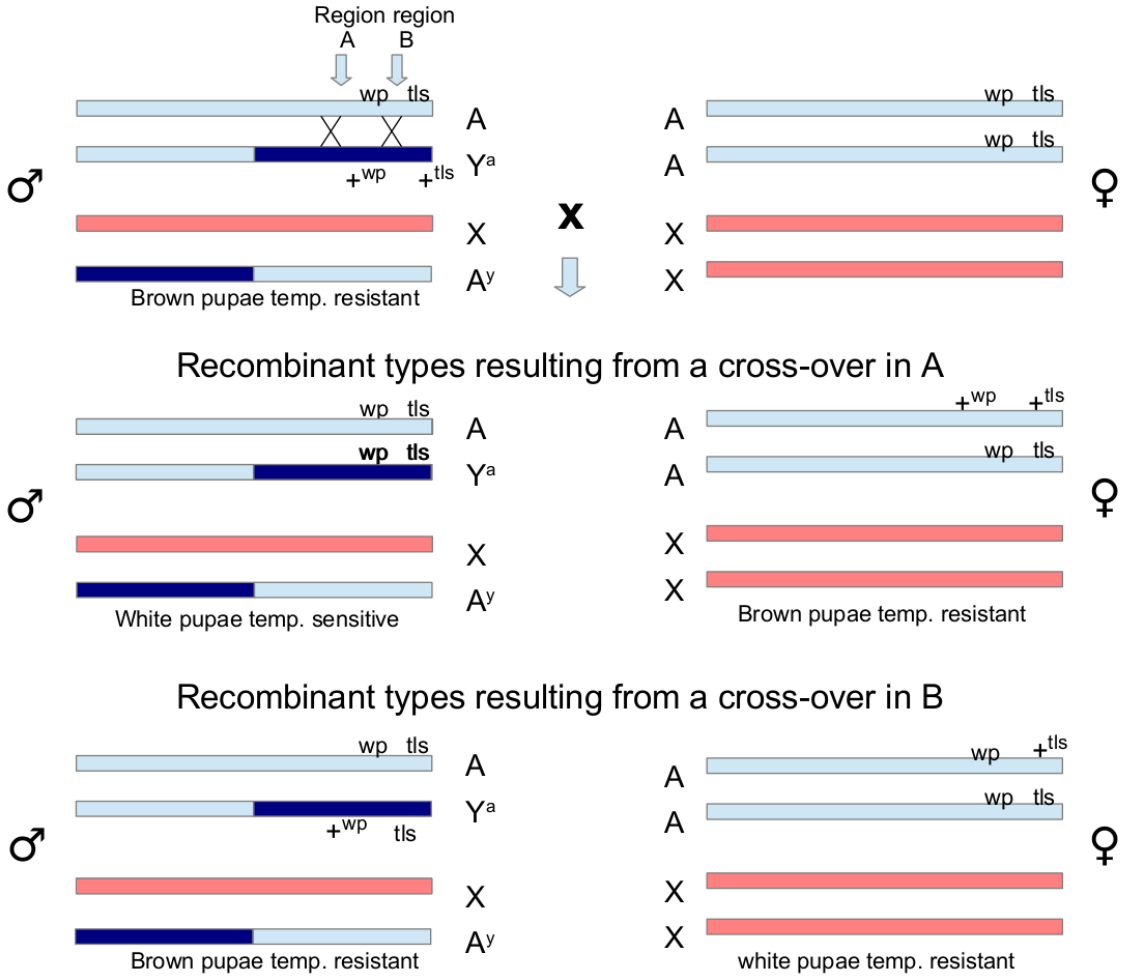


Figure1 - Medfly Genetic Sexing Strain (GSS) configuration and the effect of recombination on the phenotype of the recombinants. Wp: white pupae. Tls: temperature sensitive lethal mutation. Adapted from Caceres 2002.

gene target expression (Gossen et al.,1995) (fig. 2a).
In 2000 it was proposed a new strategy called “release of insects carrying a dominant lethal” (RIDL), and based on repressible female-specific lethality (Heinrich et al., 2000; Thomas et al., 2000).
It requires a strain of the target organism carrying a conditional, dominant, sex-specific lethal gene, introduced by transgenesis, where the permissive conditions can be obtained in the laboratory but can't be found in the wild, as a chemical additive to the diet. Gong et al. later produced a second RIDL strain inducing bi-sex lethality (Gong et al., 2005) based on tet system. As high-level expression of tTA results to be lethal during developmental stages,to semplify the system tTA were used as transactivator as well as effector (fig. 2b). In absence of tetracycine, tTA basal expression allows the synthesis of more tetracycline leading to death of both males and females, while if the antibiotic is added in the larval and adult food, tTA results to be inactivated and expressed at basal level. The strain can so be kept alive by a dietary additive but they, and their progeny, die in the wild so no offspring survive to reproduce. Similar strains are achieved also in pink bollworm (Simmons et al., 2007), in olive fruit fly, *Bactrocera oleae* (Ant et al., 2012), *Aedes aegypti* (Phuc et al., 2007).
In *C. capitata,* using alternative splicing of the sex-determining gene *transformer* (*Cctra)* (Pane et al., 2002; Fu et al., 2007) achieved female-specific lethality: the first intron of *Cctra* was inserted in the DNA sequence coding for tTA. Since the full splicing of this intron in its native gene in strictly confined to females, female-specific transcript was the only one encoding a full tTA and, as a consequence, all of the transgenic female progeny dies as larvae or pupae when reared under restrictive conditions (absence of tetracycline).
First genetically modified insect strain used in nature has been the RIDL OX1138 Pink Bollworm strain (developed by Oxitec, a an spin off company of University of Oxford (UK) in a three year program performed in cotton grown and throughout south-western USA.
Quarantine (PPQ) Center for Plant Health Science and Technology (CPHST) has been evaluated the strain for 3 years in open field trials and in mass-rearing trials in the mass-rearing facility. They showed that sterile OX1138B moths performed well in comparison with the non-transgenic strain currently used in SIT and authors state that these results provide encouragement for this strain use in SIT, and for the wider use of transgenic insects for genetics-based pest control. Oxitec has also produced the *Aedes aegypti* bisexual RIDL strain OX513A, that has regulatory approvals for import and contained testing in Brazil, Cayman Islands, France, India, Malaysia, Singapore, Thailand, USA and Vietnam. Open field trials of release of sexed sterile males have taken place in both Grand Cayman and Malaysia, and are currently also underway in Brazil.
The genomes of several pest insect have been sequenced or are ongoing (Holt et al., 2002; Nene et al., 2007; Arensburger et al., 2010) but many-specific genes remain without any functional annotation. The available reverse genetic manipulation techniques to understand new genes functions are very time consuming and laborious.
Recently, a new developed tool has been proposed, for genome editing in order to efficiently modifying any sequence of interest, which is based on a nonspecific DNA-cleaving nuclease fused to a DNA-binding domain; TALENs (Transcriptional Activetor-Like Effector Nuclease) have been carried out by fusion of the transcription

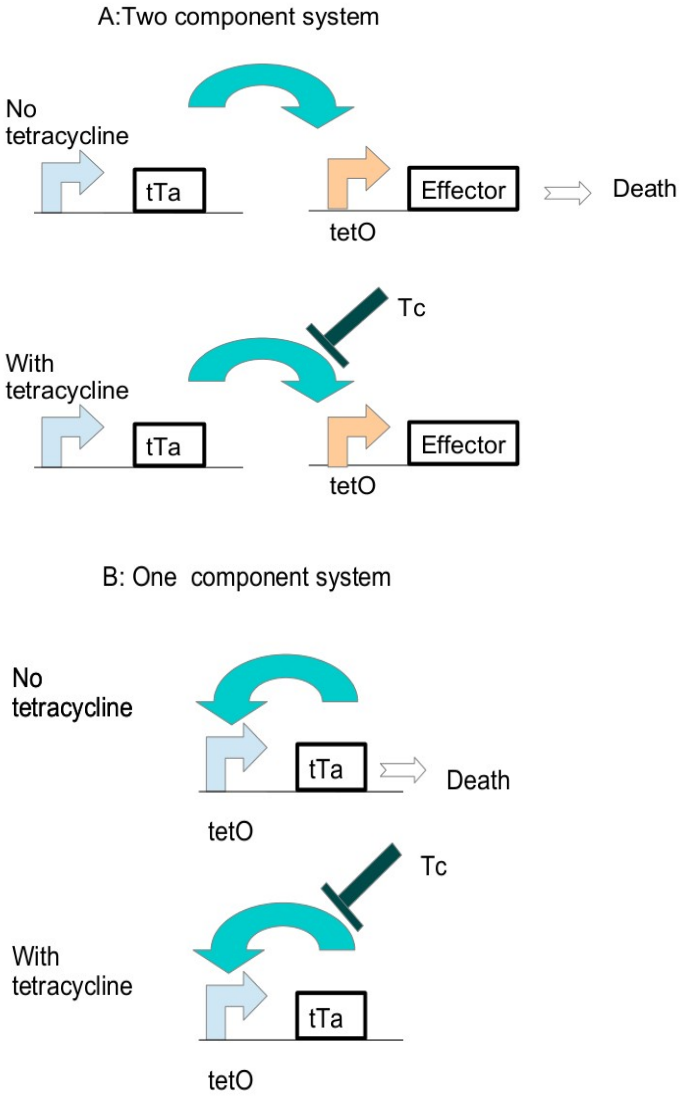


Figure 2 - Tetracycline-repressible system. A: Two component system: tTa (tetracycline-repressible transactivator) placed under the control of a suitable promoter; in absence of Tc (tetracycline) tTa binds tetO (tet operator) driving the expression of the effector gene. B: One component system. Tetracycline works as the transactivator and the effector. Adapted from Gong et al., 2005.

activator-like (TAL) effector of plant pathogenic *Xantomonas ssp*. with the *FokI* nuclease and they are able to bind specific DNA sequences due to the presence of 33-35 specific aminoacid repeats, each of which interacts with a single target nucleotide (Cermak et al., 2011)(Fig.3). FokI induces a double strand break that is naturally repaired in the cell by non-homologous end-joining inducing the introduction of an insertion or deletion mutation in the break's site. TALENs have been successfully used in several organisms, as Drosophila, *Aedes aegypti* and *Anopheles gambiae* and their capability to modify genes are expected to have great impact also on insect biotechnologies.
A second new tool to targeted genome editing is the CRISPR-CAS9, based on a bacterial CRISPR (clustered, regularly interspaced, short palindromic repeat) associated to protein 9 nuclease from *Streptococcus pyogenes* (Cas9). In this system, the Cas9 endonuclease can recognize its target site by complementary base pairing of the CRISPR (Brouns et al., 2008) and induce a double-strand breaks on the target DNA, resulting in small insertion or deletion at the target site, due to nonhomologous end joining repair (Jinek et al., 2012). The method has been successfully used in several organism, as mice, rats and silkworms (Wang et al., 2013; Li et al., 2013; Wang et al., 2013). Moreover, co-injection of Cas9 mRNA and sgRNA (a syntetic guide RNA able to recognize the target sequence and recuit the Cas9) in *Drosophila melanogaster* embryos, can efficiently induce mutagenesis; these mutations can also be trasmitted to the progeny, making possible to produce stable lines (Bassett et al., 2013).

1.3 CERATITIS CAPITATA

The invasive Mediterranean fruit fly (medfly), *Ceratitis capitata* (order:Diptera, family: Tephritidae), is one of the major economical and agricultural pests that has spread from its native range in East Africa and has now attained an almost worldwide distribution, including the Mediterranean, western regions of the Middle East, Central and South America, and the Pacific (Hawaii; western Australia) (Fig.4).
This phytophagous pest affects more than 260 different fruits and vegetables producing a devastating impact on trade and economy of the producing companies and correlated States. Damage to crops caused by *C. capitata* results from oviposition in fruit and soft tissues of vegetative parts of plants, feeding by the larvae and decomposition of plant tissues by invading secondary microorganisms. In addition to physical damage, Medfly inflicts economic damage due to costs associated with quarantine and monitoring programs, limits on export from fly infested areas, and quarantine treatments of fruit from infested areas.
Medfly is able to adapt itself to new environments and to utilising several host plants and, due to its high reproductive capacity and frequency, it possess an high invasive potential.
Adults are 4 to 5 mm long and are characterized by a pronounced sexual dimorphism (Fig.5): males possess brightly colored eyes and a pair of supra-fronto-orbital bristels, while females are bigger than males and have a pointed ovipositor 1 mm long.
Medfly's life cycle consists of four stages: adult, egg, larvae and pupae lasting 21-30

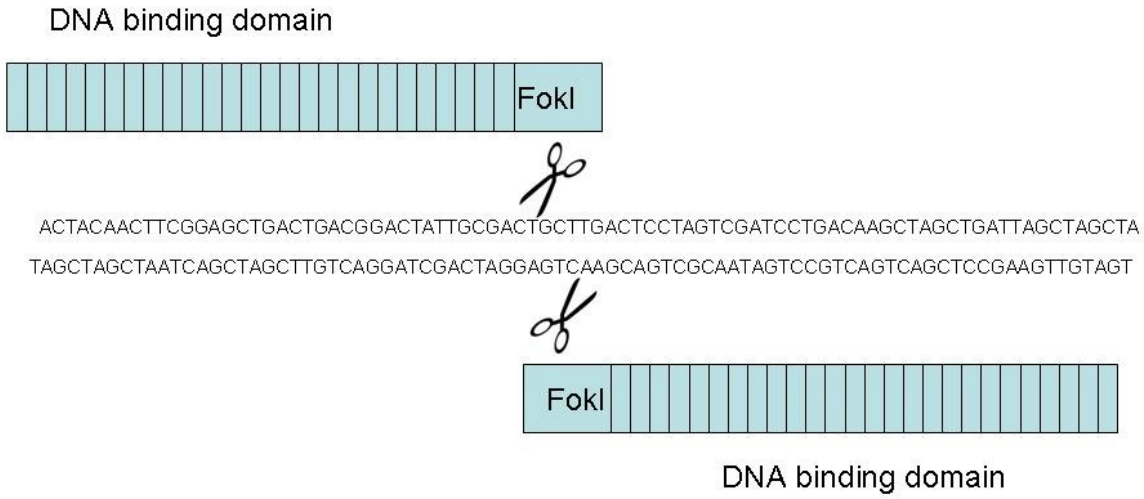


Figure 3 - TALENs structure: two monomeric TALENs bind the target site to enable the catalytic domain of the endonuclease FokI to dimerize and cleave DNA. Adapted from Cermark et al., 2011.

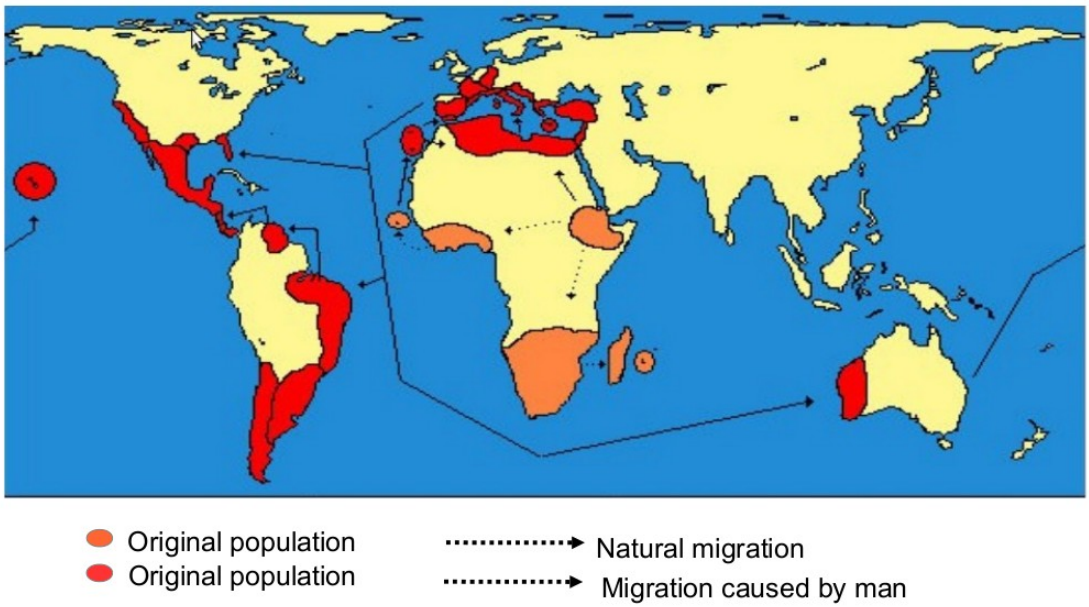


Figure 4 - Medfly worldwide distribution.

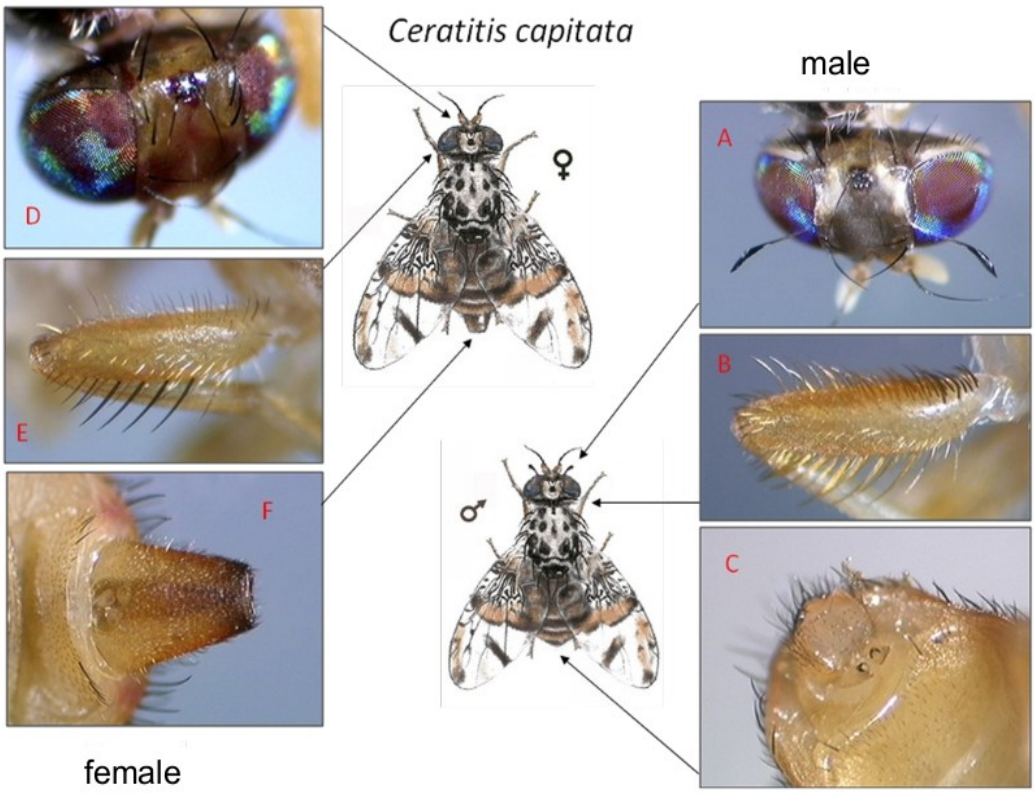


Figure 5 - Sexual dimorphism in *Ceratitis capitata.* A-B-C: male antennae, bristles and genitalia, respectively. D-E-F: absence of antennae in females, dark bristles and female genitalia, respectively.

days under cooler temperature). Females lay eggs (20 usually per day, and 300-800 during lifetime) into the fruits.
Eggs develop in almost 3 days into the first instar larvae, developing 10 more days inside the fruit, to the third and last larval instar, when they jump outside from the fruit into the soil to pupae; after almost 10 days adults emerge from pupae. The newly emerged adults require about 2-3 days to mature before starting to lay eggs.
Male medfly's courtship consists of three stages before he mounts the female: first he raises the tip of this abdomen emitting pheromone, then turns toward a female bending his abdomen ventrally and vibrating wings and finally rocks his head rapidly (Briceño et al., 1996). Head rocking seems to be associated with repeated contact between male and female aristae (Briceño & William, 2002).

1.4 DROSOPHILA SEX DETERMINATION

In highter eucaryotic species, sexual reproduction is an almost universal modality of reproduction and in insects several sex determination mechanisms among the different taxa are used. The fruitfly *Drosophila melanogaster* has been widely studied with the aim to find out and understand the molecular and genetic mechanism involved in sex determination. In *D. melanogaster* the primary sex determinant is the so called X:A ratio (the ratio of the number of X chromosomes to the number of autosomes set) (Fig.6) although it activated only transiently during the early development of females and than is maintained ON by an epigenetic mechanism (Salz, 2011). The X chromosome encodes transcriptional factors able to activate the transcription of the master regulator sex determinant *Sex-lethal (Sxl)* just when their concentration reaches a threshold, as in XX embryos. SXL protein is necessary and sufficient to start and keep an autoregolatory splicing loop, as it possess two RRM-type RNA binding domain and S*xl* pre-mRNA contains sets of SXL binding sites (Horabin & Schedl, 1993); so in males, where no SXL protein is produced, *Sxl* transcript includes the third exon which aborts translation and leads to a truncated no-fuctional SXL protein.
In XX/AA embryos SXL protein is produced, so the third male-specific exon is skipped generating only functional protein able to regulate in the soma all aspect of sex determination and fertility in the germ-line. The start of the autoregolative loop needs a primary source of SXL, produced by activation of an early alternative *Sxl* promoter, *SxlPe* (establishment promoter). *SxlPe* is active during the first 2-4 hours of XX embryonic development responding to the female dose of two X chromosomes to produce a pulse of Sxl protein that acts on the pre-mRNA products from the second promoter, SxlPm (maintenance promoter), which is activated after 4 hours, establishing the splicing-based positive feedback loop.
Sxl initiates the gene regulatory network that leads to sexually and behavioural dimorphisms regulating the choice between two alternative 3' splice sites in the *transformer* (*tra*) pre-mRNA. Only in females TRA functional protein is produced, which is able to control female-specific splicing of *doublesex* (*dsx*) and *fruitless* (*fru*) (Inoue et al., 1990) (Camara et al., 2008). In male (absence of SXL protein), a non-sex-specific splicing is followed by *tra* pre-mRNA, with production of shorter and non-functional TRA protein, leading also to a default male-specific splicing of *dsx* pre-mRNA. The male specific isoform DSX^M represses expression of female-specific

genes and active expression of male specific genes.
In females the complex TRA-TRA2 (*Transformer*-2, *tra* auxiliary gene) and RBP1 recruits different splicing factors, as SR proteins, to promote *dsx* female-specific splicing and DSX^F leads female differentiation (Saccone et al., 2002). RBP1, TRA and TRA2 are all SR or SR-like proteins possessing Arginine-Serine repeats (RS domains).The activity of SR and SR-like proteins is regulated by phosphorylation (Sandorf et al. 2003) by different kinases, including the LAMMER/CLK family (Rabinow 2012). The *Darkener of apricot* (*Doa*) locus encodes, by alternative promoters usage, at least six different isoforms with the same C-terminal kinase catalytic domain differing in N-terminal domain (Kpebe & Rabinow, 2008) of wich DOA69 (69 kDa protein) and DOA55 (55kDa protein) are highly expressed in embryos and adult heads without difference among sexes, and in the body of adult females, no restricted to the germline, while transcripts encoding other DOA isoforms (91 kDa, 105 kDa, 138 kDa, 227 kDa) are more highly expressed in males. DOA 69kDa kinase is necessary for completely normal splicing of *dsx*-F (Du et al., 1998), as able to activate Tra and Tra-2 by phosphorylation (Rabinow, Pers. Comm.).

1.5 CERATITIS CAPITATA SEX DETERMINATION

Unlike *D*. *melanogaster*, the Ceratitis homologous of *Sxl* does not have a sex determination switch function, being expressed in both sexes (Saccone et al., 1998)
while *Cctra* is not a subordinated target of *Sex-lethal* but plays an essential role initiating an autoregulatory mechanism in XX embryos. In fact, in XX embryos, a maternal *Cctra* mRNA provides fulllength CcTRA protein that initiates a positive feedback regulation and this protein drives a female-specific splicing of zygotically transcribed *Cctra* pre-mRNA so that new CcTRA protein (an RS protein) can be produced; furthemore the new synthesized protein is able to control the maintenance of *Cctra* autoregulation, acting as cellular memory maintaining the female pathway (Pane et al., 2002)(Fig.6).
Although CcTRA protein shows high sequence divergence in respect to DmTRA, it conserves its molecular function but, while *Drosophila* uses a 3' alternative splicing, *Cctra* is regulates by exon skipping (Pane et al., 2002; Saccone et al., 2011): *Cctra* gene is 4kb long and it contains a 1kb long intron wich is removed in female and included in the male-specific transcript where, due to the presence of stop codons, a truncated non-functional TRA protein is produced (Pane et al., 2002). In females, CcTRA and CcTRA-2 (*Transformer-2*) promote the skipping of the male specific introns containing the stop codon on the *Cctra* pre-mRNA. Pane et al., 2002 proposed that the *Ceratitis* dominant male determining M on Y chromosome colud be responsible, directly or undirectly, for the masculinizing activity (the molecular nature of M is yet to be identified) by preventing *Cctra* autoregulation activation. In the presence of M, longer male *Cctra* mRNA, including the first intron of the gene, is produced, while in female, where M is absent, Cctra is able to autoregulate. *Cctra* female-specific maternal transcripts are present in both XX and XY embryo; it suggest that the M factor acts inhibiting the CcTRA protein activity, so that it does not reach the threshold concentration required for female-specific splicing of all the *Cctra* transcripts (Gabrieli et al., 2010).
TRA protein directs the female-specific splicing of *dsx* and *fru* pre-mRNA as well:

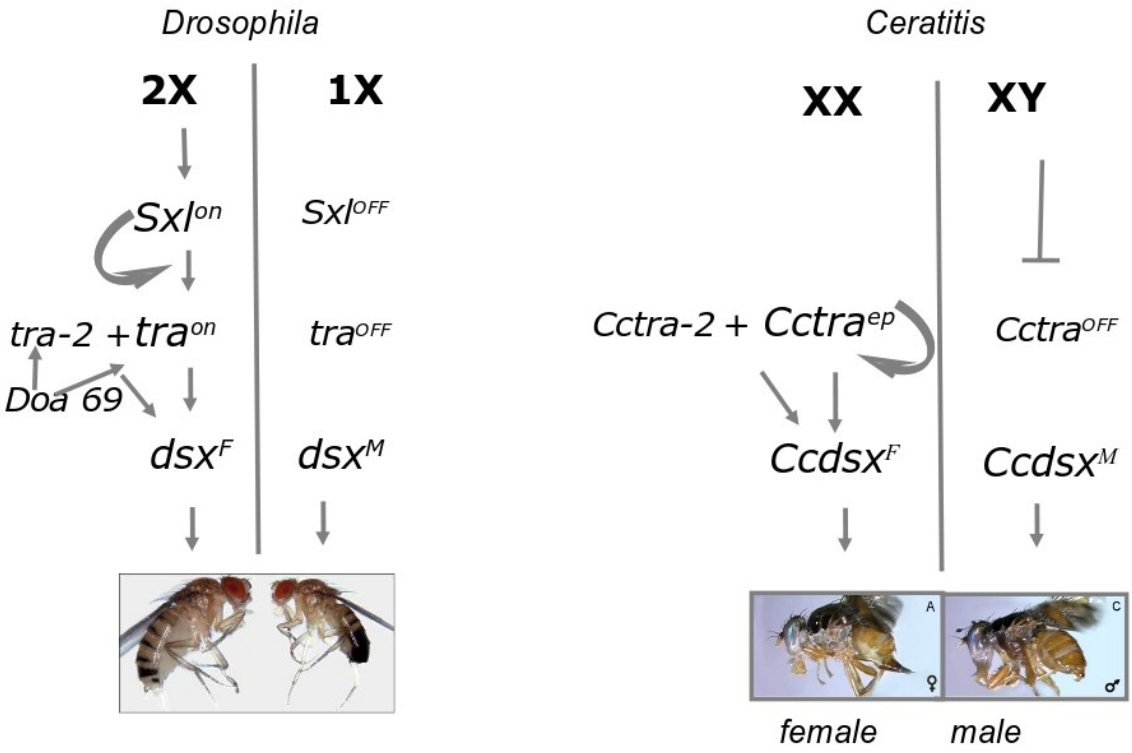


Figure 6 - Sex determination in *D. melanogaster* and *C. capitata.* In *Drosophila* the *Sxl* gene, in response to the transient primary signal constituted by the number of X chromosomes, promotes femaleness through the downstream regulatory cascade and ensures the female-specific maintenance of its activation by a positive autoregulatory feedback loop. In *Ceratitis Sxl* is not sex-specifically regulated, while the *tra* and *dsx* homologues produce, as in Drosophila, sex-specific mRNA by alternative splicing. The peculiarity of *Ceratitis* sex determination is constitute by the *Cctra* autoregulatory mechanism, which seems to be analogous to the *Drosophila Sxl* autoregulatory mechanism. *Cctra* acts as a key master switch and epigenetic memory device for *Ceratitis* female sex determination. In males instead the presence of the Y-linked male determining factor inhibits the activity of *Cctra* gene, causing a male sex determination.

TRA, forming a protein complex with TRA-2, via its argenine-serine domains (RS), is able to recognize and bind the *dsx* pre-mRNA repeat elements (*dsx*RE). CcTRA is also able to regulate sex specific alternative splicing of another downstream target gene, *fruitless* (*fru*), responsible for the male sex behaviour (Salvemini et al., 2009).
Several studies proposed *Cctra* as possible key element in SIT improvement: for example, injection of dsRNA specific for exonic regions of *Cctra* in embryos of Ceratitis produces, though *in vivo* RNAi, transient post-transcriptional silencing of *Cctra* (Pane et al., 2002) resulting in a male-only progeny.
This strategy to produce a male-only progeny has been covered by patent (WO 02/070686 A2) and proposed as a valid alternative to the GSS actually used in the biofactories: a transgenic strain containing an inducible transgene expressing dsRNA specific for *Cctra*, enables to obtain the *Cctra* gene silencing by RNAi to produce a progeny constituted by XY and XX flies, both males, that could be used in the SIT, after sterilization.

1.6 SEX DETERMINATION EVOLUTION IN OTHER INSECTS

Knowledge of sex determination in *D. melanogaster* has been a reference to identify orthologues genes in different insects.
Dsx has been identified in *Megaselia* (Sievert et al., 1997), *Bactrocera* (Sherman & Frommer, 1998), *Musca* (Hediger et al., 2004), *Anastrepha* (Ruiz et al., 2007) and Bombyx (Ohbayashi et al., 2001; Suzuki et al., 2001) and the sex-specific transcripts and the exon-intron structure result to be largely conserved.
The *fruitless* gene of *D. simulans*, *D. yakuba*, *D. pseudoobscura*, *D. virilis* and *D. suzukii* (Billeter et al., 2002) has been characterised and shows a conserved molecular structure.
Since the first *tra* hortologue has been identified in *C. capitata* (Pane et al., 2002), *tra* gene has been characterized also in *Bactrocera oleae* (Lagos et al., 2007), *Anastrepha obliqua* (Ruiz et al., 2007), *Lucilia cuprina* (Concha & Scott, 2009).
Female-specific splicing of *tra* gene involves an autoregulatory loop in all the species where it has been identified, but *Drosophila* (Pane et al., 2002; Salvemini et al., 2009; Concha et al., 2009; Lagos et al., 2007; Gempe et al., 2009; Hediger et l., 2010; Verhulst et al., 2010).
There is an evidence of a particular downstream *dsx* and *fru* genes conservation among different insect species, while the primary signal can be different.
At the moment, there are no evidence of the presence of a tra homologous in mosquitoes(Gailey et al., 2006; Nene et al., 2007;), the most important arthropod vector for the yellow fever and dengue viruses. The *Aedes dsx* orthologue has been already isolated and investigated in structure and regulation (Salvemini et al., 2011) while the *fru* gene has been recently characterized (Salvemini et al., 2013).

2 AIMS OF THE STUDY

My Ph.D. project has been focused on novel biotechnological approaches to control insect species, such as the agricultural pest *Ceratitis capitata* and the disease vector *Aedes aegypti*. During my Ph.D. activity, I have been working in collaboration (established years ago between Dr. Saccone and Dr. Alphey) with Oxitec company which is an Oxford University spin off dedicated to develop and commercialize new control methods for various insect species and with FAO/IAEA Pest insect control laboratory to test transgenic male flies (masculinized XX individuals) in competition experiments with wild type ones (XY normal males). Common scientific interests of the Oxitec, the FAO/IAEA lab and the research group (Insect Molecular Genetics) of the Department of Biology, Univ. Federico II of Naples (Drs. Salvemini/Saccone/Polito), which hosted me for the Ph.D. are the identification of sex determining/sex-specifically regulated genes of insects and their biotech use for production of male only progeny, as well as the development of novel reverse genetics tools for functional analysis and induction of specific desidered phenotypes.
In the laboratory of Insect Molecular Genetics at the Department of Biology – University of Naples "Federico II" the sex determination of the agricultural pest *Ceratitis capitata* is studied and they have been cloned the *Ceratitis* homologs of four main *Drosophila* sex determining genes: *CcSxl* (Saccone et al., 2002), *Ccdsx* (Saccone et al., 2002) *Cctra* (Pane et al., 2002) and *Cctra*-2 (Salvemini et al., 2009).
Interestingly transient RNAi against *Cctra* led to observe a complete masculinization of XX individuals and this novel method or producing male-only progeny was the subject of an international patent deposited by University Federico II of Naples (Pane et al., 2002).
Furthermore PiggyBac trasformation vector has been constructed, containing a transgene (Fig.7), that is able to transcribe, under a *Drosophila* derived heat-shock promoter (hsp70) (Pane et al., unpub. res), an inverted repeat sequence corresponding to part of the *Cctra* sequence.
Transposable element used to integration carry as marker of transformation, a gene coding for the fluorescent protein DsRed (derived from Discosoma), under the control of a constitutive eukaryotic promoter of the poliubiquitin. One out of 11 transgenic lines, (Cc5.3) that were obtained, can produce male-only progeny (Saccone, Pane and Polito; unpub. Res.). It showed a transgene-mediated maternal effect leading the XX daughters to develop as fertile males. Most probably the transposon insertion landed nearby a region having an enhancer acting also during oogenesis, inducing the production during this process of dsRNA specific for the *Cctra* gene that leads to a transient destruction of the corresponding maternal and/or zygotic *Cctra* mRNAs in both XX and XY embryos. The transient lack of the encoded early CcTRA protein induced by RNAi cause the collapse of the *Cctra* autoregulatoty positive loop in XX embryos, as mediated by the M factor in XY wild type embryos. As a result, the masculinization of XX individuals and an almost exclusive male-only progeny (1-3% of XX intersexes) is observed. Just when transgenic females are crossed with transgenic males or even not transgenic males, it's possible to produce a progeny of almost only males (both XX and XY), with a variable percentage of intersexual flies, (De Simone, PhD thesis) (Fig.8), while if transgenic males are crossed with non transgenic females, bisexual progeny is observed showing the typical 1:1 sex ratio. Moreover, by crossing hemizygous females (carrying one copy of the transgene) with

non-transgenic males, the obtained progeny is composed by 50% transgenic males and 50% non-transgenic males (in both cases, of XX and XY karyotype), as evidence that maternal effect of the dsRNA against *Cctra* acts also in the eggs which do not carry the transgene.

The first part of my Ph.D. experimental work has been focused on the study of the transgenic strain Cc5.3. The main goal about this transgenic strain, has been the identification and characterization of the sequences of the genomic regions flanking the transgene insertion point and to investigate the presence of a possible ovarian-specific enhancer that could be responsible of the maternal effect. Moreover, in order to test the ability of the XX males to compete with wild-type males for mating, competition test has been done. Another aim of my work has been to investigate how the maternal RNAi effect against *Cctra* works in detail during the embryos devolopment, as well in the adult males, analysing the t*ra* and d*sx* expression pattern.

In order to develop new sexing strategies, I tried to investigate other genes potentially involved in sex determination: due to the availability of *C. capitata* genomic scaffold and a recent defined medfly embryonic transcriptome (Salvemini, Saccone et al., manuscr. In prep.), now possible to investigate several *D.melanogaster* homologues genes. I've tried to identify in *Ceratitis* the well known DOA locus (Darkness of Apricot) and to analyze its sex biased expression levels.

Furthermore, to verify the efficiency of new available genetic transformation tools, I've tested the TALENs' action in *A. aegypti* and *C. capitata*.

The available inverse genetic tools, as RNAi, have several limits: dsRNA acts just during few hours, so it's hard to see any effect on genes acting lately during the development. TALENs enable to carry out gene silencing by stable gene mutation, so the silencing effect can be already observed on the G0 injected adult flies, as well during their development.

At last, in order to find new genes involved in sex determination in the mosquito *Aedes aegypti*, I've performed the developmental expression analysis of the *fruitless* gene in sexed samples.

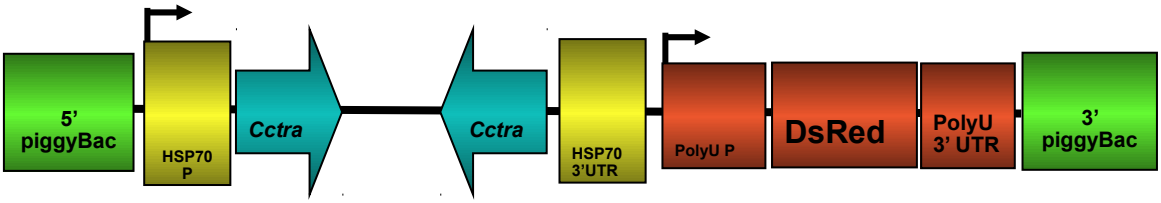


Figure 7 - Schematic representation of the transformation vector used to produce the Cc 5.3 transgenic strain.

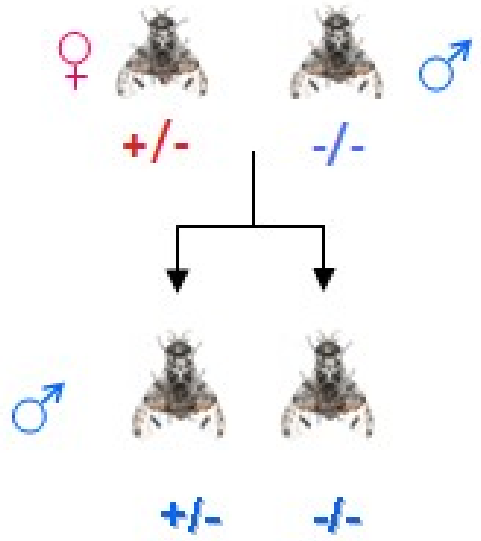


Figure 8 - Specific cross to obtain a male-only progeny. Crossing transgenic females with males (transgenic or not), it is possible to produce a progeny of almost only males (with XX or XY karyotype). About 1-5% of intersexual flies are obtained at each generation.

3 RESULTS

3.1.1 The *Ceratitis capitata* Cc5.3 transgenic strain

To identify the integration site of the transgenic construct and to investigate the presence of a possible ovarian-specific enhancer that could be responsible of the maternal effect of the transgene in the strain Cc 5.3 we took advantage of the availability at the NCBI Sequence Read Archive (SRA) of the *C. capitata* genomic sequences (Medfly Genome Project - Al Handler, USDA, US). Inverse PCR (Ochman et al., 1988) has been performed, using divergent primers on the basis of the respective transposon piggyBac ends sequences, at 3' termini of the vector following an adjustment of a protocol studied for recovery of sequences flanking piggyBac elements in *Drosophila*, by Buchholz et al. Genomic DNA extracted from the transgenic flies was digested, ligated and amplified by two nested-PCR. The gel analysis showed the presence of an intense smeared band which was directly sequenced using the sequencing primers proposed by the protocol(see materials and methods). We obtained a 96 bp long DNA genomic sequence flanking the insertion. Using this sequence to search by BLASTN the *C. capitata* SRA genomic database, a 544 bp sequence was found, containing the 96 sequence and hence covering the nucleotide position at which the transposon is inserted. This SRA has been used to try an *in silico* "genomic walking", and other 5 overlapping sequences have been found, covering a 1.7 Kb long genomic region flanking the insertion point (500 bp upstream and 1200 bp downstream) (fig. 9). These SRAs when used in BLASTN search found highly similar (identities: 84%) *Ceratitis* ESTs containing a long ORF showing similarity (identities: 20%, positives: 40%) to a Drosophila transposase (*Hoppel*-like transposon) (Reiss et al., 2003) fouded by BLASTX search.
To test if these SRA sequences correspond to effective integration site, PCR on genomic DNA from transgenic flies was performed, at 5' and 3' piggyBac integrated termini, with a primer specific for this genomic region, and the second one specific for the PiggyBac vector termini (Fig. 10) The length of the PCR products was consistent with the expected one and the identity of the fragments amplified were confirmed by enzymatic digestion.
Both flanking genomic regions (upstream and downstrem) have been analyzed for their potential transcription activity by RT-PCR and they showed to produce transcripts in wild type and transgenic adult females flies. We investigated if these transcripts are specific of the ovaries, starting to compare anterior and posterior fly regions. An RT-PCR was performed on RNA extracted from female heads and from female abdomens, showing that these specific RNAs are present in both body regions(Fig. 11).
To verify if the *Cctra*-IR transgene is transcribed in ovaries, at higher levels by the effect of a nearby enhancer belonging to this Hopper-like transposon, Real-Time PCR has been performed also for the spacer sequence of the IR and dsRed gene: the transgene is expressed in ovaries from transgenic females, while the expression levels of the transgene in thoraxes from transgenic females are very low, as well for the DsRed gene (fig. 12).
One explanation of this peculiar maternal effect as well as of the higher transgene expression in the ovary could be the position effect well known from previous Drosophila studies (O'Kane and Gehring, 1987;Henikoff, 1994).

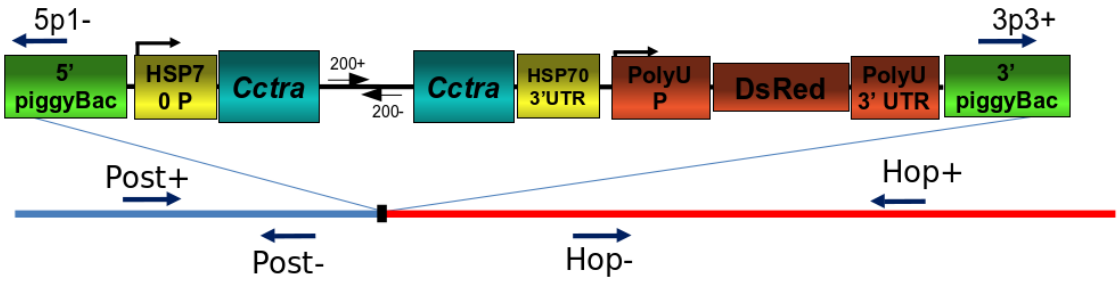


Figure 9 - Schematic representation of the insertion point of the in the Cc 5.3 strain and primers.

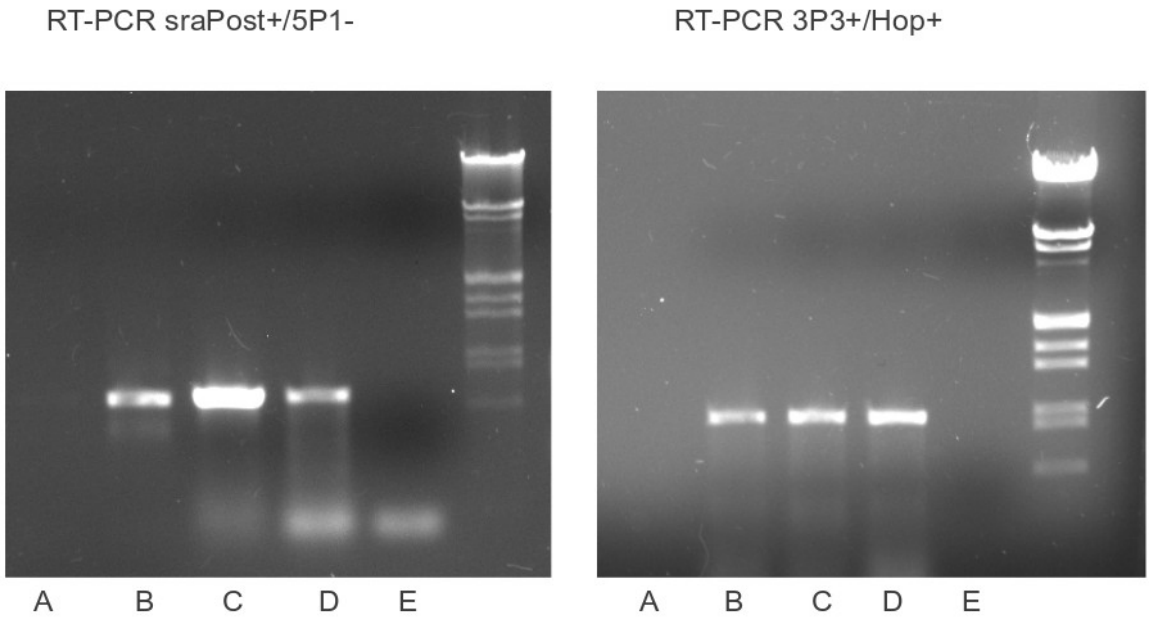


Figure 10 - Analysis of both flanking regions of the insertion site. A: wild type genomic DNA; B: homozygous cDNA from a transgenic fly; C: cDNA from a transgenic female head; D: cDNA from a transgenic female abdomen; E: negative control.

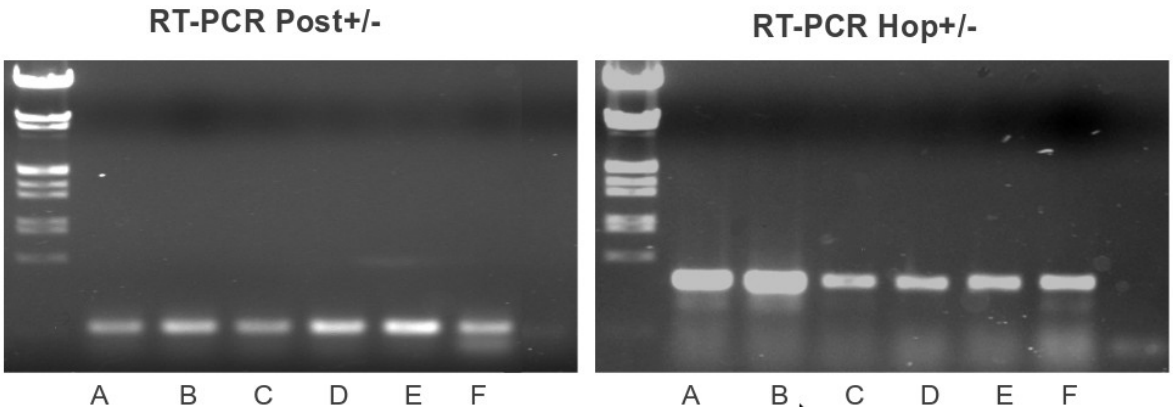
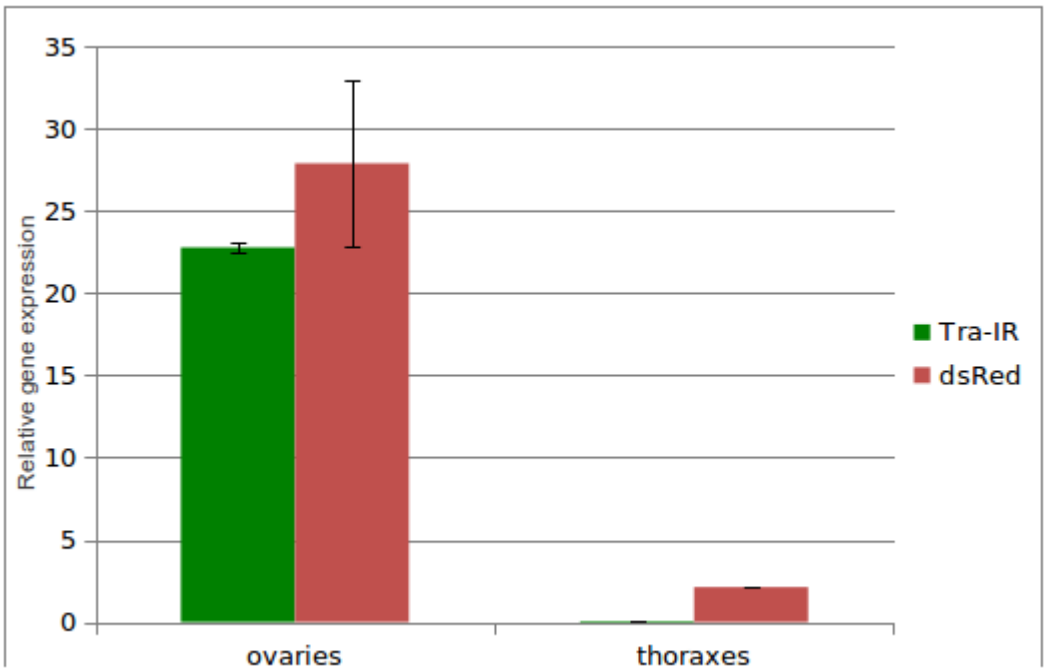


Figure11 - Expression analysis by RT-PCR of both flanking regions of the insertion site on dissected female flies. A: wild type head; B: wild-type abdomen; C-E: transgenic head; D-F: transgenic abdomen.

Figure 12 - Analysis of *transformer* Inverted Repeats cassette (Tra-IR) and DsRed



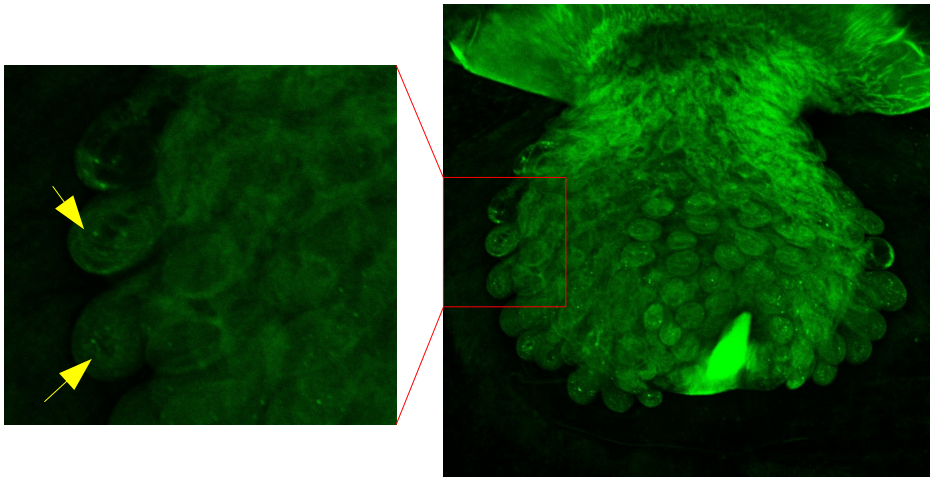
cDNA expression levels in ovaries and thoraxes from transgenic adult female flies by real time-PCR.

With the advent of the *Drosophila* germline transformation, stable position effects on transgenes were found to be very common and often caused by enhancers present nearby the integration site.

3.1.2 Are XX transgenic males competitive in mating with wild females compared to the XY males?

To test competitiveness of XX males vs XY males and to evaluate the contribution of the Y chromosome to male mating success, conventional quality control field cage tests under greenhouse conditions were performed in collaboration with the Seiberdorf FAO-IAEA Unit (Franz-Caceres), (reference FAO/IAEA/USDA Product Quality Control and Shipping Procedures for Sterile Mass-Reared Tephritid Fruit Flies), of XX versus XY males (XX transgenic fluorescent and non transgenic males versus XY non-transgenic ones), using the Medfly Argentinean strain as reference.The release of a mixed population of XX transgenic and XX non transgenic males showed in preliminary field cage test no mating abnormalities, beeing the RSI= 0.51(expected 0.50 in case of equal mating abilities) and ISI=0.19. The ISI value is in a range between +1/-1, so an ISI=0 corrispond to random matings, ISI=+1 corrispond to total mating isolation of two strains, and ISI= -1 indicates that all matings occurre between members of the opposite strain.
To confirm this result, mating tests were performed in small cages among wt-females, wt-males and XX transgenic male expressing GFP flourescent protein in the sperm. The transgenic Ceratitis line #1260 pBac{<af_attP-Ccb2t-tGFP_af<_PUb- DsRed} line was kindly provided to Dr. Saccone by E.A.Wimmer (Department of Developmental Biology, Gottingen Center for Molecular Biosciences, Gottingen, Germany).Males transformed with the construct #1260 express Cc-b2tubulin promoter-driven tGFP in the testes and produce fluorescent sperm providing the possibility to analyze adult mating physiology regarding sperm transfer, storage, competition and use (Wimmer et al., 2008).
The XX-males have been produced in our laboratory by crossing females from the 5.3 masculinizing transgenic lines and the sperm-marked transgenic line #1260.
In a collaborative project with the Dept. of Zoology of the University of Siena, crosses in small cages have been performed revealing that the XX transgenic males transferred efficiently GFP-marked X-bearing sperms into females. In single fly competitive mating experiments (1 WT female x 1 WT male x 1 XX male), WT females did not show any preference toward wild type or XX males. The total amount of adult flies used was 187 wt-female, 187 wt-males and 197 xx-males.In 143 matings in triplet (76,47%), females choose 53,85% XX males and 46,15% wild type males. Microscope observations of the spermathecae and fertilization chamber revealed motile sperm cells in almost the totality of females from both mated with wild type or with XX males (Fig. 13).
Otherwise, to test the competitiveness relatively to progeny number, small cage experiments have been done in triplicate crossing 10 wt males, 10 wt females and 10 homozygous transgenic XX males and progeny has been screened by florescence (Fig.14) and for each replicate the progeny resulted to derive respectively 27%, 44% and 48% from transgenic XX males.

A



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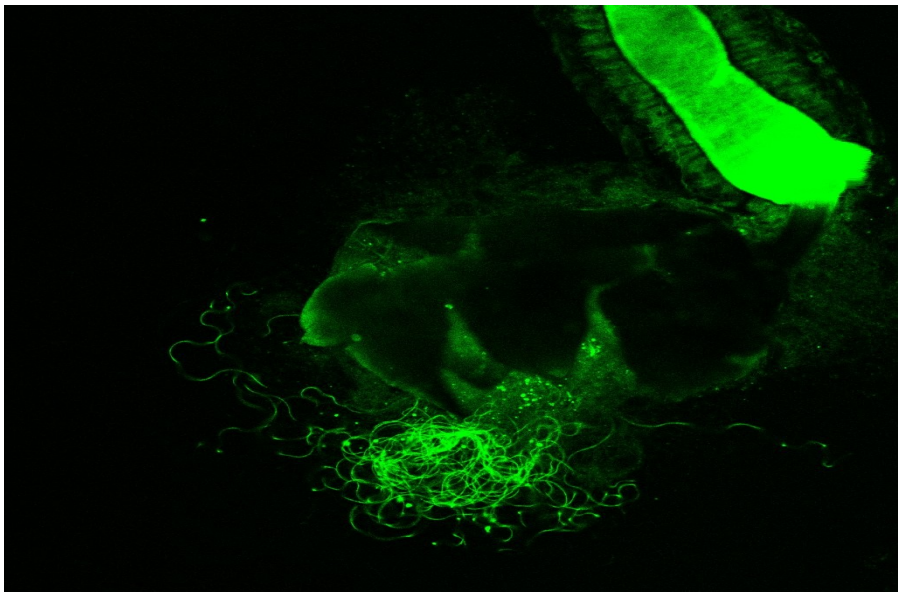


Fugure 12 – XX males are able to transfer sperms in female sperm storage structures during mating. Microscope observations of the fertilization chamber (A) and spermathecae (B) of wild-type females after mating with transgenic XX males, revealed the presence of fluorescent sperms.

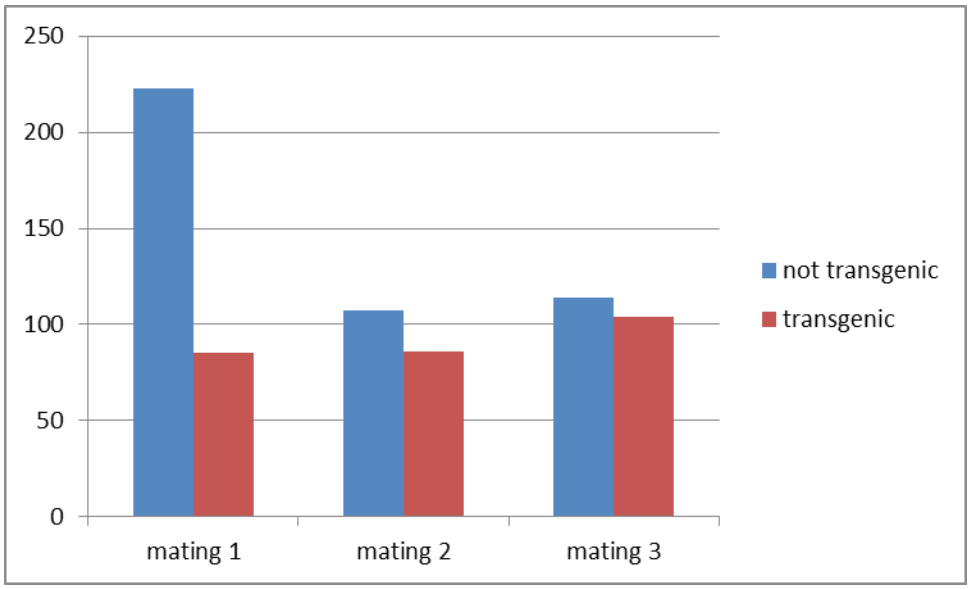


Figure 14-Mating competition performed in triplicate (mating 1,2,3) between wild-type and transgenic males, relatively to the progeny number.

3.1.3 Is the maternal RNAi affected by genetic background?

The maternal RNAi provoking a masculinization of 95-99% of XX embryos could be affected in its efficiency by the genetic background of the mother. Hence the transgenic line has been crossed with a strain of *C. capitata* derived from a South African population (SA; kindly provided to Dr. Saccone by Dr. G. Franz, FAO-IAEA Pest Control Lab). 30 transgenic (AA homozygous for the transgene) males from the 5.3 strain were crossed with 60 non transgenic females from the SA strain. 60 hemizygous transgenic (A/-) females were selected from the F1 progeny and crossed with SA non transgenic males. The progeny F2 was analyzed. Interestingly, on a progeny of 243 adults, about 54% were apparently males, 25% intersexes (female ovipositor together with male antennae or male type bristles on the forelegs), 18% malformed males (genitalia malformed), 3% of females. So there is the evidence that the maternal effect of transgene had much less masculinizing activity when the genetic background of the mothers is changed. Perhaps modificators of the endogenous RNAi efficiency exist in the genome and their different alleles are co-adapted. This finding should be taken into account when in future an RNAi-based sexing transgenic strain will be developed and selected as a candidate for rearing in the bio-factory and to be scaled up to millions of flies per week. Furthermore these data suggest that masculinizing activities of the transgene can be improved by artificial selection and bottlenecks.

3.1.4 The *Cctransformer* gene expression in the transgenic strain Cc5.3: embryonal stages.

Previous analyses (Salvemini and Saccone, manuscr. in prep.) have shown that in XY embryos *Cctra* male-specific mRNAs are detected only after 6 hours from oviposition, while in the preceeding hours only the maternally deposited ones are present (those corresponding to the female-specifically spliced ones which encode full length TRA protein). Hence the action of the Y-linked M factor starts most likely at 6 hours, blocks the maternal CcTRA protein and induces male-specific splicing of the newly transcribed *Cctra* pre-mRNAs (Gabrieli et al., 2010).
In our transgenic line, masculinization of XX individuals is promoted by the transgene producing *Cctra* dsRNA and knocking down the corresponding mRNAs by a maternal effect. The transient absence of CcTRA protein most likely causes a collapse in the *Cctra* autoregulatory loop and a permanent shift to a male-specific splicing pattern of Cctra and consequently of *Ccdsx* pre-mRNAs. I investigated at which stage of embryogenesis this molecular regulatory shift is observed in transgenic XX embryos.
The transcriptional and splicing pattern of *Cctra* and *Ccdsx* have been analyzed by RT-PCR on RNA samples extracted from unfertilized eggs and from embryos derived respectively from transgenic virgin females, and from transgenic fertilized females crossed with non transgenic XY males. Collections of XX-XY mixed embryos were performed at 4 different times AEL: 2-3h EAL, 7-8h AEL, 23-24h AEL and 47-48 AEL.
The presence of dsRNA deposited by the mother into the eggs was confirmed by an RT-PCR using primers specific for the spacer sequence of the IRs to avoid PCR suppression (Fig 15c).
We observed that the endogenous *Cctra* female-specific mRNA is present in both

unfertilized eggs and embryos until 7-8h AEL (Fig.15a). absence of male-specific *Cctra* mRNAs after 7 hours, until 8 hours, is rather surprising and unexpected in the XY embryos, which carry the M factor, and hence should normally perform male sex determination at this stage. On the other hand also the presence of *Cctra* female-specific mRNAs is surprising because the maternal effect of RNAi should provoke a consistent reduction of these transcripts.
We speculated that the maternal RNAi causes a slight reduction in the *Cctra* female-specific transcripts deposited in the eggs, leading to a loss of function effect. However the remaining amount of mRNA would be sufficient for RT-PCR amplification. Furthermore the presence of maternally inherited *Cctra*-specific dsRNA could be very effective against the first zygotic *Cctra* pre-mRNAs, leading to their degradation during various embryonic stages.
These finding suggest that the action of maternally deposited *Cctra* dsRNA molecules on the zygotically transcribed *Cctra* mRNAs causes the unexpected absence of these male-specific transcripts. Perhaps the RNAi has a stronger effect on these newly produced mRNAs, because of their relatively low abundance during early development stages, and that the reduction of zygotically *Cctra* mRNA should lead later on the permanent collapse of the *Cctra* female-specific positive feedback loop and hence to the stable production of male-only *Cctra* mRNAs.
Similarly, only *Ccdsx* female-specific mRNAs are detected in unfertilized and developing embryos until 8 hours, which is coherent with the *Cctra* splicing pattern.
Interestingly *Cctra* male-specific mRNA is observed in 23-24h AEL embryos, and it seems to decrease at 47-48 AEL.
To quantitatively evaluate endogenous *Cctra* expression level, a real-time PCR has been performed on unfertilized 0-6h AEL egg (from virgin transgenic females) and 0-6h AEL embryos from a cross of wt males and transgenic females (Fig.16). As expected, I observed a significant reduction of endogenous *Cctra* female-specific mRNAs in eggs and embryos from transgenic mothers versus non transgenic mothers.

3.1.5 The *Cctransformer* gene expression in the transgenic line Cc5.3: adult stages.

The production during oogenesis of dsRNA specific for *Cctra* gene leads most likely to a transient and partial destruction of the corresponding maternal and zygotic *Cctra* mRNAs in both XX and XY embryos. The transient lack of the maternal (early zigotically produced in XX embryos) CcTRA protein induced by RNAi causes the collapse of the *Cctra* autoregulatory positive loop in XX embryos, as mediated by the M factor in XY embryos. So male-specific Cctra and *Ccdsx* splicing in XY males was expected, because of the action of the Y-linked M factor.
The transcriptional and splicing pattern of *Cctra* and *Ccdsx* have been analyzed by RT-PCR on RNA samples extracted from single adult males respectively from 10 wt XY males, 20 XY transgenic and 20 XX transgenic males (Fig. 17). In wild type males Cctra produces mostly male-specific longer transcripts but also low amount of female-specific ones are detected by the very sensitive RT-PCR, with consistent variability between individuals. It is unclear if this is of any functional significance. The pattern observed in XX males was in most cases comparable to the wild type, with

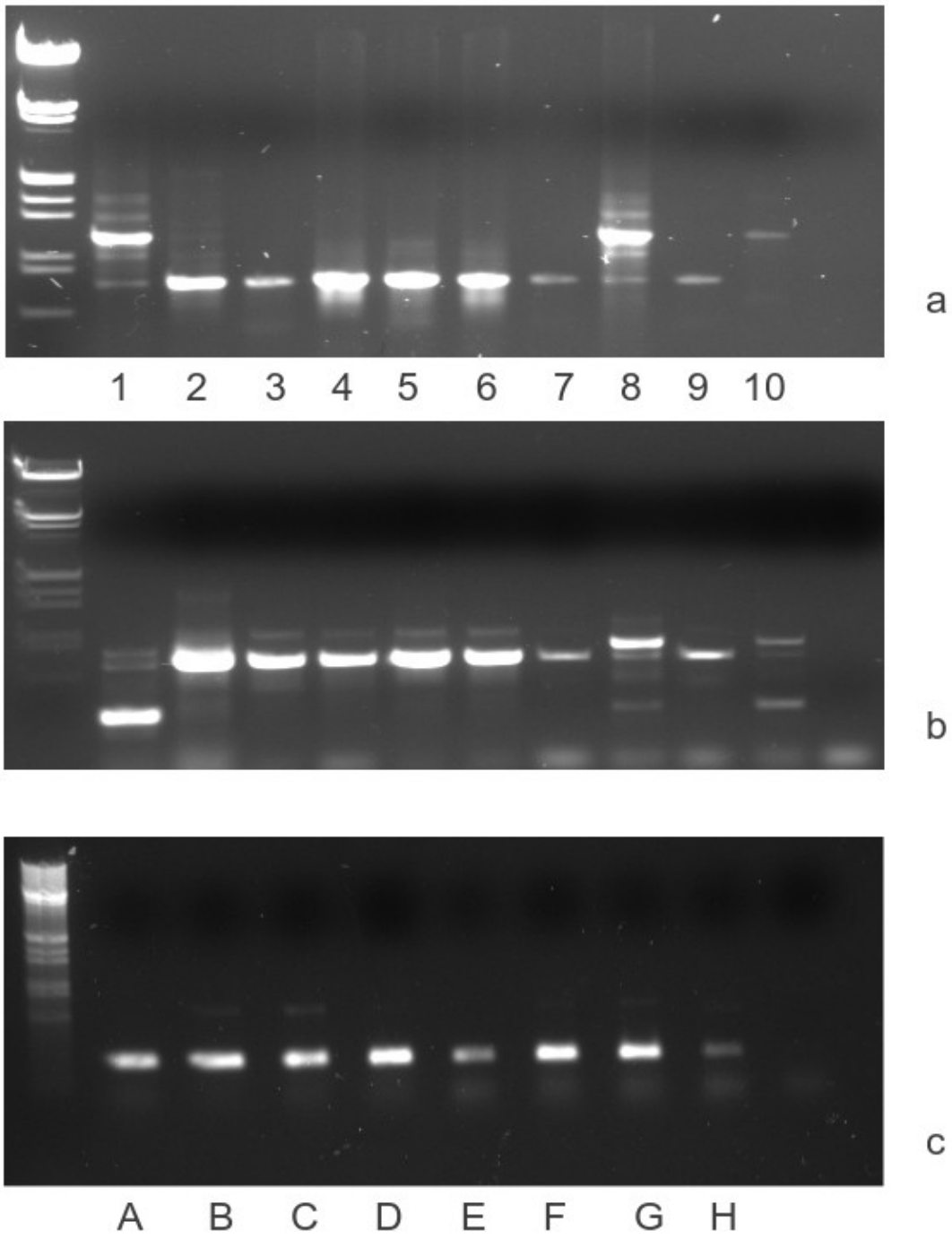


Figure 15 - Analysis of *Cctra* (a) and *Ccdsx* (b) splicing pattern in eggs and embryos from transgenic females. 1:adult male; 2: adult female; 3-5-6-7: unfertilized eggs at 2-3, 7-8, 23-24 and 47-48h AEL; 4-6-8-10: embryos at 2-3, 7-8, 23-24 and 47-48h AEL. C: Expression analysis of IR in eggs and embryos from transgenic females.: A-C-E-G: unfertilized eggs at 2-3, 7-8, 23-24 and 47-48h AEL; B-D-F-H: embryos at 2-3, 7-8, 23-24 and 47-48h AEL.

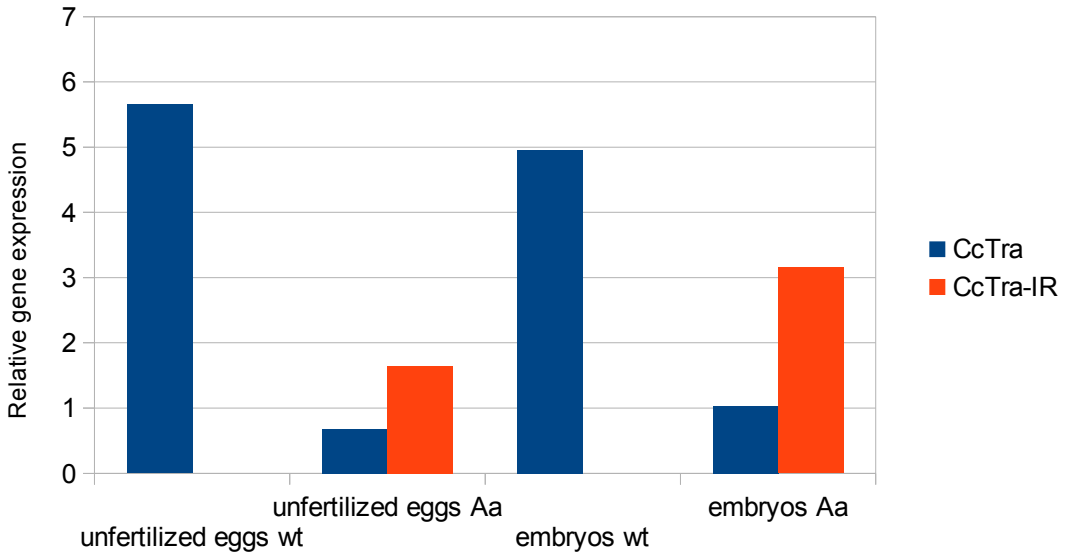
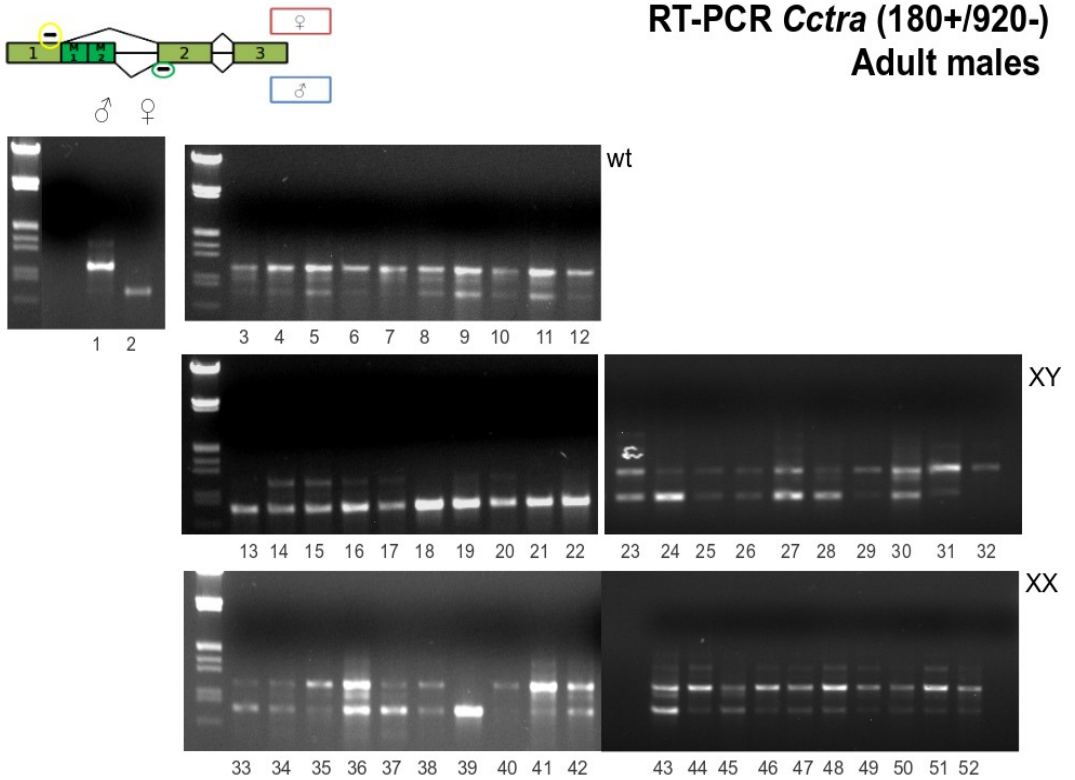


Figure 16 - Analysis of expression levels of *Cctra* and *Cctra*-IR in unfertilized eggs and embryos from wild-type and transgenic females bt Real-Time PCR. All samples are 0-6 after eggs laying.

a



b

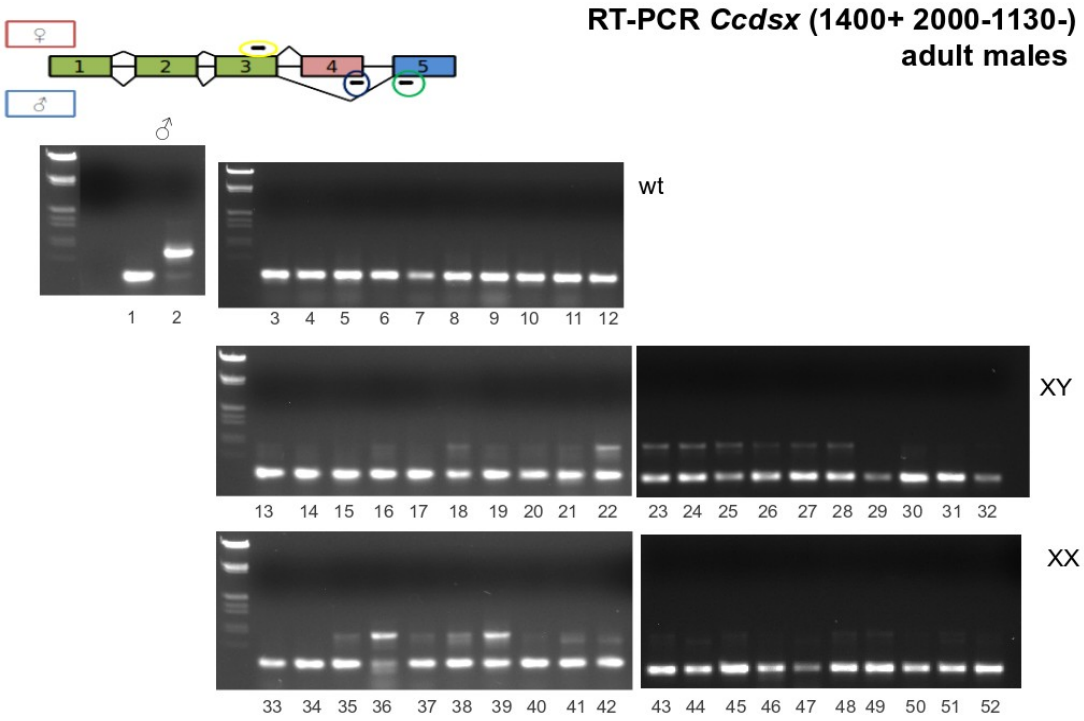


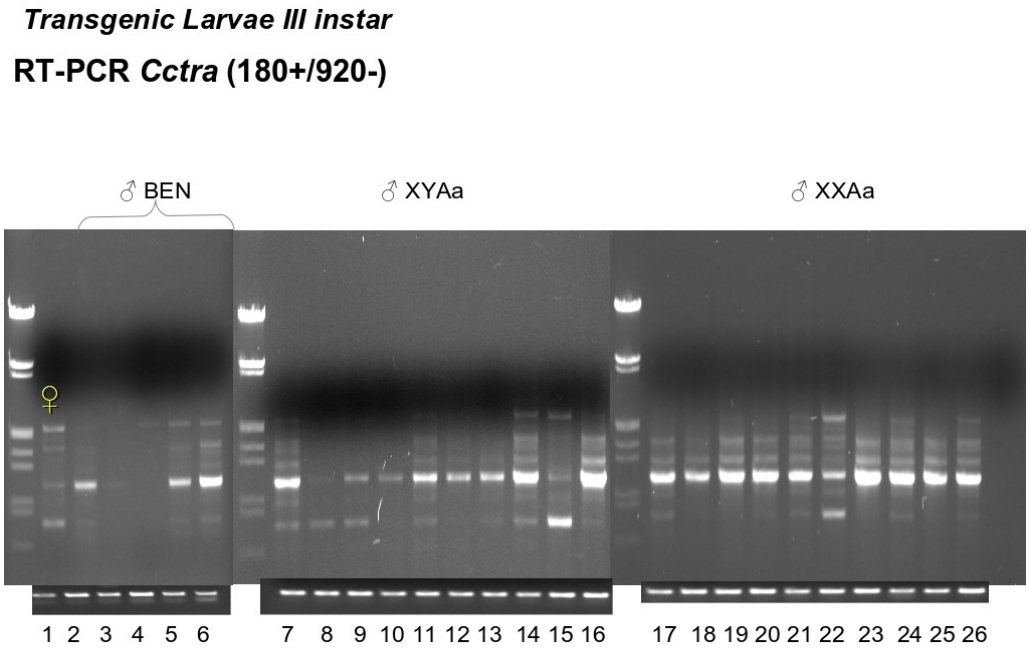
Figure 17 - Molecular analysis of *Cctra* (a) and *Ccdsx* (b) splicing pattern in adult wild-type (3-12) and XY (13-32) and XX (33-52) transgenic males.

few striking exceptions showing more female-specific then male-specific *Cctra* mRNAs (see lane 37 and 39). Very surprisingly we observed in about half of XY transgenic males the presence also of female-specific *Cctra* mRNAs, and the relative ratio was in favour of these unexpected ones, more than in XX transgenic males
The splicing pattern of *Ccdsx* is completely male-specific in wild type XY individuals. However in most of the XX masculinized transgenic flies, there are *Ccdsx* male-specific sized transcripts with very small amount of female-specific ones. This observation is coherent with the observed male phenotype but incoherent with the presence of female *Cctra* transcripts. In XY transgenic flies, I detected male-specific but also in many individuals female-specific *Ccdsx* mRNAs, which is consistent with a more pronounced effect of disturbance supposely by the transgene in the presence of the Y chromosome.
I then asked if the unexpected presence of female-specific *Cctra* and *Ccdsx* mRNAs in XY and XX transgenic males is observed also at early developmental stages.
I performed the same analysis on 30 single jumping larvae (3rd instar) which I karyotyped by Y-specific PCR: 10 non-transgenic larvae with Y chromosome; 10 transgenic larvae, heterozygous for the transgene, with Y chromosome and 10 transgenic larvae, heterozygous for the transgene, without Y chromosome.
Differently to the adults, almost all analyzed larvae showed sex-specific Cc*tra* and Cc*dsx* splicing patterns which are coherent with the corresponding sexual karyotypes. Hence most likely only later during development *Cctra* starts to produce also female-specific transcripts, indicating a regulatory shift caused by non clear causes, like the presence of the transgene or a leakness in the *Cctra* OFF state. This phenomenon is even more pronounced in XY males, which is very difficult to be explained based on the current model of *Cctra* regulation. Out of 20 larvae, one XX (masculinized) larvae and one XY larvae showed exclusively a female-specific splicing pattern for both genes, which also is surprising and not coherent with the adult phenotypes usually observed in the progeny of this specific cross (Fig. 18).
More investigations are currently underway to confirm these data and to try to pose question aiming to shed some light on the phenomenon. It's rather surprising that although XX males have mixed sex-specific splicing pattern they look apparently normal, they can mate and produce progeny.

3.2 The DOA locus analysis

The gene structure and sequence of the locus DOA in *Ceratitis capitata* have been obtained with the available bioinformatic and genomic tools: we used the *Drosophila* protein sequences of 69kDa, 105kDa, 238kDa and 55kDa isoforms, as virtual probes for a BLASTP search of the *Ceratitis capitata* genomic database (Fig.19). Real-time RT-PCR assays were performed on individuals from embryonic (0-24h AEL), larval (III instar larvae) and adult stages (Fig.20).
The CcDoa 138kDa-like results to be expressed in males, while a very low expression is observed in embryonic and larval stages and in adult females.
The CcDoa 105kDa-like isoform is expressed in both sexes of adult flies, in III instar larvae and embryos (mixed sex).
Interestingly, the CcDoa 55kDa orthologous isoform results to be expressed at higher level in males, both adults and in sexed III instar larvae, which is a novelty with the

A



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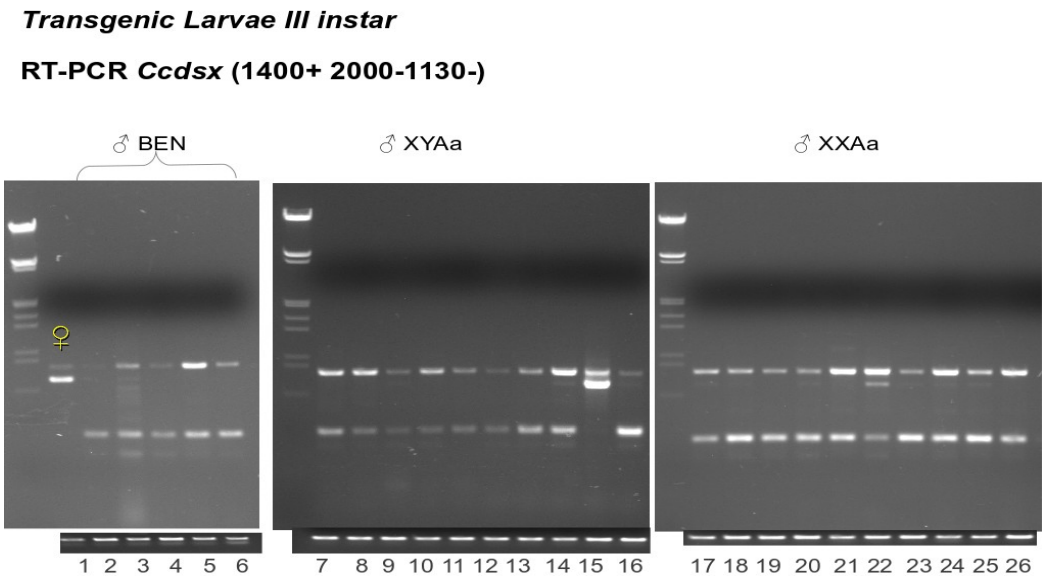


Figure 18 - Molecular analysis of *Cctra* (A) and *Ccdsx* (B) splicing pattern in III instar larvae of XY(7-16) and XX transgenic males (17-26).

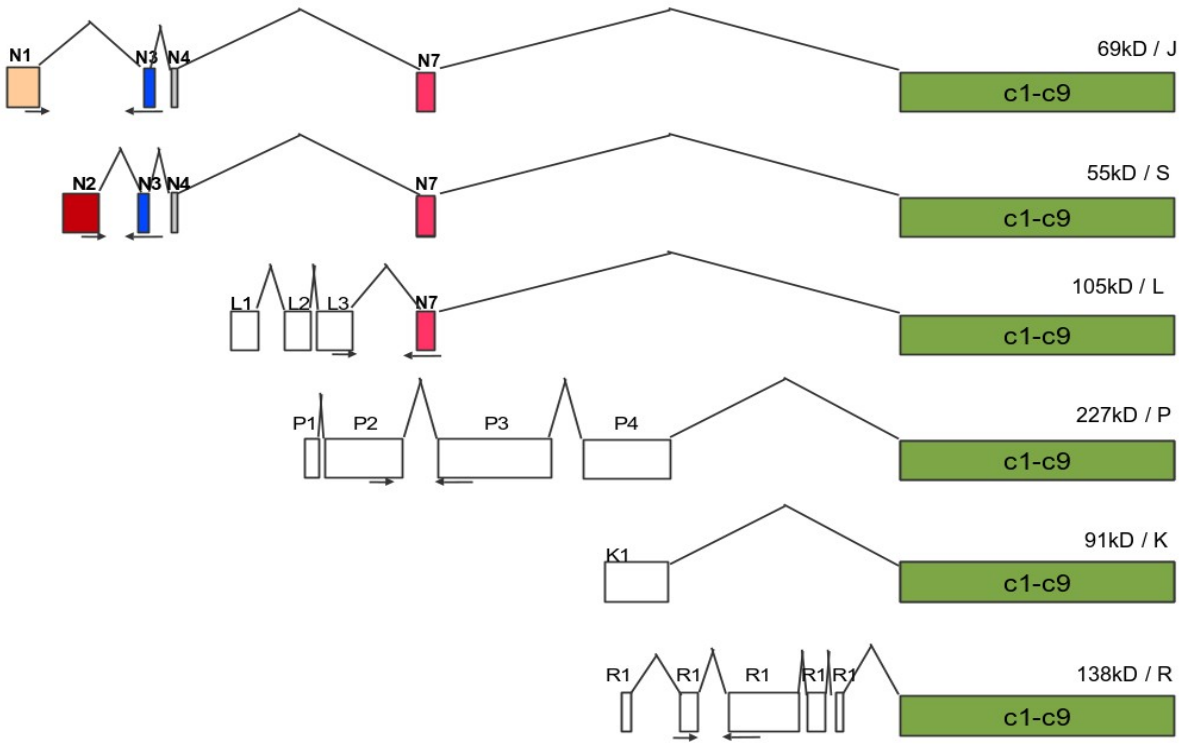


Figure 19 - Schematic representation of the CcDOA locus. Exons not to scale.

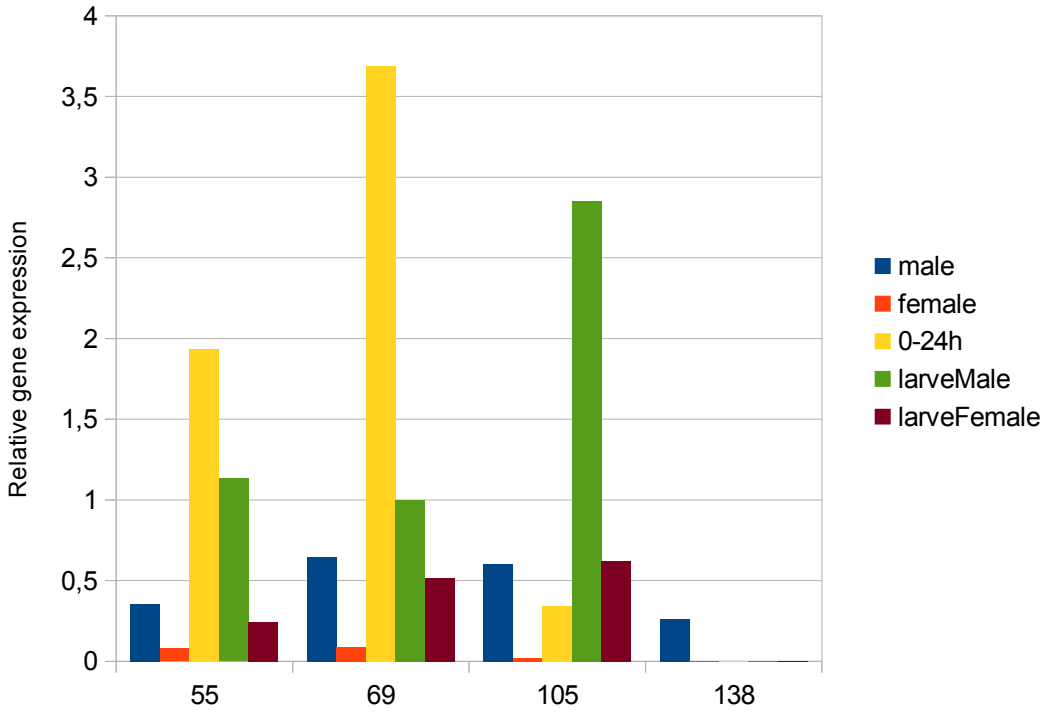


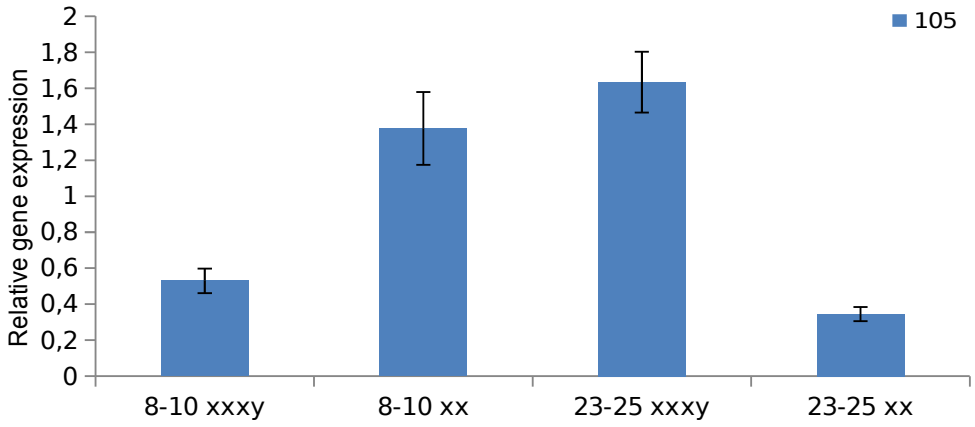
Figure 20 - Relative expression levels of DOA isoforms in *Ceratitis capitata* analyzed by real-time PCR.

respect of its *Drosophila* pattern of expression (no sex-specific). Similarly, the CcDoa 69kDa-like isoform is more expressed in adult males and III instar larvae males, differently to *Drosophila*, where is more expressed in females.
We have performed a similar quantitative analysis also during other developmental stages (Fig. 21). Very surprisingly the 55kDa-like and 69kDa-like are more expressed in females in embryos at 8-10h AEL, but during later stages (23-25h AEL) the sex bias is reversed.
To understand if the 69kDoa-like isoform is involved in sex determination as in *Drosophila*, I performed a functional analysis: a dsRNA was synthesized using a 361 bp including a region of the N1 exon. We injected dsRNA (1 μg/μl) into the posterior pole of 824 preblastoderm stage embryos of wild type *Ceratitis capitata* Benakeion strain, of which 441 larvae hatched and 187 survived to adulthood (90 males and 97 females). Only 4 flies showed some abnormalities in their phenotypes.
We observed two flies with not expanded wings, a female with malformed genitalia having female-specific *Cctra* and *Ccdsx* splicing patterns, and one male with intersexual phenotype (just one supra-fronto-orbital antenna and dark female bristol on the anterior leg) showing a *Cctra* and *Ccdsx* "intersexual" splicing pattern (Fig. 22).
We cannot draw any conclusions about the possible functions of the sex biased DOA isoforms.
We then asked if this peculiar *Ccdoa* regulation has been conserved during evolution in related species from Tephritidae family, as an indication for a functional significance. We have choosen for the analysis the Oriental fruit fly *Bactrocera dorsalis*, another well know pest insect widely distributed in the asiatic area.
Due to the observation that these DOA isoforms show a different expression pattern between *D. melanogaster* and *C. capitata*, a real-time PCR has been performed on the Oriental fruit fly *Bactrocera dorsalis*. Surprisingly, we have found that 2 out of 4 isoforms, 105 and 138, are more expressed in females, while in *Ceratitis* are respectively more expressed in males or show similar level of expression. The other two isoforms have no sex bias, including 69, which in *Ceratitis* is female-biased (Fig. 23). These data suggest that the sex-bias of *doa* isoform is evolving not only in distantly related species but also in those belonging to the same Family. Their potential functions exerted during development apparently are not stable during evolution and still need to be clarified.

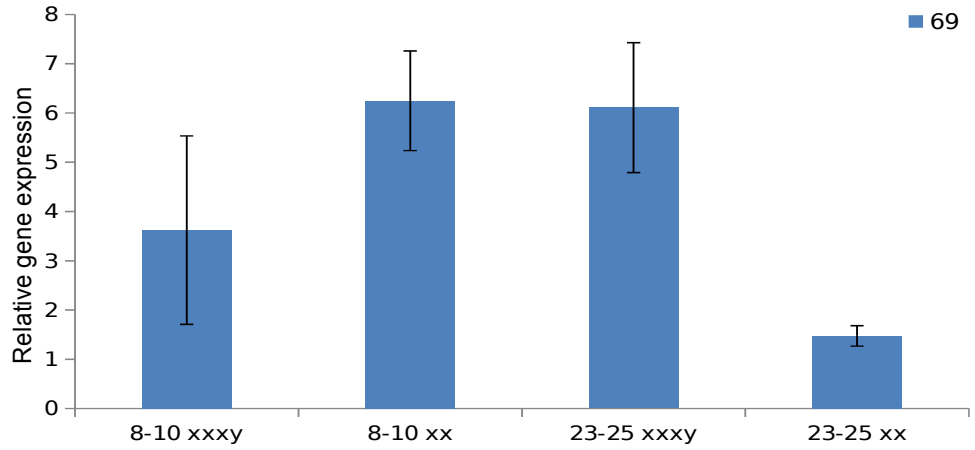
3.3 New tools for genome editing and gene silencing

The available inverse genetic tools, as RNAi, have several limits: dsRNA acts just during few hours, so it's hard to see any effect on genes acting later during the development. We decided to test in *C. capitata* and in *Ae. aegypti* the new developed TALENs and CRISPR-Cas9 methods.
We started by assembling a new TALEN for the dsRed2 gene. In collaboration with Oxitec (Oxford-UK). Two constructs (Talen-1 and Talen-2) have been produced using the SBI Kit (see materials and methods) and they have been first injected in the embryos of the *Aedes aegypti* Ox3978D strain, containing the DsRed2 and AmCyan markers which are clearly visible in larvae. 1894 embryos have been injected with a mix of two construct, OX4830 and OX4831, carrying respectively the Talen-1 and

A



B



C

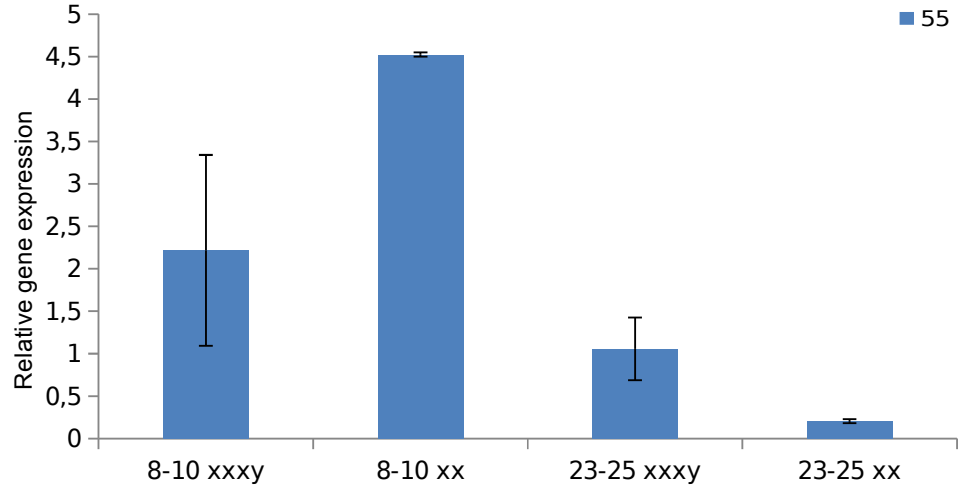
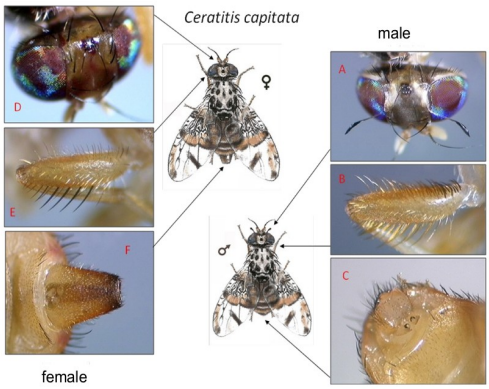
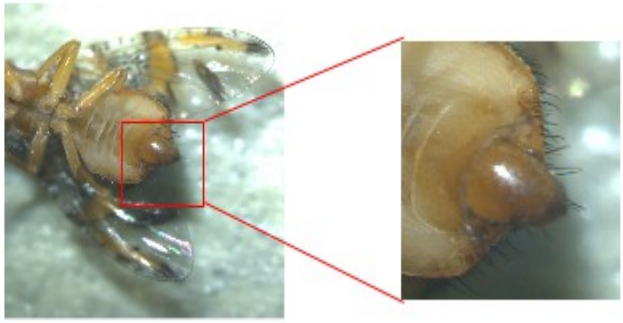


Figure 21 - Analysis of the expression levels of CcDoa 69 kDa-like (a), 55kDa-like (b) and 105 kDa-like (c) isoforms in mixed embryos (with XX and XY karyotype) and in XX-only embryos at 8-10h and 23-25h AEL by real-time PCR.

A

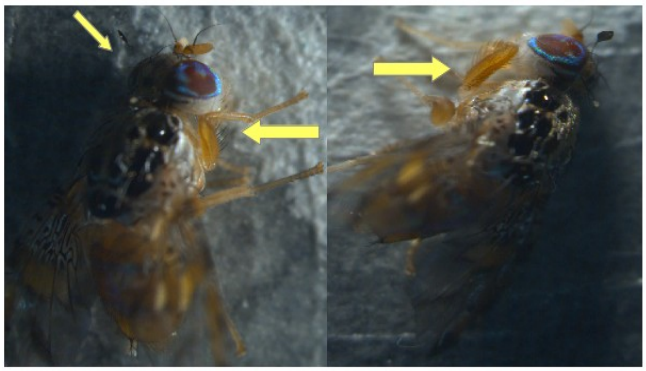


B



C

D



E.1 E.2

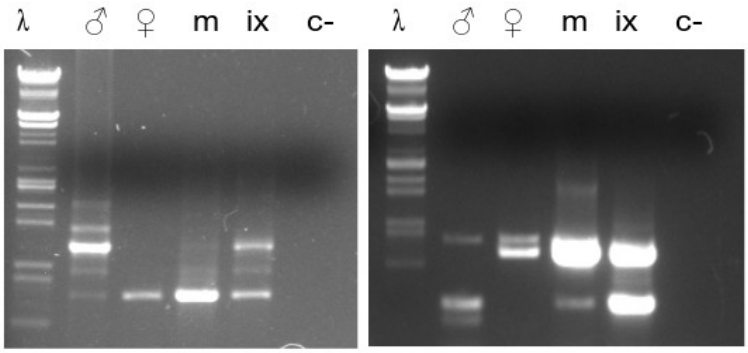


Figure 22 – Phenotypic and molecular analysys of the flies injected with CcDOA-69 dsRNA. A: *Ceratitis capitata* wild-type females exhibit prominent ovopositor and dark bristles on the anterior legs, wild-type males exhibit two additional spatulated bristles on the head and light bristles on the anterior legs. B: female fly with malformed genitalia.D: The unique intersex fly exhibit just one supra-fronto-orbital antenna and dark female bristol on the anterior leg. C: adult fly with not expanded wings. E: molecular analysis of *Cctra* (E.1) and *Ccdsx* (E.2). Ix: intersexual male. M: malformed female.

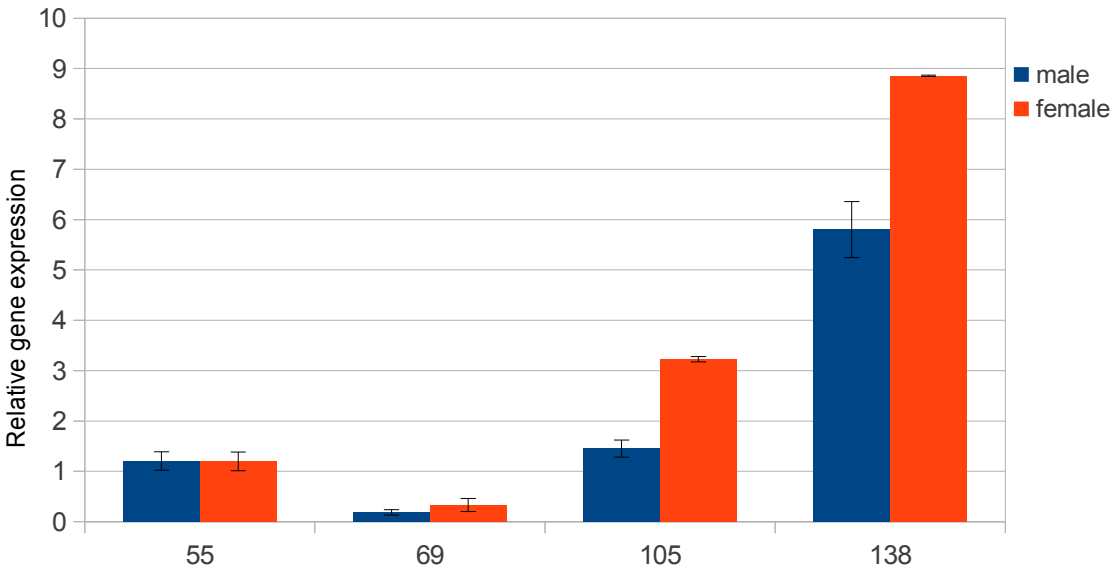


Figure 23 - Relative expression levels of DOA isoforms in *Bactrocera dorsalis* analyzed by real-time PCR.

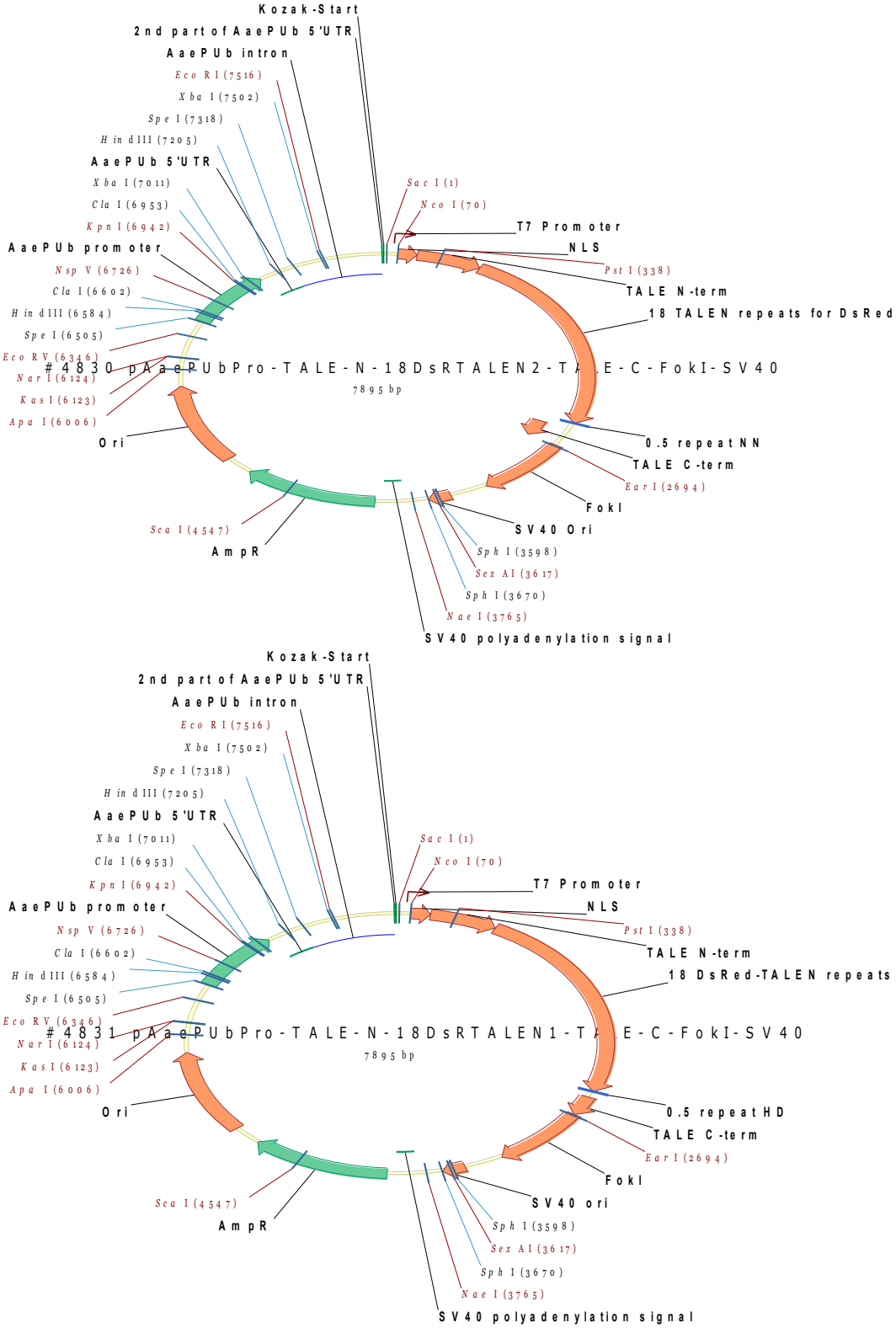
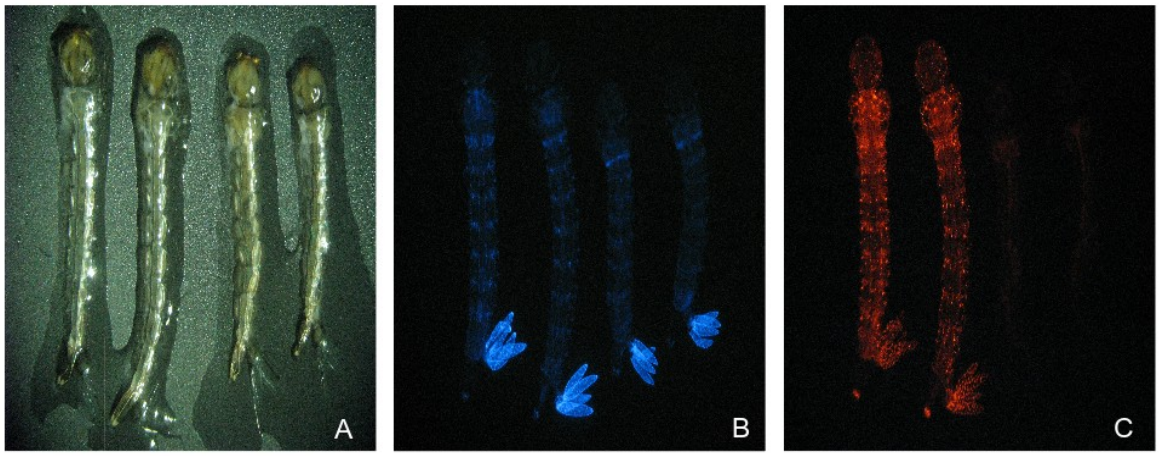


Figure 24 - Ox4830 and Ox4831 vectors carrying the DsRed2 talen-1 and talen-2.



D

```
 S   S   E   N   V   I   T   E   F   M   R   F   K   V   R   M   E   G
TCC TCC GAG AAC GTC ATC ACc gag ttc atg cgc ttC AAG GTG CGC ATG GAG GGC A
AGG AGG CTC TTG CAG TAG TGg ctc aag tac gcg aaG TTC CAC GCG TAC CTC CCG T
```

Figure 25 - Loss of DsRed2 fluorescence caused by deletion induced by TALEN injections in *Aedes aegypti* larvae. A: cold light; B: DsRed filter; C: AmCyan filter. On the left, two larvae of the original strain expressing the DsRed2 and AmCyan markers; on the right, two DsRed2 mutants (Photo credit: Pamela Gray).
D: the 6 nucleotides-long deletion induced by TALEN: Red sequences are recognized by TAL1 and TAL2 in *Aedes aegypti*. In the box are showed the deleted nucleotides.

Talen-2 sequences under the control of the *AeaPUb* promoter (Fig24), giving rise to 25 egg batches from independent parents, and a positive family (fig. 25) where AmCyan was observed, but not DsRed2; PCR and sequencing revealed a 6 bp deletion (Fig 25c).
Then the two constructs OX4820 and OX4821 (fig. 26), carrying respectively the Talen-1 and Talen-2 sequences under the control of the CcPUb promoter, has been injected into the posterior pole of pre-blastoderm stage *C. capitata* embryos of line Ox3647: this is a 4-end piggybac construct that contains Polyubiquitin-DsRed2 as the central transformation marker with the female-lethal cassette and 2 further piggyBac ends; one containing ZsGreen under a generic promoter and the other containing AmCyan under the same generic promoter. Green and Red are readily identifiable in this line (not Cyan), so that a TALEN against DsRed2 would result in a green only phenotype (Dafa'alla T.H. Et al., 2006). 1335 embryos have been injected and all the adults obtained (77) have been crossed with the wild-type strain, and the total progeny of 4685 flies has been screened quite late at pupal development, but no just green fluorescent adult has been observed.
Furthermore, we started in Ceratitis very recently a test of the targeted genome editing by CRISPR-Cas9 which is still on going in our laboratory. I've produced two synthetic guide RNA (sgRNA) specific for the *Ccwhite* gene, encoding for the wild-type eye color, (sgRNAWhite1 and sgRNAWhite2), by in vitro trascription. I utilized a PCR template produced with two different forward primer containing the T7 polymerase binding site and the gene specific sequence (sgRnaWhite1 and sgRnaWhite2) and an overlapping reverse primer containing the sequence able to recruit CAS9. Those sgRNAs will be co-injected with the CAS9 mRNA in *C. capitata* embryos heterozygous for the wild-type allele white^{+} (obtained by crossing wild-type and *white*$^{-}$ flies) to test the efficiency of the system in *C. capitata* and the survivors will be screened for eyes color, to test the efficiency of the method in *C. capitata*.

3.4 DEVELOPMENTAL EXPRESSION ANALYSIS OF THE *FRUITLESS* GENE IN *AEDES AEGYPTI*

Ae. aegypti is an arboviral disease vector. Despite it has been studied for decades, very little is known about the sex determination and reproductive behaviour. Gene involved in either sex determination and behavior could be targets for the future control strategies.
In *Drosophila melanogaster* the *fruitless* gene (*Dmfru*) encodes transcriptional factors: one of which acting as key factor in male sexual behaviour, while the other one are required for several non sex-specific developmental functions.
The fruitless gene could be a good target to develop new sex-specific splicing based sexing strategies, for instance to enable the sex-specific expression of target genes. Since fruitless is needed in males to allow courtship and mating, silencing it could lead to sterility, so it could be a new tool to induce sterility.
My research group has recently identified the *fruitless* gene in *Aedes aegypti (*Salvemini M. et al., 2013): it consist of six non sex-specific exons encoding for the C- terminus FRU, the *fru*P1 exon, divided in two sub-regions (*fru*P1^{m} , common but encoding just the male-specific N-terminus, and the female-specific *fru*P1^{f} , carrying multiple stop signals) and the exon P2, encoding for a short non-sex-specifc N-

teminus (fig. 27). P1 and P2 are mutually exclusives, possibly because they derive from different promoters. RT-PCR have been performed on total RNA extracted from different stages (embryos 0-36h old, early larvae, late larvae, pupae, adults) mixed and sexed, using *rp49* gene as positive control (Fig . 27B-27D1) while the single larvae and pupae have been sexed using the sex-specific spliced *Aeadsx* (fig. 27D1), confirming the presence of two classes of transcripts: the first class derives from the P1 promoter and is detected III instar larvae till adults, producing a transcript alternatively spliced in a sex-specifc manner (fig. 27C1, 27D2). The second class of transcripts, produced by P2 promoter, is non-sex-specific and is transcribed in all the developmental stages analyzed (fig. 27C-2).

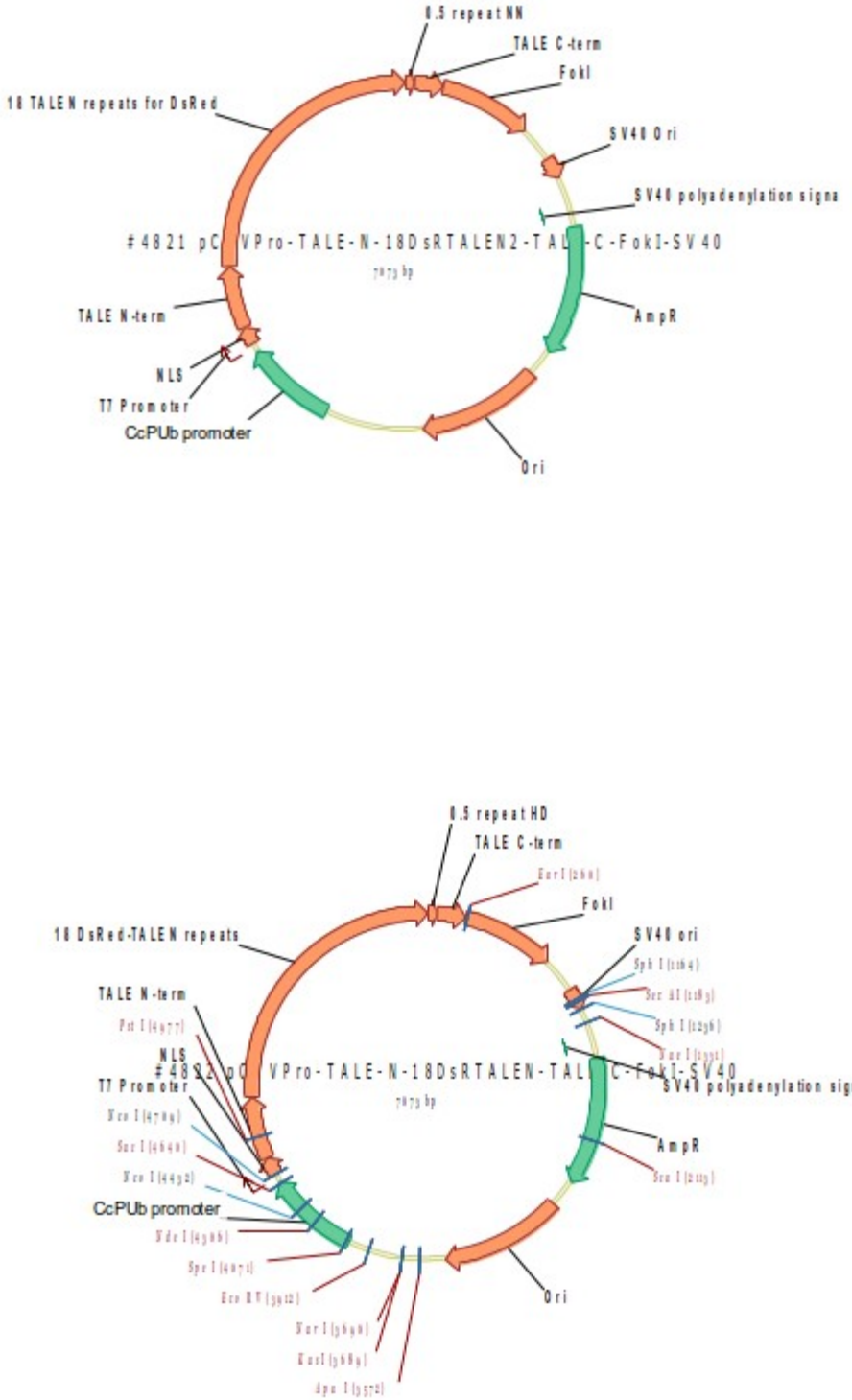


Figure 26 - Ox4821 and Ox4822 vectors carrying the DsRed2 talen-1 and talen-2.

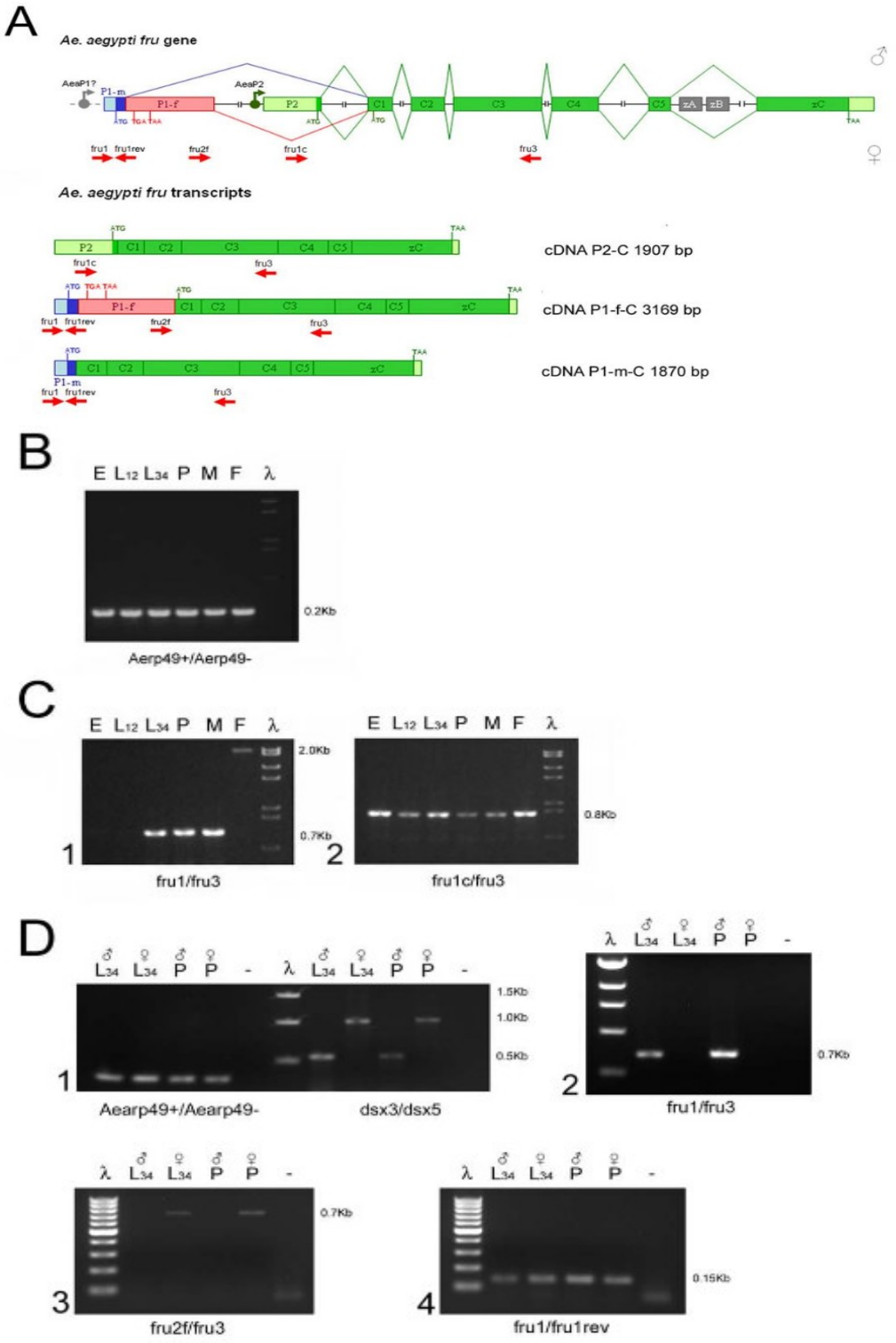


Figure 27 – *Aeafru* gene and transcripts and their developmental expression. (Salvemini et al., 2013). A: Exon P1 constists of two sub-regions: a male-specific *fru*P1^{m} (in blue), and a female-specificthe *fru*P1^{f} (in pink). Green boxes represent common non-sex-specific exons. B: *rp49* positive control. C: P1 and P2 developmental expression patterns. D: expression pattern on single sexed larvae.

4. Discussions

The main aim of my work has been to study the medfly transgenic strain Cc5.3 in order to test the possibility to use it in SIT field programs. Quality control field cage tests under greenhouse conditions and mating testes performed in small cages to test competitiveness of XX males vs XY males revealed that XX males are competitive and compatible in comparison to wild-type males. These results confirm that those males can be released, after sterilization, in the field within SIT program, as alternative to current genetic sex separation strategies. However the evidence that the maternal effect of transgene had much less masculinizing activity when the genetic background of the mothers is changed, should be taken into account when in future an RNAi-based sexing transgenic strain will be developed and selected as a candidate for rearing in the bio-factory and to be scaled up to millions of flies per week. Moreover, the polyandry is a frequent behaviour (Bonizzi et al., 2006): "re-mating" experiments to evaluate the tendency of the females to polyandry in presence of XX males, will be performed in collaboration with the Dept. of Zoology of the University of Siena.
In order to better understand the molecular effects of the *Cctra*-IR transgene by which it masculinize XX embryos, its activity as well as it effect on the endogenous *Cctra* mRNAs were monitored. RT-PCR analyses performed on unfertilized eggs and early embryos deposited by transgenic mothers releaved the expression of the *Cctra*-specific dsRNA molecules. Furthermore the quantity of the endogenous *Cctra* mRNAs results to be significantly reduced in eggs and embryos from transgenic mothers. This result indicates that the dsRNA production in the transgenic mothers induces an RNAi against *Cctra* which is effective already during oogenesis and most likely continues during embryogenesis. In wild type XY males Cctra is switched OFF by the Y-linked M factor by presently unknown mechanism leading to male-specific splicing and the inclusion of a stop-containing exon (Pane et al., 2002; Salvemini et al., 2009). This regulatory event leads to the presence of a short most likely non functional ORF for CcTRA in males.
In wild type females, *Cctra* is able to promote female-specific splicing of *Cctra* itself and *Ccdsx* pre-mRNAs. In transgenic mothers, the production of dsRNA against *Cctra* affects its ability to start this positive feedback loop leading to a shift into the male splicing mode and hence to the *Cctra* OFF state. A transient RNAi against either *Cctra* or *Cctra-2* provokes a stable change into the male splicing mode for both *Cctra* and *Ccdsx* until adulthood in XX individuals (Pane et al., 2002; Salvemini et al., 2009). The XX males can mate and exert an apparently normal sexual courtship behaviour. We investigated how the splicing pattern of *Cctra* and *Ccdsx* is affected at larval and adult stages in transgenic XX and XY males derived from transgenic mothers. Surprisingly we have found an instability in the male-specific splicing shift caused by the RNAi and we detected the presence also of a variable amount of female-specific *Cctra* and *Ccdsx* transcripts in adult stages but not so pronounced in larval stages. Very surprisingly the splicing pattern instability appears to be much more pronounced in XY rather then XX individuals. This is very unexpected considering that the presence of the Y chromosome should lead to a male-specific *Cctra* and *Ccdsx* splicing and that the RNAi against *Cctra* should only slightly reduce the quantity of *Cctra* male mRNA which however is expected to be non functional. Our present hypothesis that could explain these data is that the *Cctra* male-specific

isoforms (short male-specific peptides) also could play a role in the maintenance of the male-specific pattern, analogously to the female specific ccTRA protein required to maintain the *Cctra* female-specific splicing. These male-specific CcTRA peptides could interact with the Y-linked M factor to maintain the *Cctra* male-specific splicing, and hence an RNAi could affect a stechiometric equilibrium which could underlay the bistable ON/OFF of this gene. Additional analysis are underway to better clarify this unusual splicing pattern in adult males.
As *Cctra* and *Ccdsx* splicing pattern instability is observed mostly at adult stages, when the sexual differentiation is already completed, it is not surprising that the XX flies looks as normal males, although they hide a molecular mosaicism. It is hence auspicable in the future to develop novel sexing transgenic strains in which is possible to provoke a transient RNAi during oogenesis and early embryogenesis against *Cctra* to obtain a more stable splicing pattern shift..
The DOA locus has been identified in *Ceratitis capitata* as well as in *Bactrocera dorsalis*. The CcDOA 69kDa-like show a peculiar expression pattern: while in *Drosophila* this isoform result to be essential for female sex determination, in *Ceratitis capitata* it results to be more expressed in 23-25h embryos, III instar and adult males, but is more expressed in females in 8-10h embryos. RNAi experiments to CcDOA-69 post transcriptional silencing failed to provoke alternations in sexual differentiation or sex ratio. TALENs and Cas9 reverse genetics approaches will help to clarify in future if *DOA*-69 is involved in sex determination in *Ceratitis capitata.*
Aedes aegypti is one of the main vectors of dengue, chikungunya and yellow fever viruses. To find new genes involved in sex determination in the mosquito *Aedes aegypti*, that could be used to develop new transgenic strains in order to reduce disease spread, I've performed the developmental expression analysis of the *fruitless* gene, responsible for sexual behaviour. A male-specific *fru* isoform has been identified, and further studies will be needed to test if it's responsible for male abilities in mating. Due to the limits of available reverse genetic tools, many efforts are being made to develop new genetic transformation strategies. Recently TALEN and CRISPR-Cas9 have been proposed to induce a site-specific modification to gene silencing. I've established that TALEN can work in *Aedes aegypti*, while new test will be need in *Ceratitis capitata,* as well as for CRISPR-Cas9.
Being essential for mating, *fruitless* gene could be a good candidate for the development of new genetic control strategies for insect pest species. *Fru* function has not yet been studied in vivo in mosquitoes to date, also for the difficulty to produce isoform-specific mutant or isoform-specific RNAi silencing at larval or adult stages. Since TALENs has resulted to be an efficient gene destrupting tool in *Aedes aegypti*, this new technique could make possible to induce a specific non-coding mutation in the *fru* male-specific exon, opening the way to the first in vivo study of *fru* gene in a mosquito species. *Fru*-based strategies for population suppression of pest species can be envisioned in the future through the production of a transgenic strain able to transmit in natural populations a conditional *fru*-silencing transgene, able to lead to sterility of next generation-males, bacause of altered sexual behaviour.

5. MATERIALS AND METHODS

Rearing of *C. capitata* strains

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

Rearing of *Ae. aegypti* strain

The *Aedes aegypti* strain originated from Malaysia in 1977, was reared at 27°C and 70% relative humidity. Larvae were fed on crushed dry fish food, while adults on 10% glucose supplemented with 14 μg/ml penicillin and 14 μg/ml streptomycin. Females were fed on horse blood before eggs collection.

RNA and DNA extraction

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

DOA69 RNA interference

DOA69 dsRNA was obtained and injected as described for *Drosophila* (Kennerdell and Carthew, 1998). A fragment from position 1 to 528 was amplified with primers that introduced a T7 promoter sequence at each end. This template was used to produce dsRNA fragments by in vitro transcription with T7 RNA polymerase using the Megascript Kit (Ambion). The dsRNA was precipitated with ethanol and resuspended in injection buffer (Rubin and Spradling, 1982). Embryos were collected 30 minutes after egg laying, hand dechorionated and microinjected with 1 μg/μl dsRNA. Injected embryos were allowed to develop at 25°C.

DsRed2 TALEN-Assembly.

For the Talen-1sequence, the following Repeat Variable Domains (RVDs) has been produced:
HD HD NG HD HD NN NI NN NI NI HD NI NG HD NI HD.
For the Talen-2 sequence, the RVDs choosen have been: NN HD HD HD NG HD NI NG NN HD NI HD HD NG NG NN.
The assembly and cloning of Talen into the vectors have been carried out using the EZ-TAL Assembly Kit (SBI System Biosciences) according with the procedures proposed by the producer.

Talen microinjections

The injection mix had 500 ng/ul of each DNA construct (Tal-1 and Tal-2).
For *Aedes aegypti*, eggs were collected on a wet paper filter, sliced and trasferred on a cover slip, covered with Holocarbon oil and injected at the preblastodermal stage.The cover slips of injected embryos were placed vertically into water in order to drain the oil for at least an hour, and then immediately placed vertically in a sealed

humid box for 4 days.
For *Ceratitis capitata,* embryos were collected at 0-1h after deposition, dechorionated using 1.5-2% hypochlorite solution, sliced on a cover slip and covered with holocarbon oil. Injected embryos were allowed to develop at room temperature.

Reverse transcription PCR
RT-PCR was performed using RNA (after DNAsi treatment) from different stages with Euroclone MMLV. Starting from a maximum concentration of 2,5 ug of RNA from each sample, add 1 µl of oligo (dT), incubate the sample at 70°C for 5 min. then the sample is mixed with 5X Reaction Buffer, dNTP mix, RNAse inhibitor, MMLV reverse transcriptase in the total volume of 20 µl. The mixture was incubated in a thermal cycle at 42°C for 1h and 94°C for 10 min.
The primer pairs RpP1+/RpP1- was used as the positive control. The following PCR cycles were performed: 5 min at 94°C, 35 cycles with 1 min at 94°C, 1 min at 60°C, 1 min at 72°C, 10 min at 72°C.

Inverse PCR
Inverse PCR at 3' termini of the integrated PiggyBac vector, has been performed adapting the protocol available at http://flystocks.bio.indiana.edu/Browse/in/exel-ins.htm. Genomic DNA (1ug) from homozygous transgenic flies has been digested with *Hae*III restriction enzyme and ligation has been performed with NEB T4 DNA Ligase. First and second rounds iPCR have been performed by using Eurotaq (Euroclone) with, respectively, the primers pb3F1 and pb3R1, and pb3F2 and pb3R2. The following PCR cycle was performed: 1) 95°C 5 min ,2) 95°C 30 sec, 3) 55°C 1min, 4) 72°C 2 min, 5) GOTO 2 x34, 6) 72°C 10 min, 7) 12°C hold. Sequencing was performed using an automated 377 DNA sequencer (Applied Biosystem), using the primer pB-3SEQ. Sequence comparison were performed using the BLAST search algorithm at the NCBI.

Real-time PCR
Real-Time PCR was conducted using a 40-cycle, two step PCR in an ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA) using SYBR Green Master Mix (ABI) and 200 nM primers. Reactions were performed in triplicate. Primer efficiency and Ct were calculated using the real-time PCR Miner software (Zhao et al., 2005) available on http://www.miner.ewindup.info/. The datas have been analyzed by $\Delta\Delta$Ct method.

Relative Sterile Index (RSI) and Isolation Index (ISI)
Relative Sterile Index (RSI, an index of male sexual competitiveness) and Isolation Index (ISI, a measure of mating compatibility) have been calculated. The RSI is the major index of male sexual competitiveness, as represented by the formula: RSI= SW/SW+WW (S= sterile or transgenics insects; W=wild tipe insects), RSI value can vary from 0 to 1, where 0 indicates that all of the wild females that mated in the cage mated with wild males, 1 indicates that they all mated with sterile males, and 0.5 indicates that half mated with sterile males and half with wild males and that sterile males are equally competitive with wild males. The ISI value can be calculated by the formula ISI=/SS+WW)-(SW+WS)/SS+WW+SW+WS and its values range from -1 (complete negative assortative mating; i.e., all matings are with members of the opposite strain) through 0 (random mating) to +1 (complete positive assortative

mating; total mating isolation of the two strains).

sgRNA production

PCR were performed with no template, using the forward CRISPR _FCcwhite1 and CRISPR _FCcwhite2 primer, containing the T7 polymerase binding site and the target sequence, and the reverse common primer sgRNA_R. The PCR products have been purified with Wizard pcr clean up Kit (Promega). In vitro transcription was performed with the Megascript T7 kit (Ambion) using 300 ng of purified template.

List of primers

N1dsT7+	TAATACGACTCACTAGTTTGCGCCTAGACGCGAAG
N1ds+	GTTTGCGCCTAGACGCGAAG
virilizer-	AGAGCGAATTTGGTACGTGTGA
virilizer+	GAAGGCATACGTTATTTCCTAATTGG
Deadpan-	AAGCCGTGTGGATTCGACAT
Deadpan+	ATTCGGACGACGATTTCGAT
sisterlessA-	CCTCTCTGCTTCCTGCAGTTG
sisterlessA+	TGCATCCTGCCGTAAATCAC
DsRedReal-	CCGCTCCTTTCGCTTTCTT
DsRedReal+	AGGGAGCCCCCGATTTAGA
tra-2real-	ACTACTTGTATCCTCTCGATTGGT
ra-2real+	ACCGTTGTATAGGAGTTTTTGGT
69dsT7-	TAATACGACTCACTAGGTGAGCGCTAAAAACGGTG
69ds-	GGTGAGCGCTAAAAACGGTG
atubulin-	GGGCACCAAGTTAGTCTGGA
atubulin+	CGCATTCATGGTTGATAACG
qbd 138-	CGACTACTGCTGCTGTCTGC
qbd 138+	ACGGACTCATGCACTCATCG
qbd105-	GCCGTCTCCGTGAACTTGAA
qbd105+	CTTTGGAGCCCACGACAAAC
qbd55+	TGGGTAGGAGAGCCAATCAA
qbdN3-	GAGTCCACTCTTCTGCGCTT
qbd69+	ATCTACCTGCTTCGCCGTTT
200-	GGTTTGGTGGGGTATCTTCA
200+	CACAACCAATTGCCTCCTCT
SOD-	TCATCGGTCAATTCGTGCAC
SOD+	TGCTCCGAGAACGTTCACG
190-	ACCAAATCTACGTTTAATAAC
200Real-	AGGAGGCAATTGGTTGTGAAA
200Real+	GCAGGTCCGAACCTCATAACA
TraReal-	CGGAGGCGGCGTCAT
TraReal+	CATATTCCACTTGGCCAACTCA
CcYR	GACGGTAAGTGCCATTCGTT
CcYF	GCTCGAAGACATGCATTGAA

post-	GTGCTCAAAATATCCAATGC
post+	CTTCCTTGAACAAACCCCTG
Hop-	GAGACCGTTCCATCCACAG
Hop+	AACGCGGAATGTCATAGACG
pb3seq	CGATAAAACACATGCGTCAATT
pb3R2	TGCATTTGCCTTTCGCCTTAT
pb3R1	GTCAGAAACAACTTTGGCACATATC
pb3F2	CCTCGATATACAGACCGATAAAAC
pb3F1	CAACATGACTGTTTTTAAAGTACAAA
RPP1-	AATCGAAGAGACCGAAACCC
RPP1+	TTGCGTTTACGTTGCTCTCG
5P 1-	TCACCAACAAGCTCGTCATC
3P 3+	AAATGAAGTGCCTGGTACATC
DSX 2000-	ACGACGGCATGACCTTTAAC
DSX 1130-	CTGGTGGTGACATCGTATCG
Ccdsx1400+	TGAAGAGGCATCAAGGCGTAT
Cctra180+	ATAGAGCGCAGTGTCAATCC
Cctra920-	TCCTGTTCTTCCGATCTGTG
q69+	CTGCCTCACCGTTTTTAGCG
q69-	CAAAGGCGATGCTTCTTCCG
q105+	GGTAAGACCTCACTGGCGAC
q105-	AAATGTACCCTCACCCAGCG
q227+	CCAGAAATCGGCAACAACGG
q227-	CCTTTTGCTGGGCAACCAAT
q138+	AAAGTGCGACAGCCGTATCG
q138-	CGCCAGTTGCGGTGGG
q55+	GTGAGCGAAAAGAAAGAAACCGA
q55-	AAAGGCGATGCTTCTTCCGA
qRPP1+	GGCTTTGGAAGGTATCAACGTT
qRPP1-	TCTGGTTCTTCCTCCTTCTTCTTCT
CRISPR F Ccwhite2	GAAATTAATACGACTCACTATAGGGTGGCGAGCGTAAGC GACGTTTTAGAGCTAGAAATAGC
CRISPR _F Ccwhite1	GAAATTAATACGACTCACTATAGGGGTGCCGGGTCGGGT GAAGTTTTAGAGCTAGAAATAGC
sgRNA_R	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACG GACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

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