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Generic approach for the development of genetic sexing strains for SIT applications

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Contents

Summary:

The application of the Sterile Insect Technique (SIT) in area-wide integrated pest management (AW-IPM) programmes continues to increase in response to requests from Member States. These requests include the development and refinement of SIT packages for programmes to control populations of different insect pests of agricultural, veterinary, and human health importance. The development and operational application of such programmes with an SIT component against insect pests and disease vectors continue to reveal research areas where new technologies could further improve efficiency and thus lead to more efficacious programmes.

One such critical area, where important advances need to be made to increase the cost-effectiveness of the technique, or where it is a prerequisite before any SIT application is conceivable, concerns the development of genetic sexing strains (GSS). In SIT programmes against agriculture pests, the release of both sexes is primarily of economic concern; however, in SIT programmes against some insect disease vectors (e.g. mosquitoes), it is an essential prerequisite to release only males since females are blood feeders and may potentially transmit disease even if sterile.

This CRP focuses on the development and evaluation of generic approaches for the construction of GSS to be used for sterile insect technique (SIT) applications, as part of AW-IPM programs, to control populations of agricultural pests and disease vectors. Significant progress has been achieved so far, which can be summarised as follows:

(a) traits such as white pupae, black pupae (ebony), temperature-sensitive lethal (tsl), slow development (sd), slow larvae, white eye, red eye (cardinal), yellow have been identified as suitable to be used for generic strategies for the construction of GSS. The genes responsible for most of these traits have been isolated and/or exploited as markers [*white pupae, black pupae* (*ebony*), *tsl, white eye, cardinal, yellow*]. In addition, it has been suggested that genes involved in salinity tolerance, desiccation/UV tolerance, auxotrophy, insecticide resistance, and phototaxis may be worth exploring;

(b) in respect to the development of generic approaches for the construction of GSS for SIT targeted agricultural pests and human disease vectors, a number of mutant strains have been established including white pupae strains in *Bactrocera correcta, B. oleae, B. tryoni,* and (new strains for) *Ceratitis capitata* and *B. dorsalis*; black pupae (ebony) strains in *B. tryoni* and *D. suzukii,* while black pupae strains are already available in *Anastrepha fraterculus, A. ludens, A. obliqua*; red-eye (cardinal) strains in *Aedes aegypti* (new strains were also developed), while such strains are already available in *Anopheles gambiae, Culex pipiens, Ae. albopictus* and *Plutella xylostella*; yellow strains in *Ae. albopictus and B. tryoni,* while such strains are already available in *Spodoptera litura (yellow-y)*. In addition, three genes were identified as being able to induce temperature-sensitive lethal phenotypes, and novel *C. capitata* lines with temperature sensitive phenotype have been developed. Knock-in of a fluorescent marker protein on the Y-chromosome of *C. capitata* was initially achieved by CRISPR-based HDR, but the strain was lost after two generations due to loss of fluorescence. Transgenes have been integrated randomly near the M loci of both *Aedes aegypti* and *Aedes albopictus* using the *piggyBac* transposons. In parallel, the sequencing of several insect (*A. fraterculus* sp.1, *A. ludens*, *B. dorsalis*, *C. capitata* and *Z. cucurbitae*) genomes and transcriptomes is ongoing aiming to facilitate the isolation of selectable markers and to identify suitable regions for knock-ins on Y chromosomes of SIT targeted species;

(c) new *C. capitata* GSS were developed based on an X-autosome translocation, and quality control analysis indicated that they do not differ from the Y-autosome translocation lines. In addition, this system offers an easy, fast, and generic approach for the genetic refreshment of mass-reared colonies. Quality control analysis was also performed on GSS constructed with the newly established medfly white pupae mutant lines, and no difference was observed when compared with the original ones, and

(d) a *C. capitata* cage population of XX only flies (lacking the Y chromosome) having an artificial MoY (male determining factor) has been established and maintained for twelve generations to further investigate XX fertile male individuals and the masculinization strategy toward the construction of novel GSS.

Background

Scientific status and problems to be researched: Insects are the most abundant, speciose, and diverse animal group on this planet. Although most insect species are beneficial or harmless, there is a small number of them that are considered major pests to agriculture, livestock or human health, and their populations need to be managed. Conventional methods are primarily based on insecticides. However, there are increasing concerns about their negative impact on human and environmental health, as well the inevitable selection of insecticide resistance due to their extensive use. The Sterile Insect Technique (SIT) represents a species-specific, non-polluting, and environmentally benign approach that has been extensively used during the last 50-60 years to control populations of insect pests and disease vectors as a component of area-wide integrated pest management (AW-IPM) programmes. Due to its successful use against different target species, the requests of the application of SIT continue to increase from FAO/IAEA Member States (MS). Programme efficiency, cost-effectiveness, as well as safety and biosecurity, depend on the availability of genetic sexing strains (GSS) which can allow male-only releases. It is now possible to develop such GSS by both classical and modern biotechnological approaches that are presented later.

Targeted species: The pests targeted for SIT applications include species of agricultural, veterinary, and human health importance. Potential targeted SIT species of agricultural importance are the following fruit fly species: *A. fraterculus* (species complex), *A. grandis, Anastrepha ludens, A. obliqua, A. suspensa, Bactrocera carambolae, B. correcta, B. dorsalis, B. jarvisi, B. oleae, B. tryoni, B. zonata, Ceratitis capitata, C. fasciventris, C. quilicii, C. rosa, Drosophila suzukii, Zeugodacus cucurbitae* and *Z. tau*. The following moth species are also considered major agricultural pests and potential targets for the SIT: *Cydia pomonella, Diatraea crambidoides, D. saccharalis, Ectomyelois ceratoniae, Grapholita molesta, Pectinophora gossypiella* and *Plutella xylostella.* The following species of human health and veterinary health importance are also considered potential targets for the SIT: *Aedes aegypti, Ae. albopictus, Ae. polynesiensis, Anopheles albimanus, An. arabiensis, An. darlingi, An. gambiae, An. stephensi, Cochliomyia hominivorax, Culex pipiens, Glossina* species, *Lucilia cuprina* and *Musca domestica*.

1. Genetic sexing methods: These methods to develop a GSS can be classified into two categories: (a) using classical genetics and (b) molecular engineering methods. The GSS developed several decades ago in the Mediterranean fruit fly, *Ceratitis capitata*, are good examples of classical, sophisticated applications of standard genetic manipulation and successful integration of these strains into operational programmes. For this species, a series of strains bearing a *temperature-sensitive lethal* allele (combined with the *white pupae* marker) were developed by means of irradiation and classical genetics linking the wild-type alleles of these genes to the male determining locus and the Y chromosome. Several of these strains (VIENNA-7 and VIENNA-8) have been thoroughly evaluated and are currently being used in mass-rearing facilities for large-scale AW-IPM programmes that include an SIT component. In addition to classical genetic approaches for the development of GSS, transgenic approaches transferring at least one non-host DNA sequence into the target genome are being explored. Transgenic insects herein are defined as insects whose genetic material has been altered in a heritable way through the techniques of genetic modification, all of which allow for the combination and/or introduction of foreign genetic material into host insect genomes in a way that does not occur naturally by mating, and/or natural recombination. It should be noted that the regulation of transgenic technology and public acceptance remains a major issue for the implementation of this technology which may be restricted by governmental or regional mandates.

2. Available sexing technologies for application: Pest control strategies that include an SIT component are currently applied against several insect species. The development of a genetic sexing system in the medfly led to a significant improvement in the cost-effectiveness and efficiency of the SIT in the field and showed that other insect pests could benefit from it. Thus, there is a widely recognized need for the development of sexing systems for SIT programs of other species. In addition, this is a prerequisite for mosquito SIT since females are the transmitting sex of major human pathogens.

2.1. Tephritids: The VIENNA-8 (and VIENNA-7) GSS of *C. capitata* carry the *white pupae* (*wp*), *slow development* (*sd*) (Porras et al., 2020) and *temperature-sensitive lethal* (*tsl*) mutations as well as a Y-autosome

translocation that includes wild-type functional copies of these genes. Via an embryonic elevation in temperature, females can be eliminated in an early stage of development (Franz et al. 2021; Augustinos et al. 2017). GSS have been developed for *B. dorsalis* (McCombs & Saul 1995; Isasawin et al., 2012) and *Z. cucurbitae* (McInnis et al., 2004) based on *white pupae* mutations. Similarly, GSS for *A. fraterculus and A. ludens* have been developed based on a *black pupae* mutation and this mutation has also been isolated in *A. obliqua* (Zepeda et al., 2014; Meza et al., 2020). These pupal colour markers have the disadvantage that females have to be reared up to the pupal stage before sexing by sorting can be achieved. Transgenic technologies have been used to develop novel sexing systems originally in *Drosophila melanogaster* as a proof-of-principle, and later in several insect pest species. One approach uses an autoregulated tetracycline-suppressible (Tet-off) transcriptional activator (tTA) as a lethal effector that was made female-specific by integration of a sex-specifically spliced gene intron from a *transformer* (*tra*) gene, resulting in female-specific lethality in the absence of dietary tetracycline (Fu et al., 2007). Similar to the colour-based marker, this system is suboptimal in that the female lethal phase is late in development. A subsequent improvement was made through the development of new Tet-off sexing strains, with lethality acting in early embryogenesis, in *C. capitata*, *A. suspensa*, *A. ludens*, *L. cuprina*, and *C. hominivorax* that also make use of a *tra* intron1 for female-specific lethality (Schetelig & Handler 2012; Ogaugwu et al., 2013; Schetelig et al., 2016; Yan & Scott 2015; Concha et al., 2016). With these systems, female progeny are eliminated during early embryogenesis and most of these systems do not require high dietary concentrations of tetracycline during mass rearing. In addition, a Cctra-RNAi transgenic sex-reversion line of *C. capitata* that shows 95% conversion of XX individuals into fertile XX males (with 50% non-transgenic), with 5% intersexes was generated showing a masculinizing maternal effect (Saccone et al., 2007) Alternatively, the positive *tra* autoloop can be targeted in the female germline to generate ¨arrhenogenic¨ females, which produce only-male progeny. Many higher dipterans depend on maternal provision of *tra* to engage the *tra* autoloop in the zygote. Depletion of maternal *tra* will prevent zygotic activation of *tra* and male development will follow. Prezygotic repression of maternal *tra* has been observed and documented in the housefly *M. domestica*. For instance, the *Ag* mutation in *M. domestica* acts as a dominant *tra* loop breaker in the female germ line and gives rise to male only progeny (Hediger et al., 2010). Also, transplantations of M carrying germ cells into a female host will give rise to only-male progeny demonstrating that expression of M in the female germline can be used to pre-zygotically block the female promoting activities of *tra* (Dübendorfer and Hediger, 1998 doi: 10.1093/genetics/150.1.221.). In other tephritid species like *A. suspensa*, the transient dsRNA knock-out of *tra* and *tra2* did lead to 98% female sex-reversion, but while those XX males had the advantage of being sterile, their *dstra-2* treated XY siblings were fertile (Schetelig et al., 2012). Thus far *tra-2* function is required for male fertility in XY drosophilids, including the *D. suzukii* pest species, but not in tephritid species that would require an independent male sterility system when *tra-2* suppression is used for sexing.

2.2. Mosquitoes: Female mosquitoes are solely responsible for biting humans and transmitting pathogens, therefore, they must not be released by SIT programs, since they could contribute to local disease transmission (Kojin et al., 2022). This places unique constraints on any efforts to optimize SIT for mosquitoes, as GSS would significantly contribute to the elimination of females, ideally at any early developmental stage. The first mosquito GSS were developed in the 1970s, using classical genetic approaches involving mutagenesis and chromosomal translocations. These strains relied on the use of insecticide resistance genes which were translocated to the Y chromosome, linking resistance exclusively to males. Using this approach *Anopheles albimanus*, *An. arabiensis* and *An. gambiae* GSS based on dieldrin resistance were developed in the 1970-80s (Kaiser, 1978, Robinson, 1986, Curtis. 1978, Lines, 1985). However, these strains were eventually deemed unsuitable because of high genetic instability, and they are no longer available. An *An. arabiensis* GSS was developed based on dieldrin resistance by IPCL colleagues, but due to low fertility and concerns over dieldrin residues in adult males and subsequent environmental bioaccumulation, the strain was considered not suitable for field use (Yamada, 2012, 2015). Similarly, one of the first GSS was constructed in *Ae. albopictus* by linking *rdlR* gene, conferring resistance to dieldrin, to maleness (Dandalo et al., 2018; Lebon et al., 2018). This strain, together with a second strain obtained using the same approach (Tortosa, personal communication) allow producing >98% of males following dieldrin treatment of larvae and are easily maintained in the insectary. Promising research results and technologies have been reported: (a) A first generation GSS has been developed for *Ae. aegypti* in IPCL using classical genetic approaches; (b) Sorting of fluorescent larvae: sex-specifically marked larvae can be sorted by a COPAS sorting machine (Catteruccia, 2005; Lutrat et al., 2022; Ntoyi et al., 2022); (c) Female lethality system acting in late larval/pupal stage called ´female-specific RIDL´ (fsRIDL) (Fu

et al., 2007); (d) Sex distortion: A "sex-ratio distortion" approach was developed for *Anopheles gambiae*, which destroys X-bearing sperm that resulted in 95-97% male progeny (Galizi et al., 2014) and (e) Sex conversion: Sex conversion approaches have been successfully developed in mosquitoes (Aryan et al., 2020; Lutrat et al., 2022; Zhao et al., 2022). Approaches (d) and (e) are, in theory, more efficient than female lethality, as they could double the total number of male progeny produced per parental population, by replacing females with converted males. Recently, the targeting of the *A. gambiae* femaleless (*fle*) gene, related to Tra2 splicing factor, led to masculinization and death of genotypic females showing its involvement in female sex determination and dosage compensation (Krzywinska, et al., 2021). The authors showed that *fle* is an evolutionary conserved gene in anophelines and proposed that it could be used as a "universal molecule", could be targeted in genetic control to eliminate females of all malaria vector species.

2.3. Lepidoptera: The available sexing mechanisms developed for Lepidoptera have been based either on the construction of balanced lethal (BL) strains or W-linked selectable markers. Unfortunately, the use of BL strains for genetic sexing is not easily applicable under mass rearing conditions. Suitable W-linked markers are only available for *Bombyx mori* (Marec and Vreysen, 2019). A GSS with a W-linked dominant conditional lethal mutation (DCLM) would permit the maintenance of both sexes under permissive conditions and the elimination of the female moths under restrictive conditions. However, to date, no DCLM has been identified in Lepidoptera. Alternatively, modern biotechnology methods could be used to introduce a DCLM into the W chromosome. An advantage of this approach is that only female progeny will have the transgene, but not the released males, which will have a fully wild-type genome (Marec et al., 2005). Recently, successful insertion of the DsRed fluorescent marker into the W chromosome of *B. mori* has been achieved using piggy*Bac*-mediated transgenesis, demonstrating that this approach is feasible (Ye et al., 2023). Using another strategy, transgenic sexing strains of *B. mori* and pink bollworm have been made that overexpress tTA in females when raised in the absence of tetracycline in the diet. Sex-specific expression was achieved by using the splicing signals from the pink bollworm *doublesex* (*dsx)* gene (Jin et al., 2013; Tan et al., 2013).

3. Sex determination: Knowledge on sex determination pathways of the SIT-targeted insect species can be very useful for the construction of a GSS (Saccone, 2022). Sex determination is well characterised in *Drosophila melanogaster*, in which two doses of a set of X-linked transcriptional regulators activate the master gene *Sex-lethal* (*Sxl*), which determines the female fate in XX embryos and represses dosage compensation. In XY embryos (males), which contain only one dose of X-linked transcriptional activators, *Sxl* remains inactive. In *Ceratitis capitata*, the *tra/tra-2 > dsx/fru* module of this sex determination pathway is conserved at the structural and functional level (Pane et al., 2002; Bopp et al., 2014). However, the *Sxl* homolog in *C. capitata* (*CcSxl*) is not acting as the upstream regulator of the *tra* homolog (*Cctra*) (Saccone et al., 1998; Zhang et al., 2014). Instead, activation of *Cctra* functional gene products require the presence of maternal *Cctra*, that acts together with the *Cctra-2* gene product (Salvemini et al., 2009), to maintain the epigenetic autoregulatory function resulting in female sexual differentiation. When *Cctra* female activation is prevented by the male determiner (M factor), or artificially by dsRNA, male sex determination and male differentiation results (Pane et al., 2002). This mode of *tra* autoregulation and its embryonic RNAi sensitivity appears to be widely conserved in many other Tephritidae, including *Bactrocera oleae*, *B. tryoni*, *B. jarvisi*, *B. dorsalis*, *B. correcta*, *Anastrepha suspensa*, the Calliphoridae, *Lucilia cuprina*, and the Muscidae, *Musca domestica* (Hediger et al., 2010; Sanchez, 2008; Nagaraju J, and Saccone G., 2010, Laohakieat et al., 2016, 2020; Saccone, 2022). Inactive Cas9 targeting Medfly *tra* without inducing a mutation led to full masculinization indicating that transient repression of *Cctra* transcription during early embryogenesis also affects the establishment of the female-determining *Cctra* autoregulatory loop (Primo et al., 2020). In *L. cuprina*, knock-down of *tra* by Cas9 also interferes with dosage compensation, an effect that could be used for female killing (Williamson et al., 2021).

One of the several M factors present in wild populations of *M. domestica*, *Mdmd*, has been isolated (Sharma et al., 2017). The *Mdmd* gene originated from a duplication of a highly conserved autosomal gene, CWC22, encoding a spliceosome protein, suggesting that *Mdmd* has a direct role in repressing female-specific *Mdtra* splicing. The male-determining factor in *C. capitata* has been previously mapped on the long arm of the Y chromosome (Willhoeft and Franz, 1996). The M factor has been molecularly isolated in the Medfly, named *Maleness-on-the-Y* (*MoY*) and found to induce male-specific splicing of *Cctra* within hours during embryogenesis of XY (Meccariello et al., 2019). *MoY* orthologues are widely conserved in Tephritidae species of *Bactrocera* and *Zeugodacus* genera and shown to be functionally conserved in the olive fruit fly, *B. oleae*,

and the oriental fruit fly, *B. dorsalis*. Recently, injections of *MoY* gene in *M. domestica* XX embryos induced male-specific splicing of *Mdtra* gene, indicating the Medfly gene can masculinize even non-Tephritidae species having different Maleness factors. Hence, *MoY* is a promising tool for a tephritid generic transgenic strain sexing by masculinization of XX individuals (Meccariello et al., 2019).

A novel molecular mechanism for male sex determination was revealed in *B. dorsalis* (Peng et al., 2020). An autosomal miRNA, miR-1-3p, showed XY-biased expression in early embryos, and targeting *Bdtra* mRNA led to its male-specific splicing pattern and male sex determination. The male-biased expression of miR-1-3p is likely to be under the direct or indirect control of *BdMoY*. RNA interference as a natural sex determination mechanism has been described only in lepidopteran species (Kiuchi et al., 2014), but it is a novelty for dipteran species and its potential evolutionary conservation should be explored.

Genetic evidence suggests that a Y-chromosome or M-locus linked *M* factor initiates male development in *Anopheles* and *Aedes* mosquitoes, respectively (Gilchrist and Haldane, 1947, Baker and Sakai, 1979). Recent studies isolated the male-determining factor *Yob*, encoding a novel short protein, in *An. gambiae* (Krzywinska et al., 2016), *Guy1* in *An. stephensi* (Criscione et al., 2013, 2016; Qi et al., 2019), and the primary sex-determiner *Nix* in *Ae. aegypti and Ae. albopictus* (Hall et al., 2015, Gomulski et al., 2018, Lutrat et al., 2022), and a putative male-determining gene, *Nix*, in *Ae. albopictus* (Lutrat et al., 2022). *AsuMf*, a male-specific duplication of an autosomal splicing factor in *Armigeres subalbatus*, is required for male development (Liu et al., 2023). In these mosquito species, downstream genes such as *dsx* and *fruitless* (*fru)* have been identified and are regulated by a partially conserved sex-specific alternative splicing mechanism (Scali et al., 2005; Gailey et al., 2006; Salvemini et al., 2011; Salvemini et al., 2013). At the same time, a *transformer* homolog in both species is either absent or remains to be identified. No genetic information is available for the upstream splicing regulators of the *dsx* and *fru* genes, controlled in males by the primary signals *Yob* and *Nix*.

In Lepidoptera, the chromosomal mechanism of sex determination is the heterogametic WZ type. It was shown by Kiuchi et al. (2014) that the feminizing factor in *B. mori* is a W-encoded small PIWI-interacting RNA named *Fem* piRNA. The authors also showed that the *Fem* piRNA down-regulates the expression of a Z-linked gene, *Masculinizer* (*Masc*), which promotes male development in the absence of a W chromosome. The *Fem* piRNA therefore controls female-specific splicing of the *B. mori doublesex* (*Bmdsx*) gene by down-regulating expression of the *Masc* gene (Kiuchi et al., 2014). In addition to the sex-determining function, *Masc* also induces dosage compensation (Tomihara et al., 2022). Several recent studies suggest that the role of *Masc* is conserved in Lepidoptera sex determination (Lee et al., 2015; Fukui et al., 2018; Wang et al., 2019; Harvey-Samuel et al., 2020; Deng et al., 2021; Visser et al., 2021). However, it is not yet known whether the Fem piRNA-*Masc* sex-determining pathway is conserved in other lepidopteran species having WZ sex determination. For example, deep sequencing analysis identified no female-specific small RNA that mapped onto the *Masc* mRNA in *Ostrinia furnacalis* (Fukui et al., 2023). Recently, a W-linked locus that is a source of small silencing RNAs targeting the *Masc* sequence has been identified in *P. xylostella* (Harvey-Samuel et al., 2022), suggesting convergent evolution of a *Fem*/*Masc* pathway in this phylogenetically distant species. On the other hand, the presence or absence of the W chromosome plays no role in sex determination of wild silkmoths (*Samia cynthia* ssp.), and their sexual development depends on the Z:A (A = autosome) ratio (Yoshido and Marec, 2023).

In Hymenoptera, the core *tra*-splicing-autoloop is started by very different means, such as heterozygosity at a complementary sex determining locus in *Apis mellifera* (Gempe et al., 2009) or a transcriptional activator with a parent-of-origin effect in *Nasonia vitripennis* (Zou et al., 2020). The insect sex determination pathway based on sex-specific splicing of *tra* and *dsx* seems ancestral to the Holometabola (Wexler et al., 2019), whereby *tra* serves as transducer of the primary signal and *dsx* as executer for sex determination. However, the primary signal differs widely (Hopkins and Kopp, 2021).

Wexler et al. (2019) isolated *dsx* orthologues in species belonging to three hemimetabolous insect orders, including Hemiptera and Blattodea, and found *dsx* sex-specific splicing regulation in two of them. Interestingly, these orders include major pests such as *Blattella germanica* (L.), and the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae), which negatively affect several vegetable crops that are grown in greenhouses. Furthermore, hemipteran species, such as the brown marmorated stink bug *Halyomorpha halys*, a public health pest worldwide, and the green vegetable bug *Nezara viridula*, have been considered as potential candidates for

SIT (Bourtzis and Vreysen, 2021).

Recent advances in the Hemiptera genetics of sex determination revealed that nymphal RNAi of the brown planthopper (BPH) *Nilaparvata lugens* - one of the most devastating rice pests in many Asian countries - *tra2* orthologue (*Nltra2*) led to a phenotypic sexual reversal of females into sterile males likely by controlling *Nldsx* splicing (Zhuo et al., 2017; idem, 2018). Zhuo et al. (2021) identified two novel splicing regulators of *Nldsx* in this species, named *Nlfmd* and *Nlfmd2* (female determinant factors), belonging to the hnRNP40 family gene. interestingly, *Nlfmd* produces female-specific and non-sex-specific isoforms via alternative splicing by exon skipping. Female-specific NlFmd is 613 aa long, and the non-sex-specific form is shorter (449 aa). *NlFmd* shows very low similarity to the hymenopteran protein *A. mellifera* Feminizer (AmFem) over a very short region, conserved among *AmFem* and *CcTra* (Hasselmann et al., 2008). Knockdown of *Nlfmd* in female nymphs resulted in masculinization of somatic morphology and *dsx* splicing. The female-specific isoform of *Nlfmd*, *Nlfmd-F*, is maternally deposited and zygotically transcribed. Depletion of *Nlfmd* by maternal RNAi or CRISPR-Cas9 resulted in female-specific embryonic lethality. Knockdown of *Nlfmd2*, also conferred masculinization. Like TRA, the NlFMD protein is an SR-rich protein that does not have a predicted RNA binding domain. The authors suggested that LlTRA2 interacts with NlFMD/TRA like in *Drosophila melanogaster* and possibly other dipteran species.

4. Recent developments:

4.1. The applications of 'big data' for molecular genetics: The community can overcome major bottlenecks in research by the application of next-generation sequencing (NGS) technologies to genetic problems. The available technologies have a number of applications that range from whole genome sequencing to gene expression analysis. Currently, there are two main sequencing platform types (a) "Short read" (50-500 bp) sequencers e.g. Illumina and Ion torrent, and (b) "long read" (>5 Kb up to 2 Mb) e.g. PacBio and Oxford Nanopore. There are also a number of applications that leverage the high throughput of the Illumina machines to provide long pseudo-reads of up to 150 Kb, e.g. Chromium 10X Genomics, and genome scaffolding e.g. Chromium 10X and HiC. We now have the capability of rapidly obtaining such whole genome sequences from a species, a strain, and even a single individual. In addition, using a series of tools, we have shown that we have the potential to improve assemblies by integrating linked read and long read data. We are also able to generate haplotype-specific assemblies for diploid species using these technologies. Another important development is the application of long-read technologies (Nanopore and PacBio) for transcriptome sequencing and assembly, which can be used to enrich genome annotation efforts. Taken together, these technologies with bioinformatic analysis allow us to produce a wealth of ancillary data that play an increasingly prominent role in the identification of target (marker) genes, including their mode of regulation. An example of these recent developments, both in terms of sequencing chemistry and in its bioinformatic analysis, has been the discovery and subsequent characterization of Y-chromosome sequences, including Y-linked M factors in mosquitoes, *Ceratitis capitata* (*MoY*; Meccariello et al., 2019), *Bactrocera oleae* (Bayega et al., 2020) and in *Musca domestica* (*Mdmd*; Sharma et al., 2017). These tools in combination with methods described below for genome manipulation, have made it possible to build novel types of GSS in any species targeted. Furthermore, they are currently employed to molecularly identify the loci responsible for many of the GSS-based mutations described above. In addition, a marker-assisted mapping approach was successfully used to help identify the causal genes of both dominant and recessive phenotypes in *Aedes aegypti*, overcoming the significant challenge posed by vast recombination deserts in this species (Chen et al., 2022). Therefore, access to such tools will likely underpin a new type of capability that will greatly enhance the toolkit available to the SIT community (Papanicolaou et al., 2016; Matthews et al., 2018; Turner et al., 2017; Van't Hof et al., 2016; International Glossina Genome, 2014).

4.2. New era in cytogenetics and chromosome manipulation: In the era of NGS, laser microdissection seems to be a particularly useful tool for preparation of sex chromosome-specific DNA libraries. In insects, this technique was first demonstrated in the codling moth, where it was used for the development of W-chromosome painting probes and for obtaining first sequence information on the composition of this heterochromatic chromosome (Fuková et al., 2007). Using laser microdissection, highly specific X- and Y-chromosome-painting probes were prepared and used for cytogenetic research in the olive fly, *Bactrocera oleae* (Drosopoulou et al., 2012). In the

flour moth (*Ephestia kuehniella*), high-throughput sequencing of laser microdissected sex-chromatin bodies provided the first complex information about the DNA composition of the lepidopteran W chromosome (Traut et al., 2013). Especially in tephritid fruit flies, GSS constructed using classical genetics carry a translocation of an autosomal segment on the Y chromosome and sometimes an inversion that was introduced to reduce recombination. Cytogenetic methods were used to determine the origin and size of the translocated segment, localize translocation breakpoints or map the extent of inversions, which is critical for the stability and fitness of the strains (Franz, 2002). The identification of breakpoints and delimitation of inversions was facilitated by polytene chromosome maps available in most tephritid pests (Stratikopoulos et al., 2008; Drosopoulou et al., 2014). In Lepidoptera with small and numerous holokinetic chromosomes, specific patterns of longer meiotic bivalents in pachytene allowed the identification of sex chromosomes and characterization of radiation-induced chromosome rearrangements (Traut et al., 2007). Cytogenetic research has been greatly accelerated using advanced tools of molecular cytogenetics that are currently available for detailed analysis of insect chromosomes. Various modifications of fluorescence *in situ* hybridization (FISH), such as FISH mapping of repetitive sequences and multigene families (e.g. rDNA and histone genes), genomic *in situ* hybridization (GISH) and comparative genomic hybridization (CGH) were used for the identification of sex-determining regions to which selectable markers should be linked and for the characterization of DNA content of the Y or W chromosomes (Willhoeft and Franz, 1996; Willhoeft et al., 1998; Fuková et al., 2005), which was relevant to the GSS stability and provided useful data in species with poorly understood karyotypes (Nguyen et al., 2013; Šíchová et al., 2013). Recent advances in insect genomics has led to the development of new molecular cytogenetic methods required for the construction of high-resolution physical maps, such as BAC-FISH (FISH with bacterial artificial chromosomes as probes) and TSA-FISH (FISH with tyramide signal amplification), which represent an important framework for improving the quality of genome assembly, annotation, and analysis (Nguyen et al., 2013; Carabajal Paladino et al., 2014; Yoshido et al., 2014).

4.3. Genome editing - new tools for modifying genotypes: Genome editing allows the precise modification of genomic DNA sequences *in vivo* and can be achieved using three available technologies – Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Nucleases (TALENs), and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR). These technologies can be used to induce DNA double-strand breaks (DSBs) at predetermined target locations in the genome. In the case of ZFNs and TALENs, DNA endonuclease domains are attached to proteins whose amino acid sequences have been designed to bind to specific sequences. In the case of CRISPR, the Cas9 protein (or equivalent ones like CPF1) is directed to cut predetermined target locations in the genome by providing it with single-guide-RNAs. Double-stranded breaks in genomic DNA can be repaired either by non-homologous end-joining (NHEJ), resulting in possible disruption of the target sequence through insertion or deletion of nucleotides (creating knockout lines; Meccariello et al., 2017) or by homology-directed repair (HDR) that can be used to insert DNA sequences at the target locus via homologous recombination (HR) producing knock-in lines (Li and Handler, 2017; Aumann et al., 2018; KaramiNejadRanjbar et al., 2018). Directed knock-in lines can also be produced by NHEJ (Farnworth et al., 2020). RNA-guided *piggyBac* transposition, which has been successfully employed in human cells (Hew et al., 2019; Rezazade Bazaz et al., 2022), could also be trialled. Gene editing technologies provide precise mutagenesis capabilities which were previously limited to nonspecific chemical agents - (e.g. EMS) or radiation-based (e.g. X-rays) methods for the creation of insect genotypes needed for effective GSS (e.g. VIENNA-8). CRISPR/Cas9 technology has been limited so far to arthropod species for which an embryonic microinjection protocol exists. However, a recent technical improvement called the ReMOT system (Chaverra-Rodriguez et al. 2018) promises to overcome this technical limitation, allowing GSS development for species for which *in vivo* reverse genetics tools are still not available, for example, the tsetse fly. Chemical- and radiation-based mutagenesis must be coupled to large genetic screens designed to detect and recover the desired genetic alterations, and the 'classical' approaches, while demonstrably effective in some cases, depend on chance occurrences of the mutations or chromosomal rearrangements of interest and can require many person-years of effort to produce desired genotypes. The high precision and accuracy of gene editing technologies enable the creation and assembly of genotypes identical to those created and assembled using 'classical' mutagenesis and genetic approaches, but crucially, without necessarily requiring large genetic screens or many person-years of effort. This is a clear benefit of using genome editing technologies for the creation of GSS. Because the organisms produced using gene-editing technologies can be genetically identical to those produced using 'classical' approaches, their transition from the laboratory to the field and adoption by end-users could follow current technology transfer strategies for non-transgenic organisms, another potential benefit of using gene-editing technologies.

4.4. New developments on RNAi for pest control: RNAi is a powerful tool for experimental studies that aim to determine gene function. This commonly involves the microinjection of dsRNA into the target organism. However, administration through feeding is also possible. The dsRNA is cut by endogenous Dicer proteins into a population of small interfering RNAs (siRNAs), which in turn bind and degrade complementary mRNA sequences. In plants and some invertebrates (e.g. *C. elegans*), the efficacy of RNAi is improved through a combination of signal amplification and systemic spread, such that the entry of one dsRNA or siRNA molecule into a single cell can lead to effective silencing of the target gene throughout the target organism. In most insects, RNAi appears to be cell-autonomous, with no amplification or cell to cell transfer of the gene silencing signal. The lack of a mechanism for amplification and systemic spread of a dsRNA signal in fruit flies and mosquitoes has implications for the development of RNAi as a control tool for insect pests. To achieve effective control, dsRNA/siRNA delivered through the environment (environmental RNAi, eRNAi; Ivashuta et al., 2015) of the pest must somehow be delivered to the appropriate tissue in the target pest at a sufficient dose to produce the necessary level of gene silencing to achieve the desired objective, usually mortality. There is a considerable variation across insect species in their sensitivity to eRNAi, and the evidence to date suggests that this is largely due to the relative uptake, stability and transport efficiency of dsRNA or siRNA among insects (Ivashuta et al., 2015; Mamta and Rajam, 2017). The effectiveness of eRNAi could be improved by technologies that provide (a) more effective transport across the integument (cuticle or gut), (b) greater protection against degradation by UV and enzymes, and/or (c) active transport to the target tissues. Microorganisms constitute one of methods for dsRNA delivery in insects. This system was initially utilized in *C. elegans* (Timmons and Fire 1998; Timmons et al., 2001) but has since been extensively applied to insects as well. Viruses are extremely efficient at delivering nucleic acid material into the intracellular environment; however, the *in vivo* application by viruses has not been widely investigated yet, probably due to the many safety issues that accompany their delivery (Kolliopoulou et al., 2017). Nanoparticles have also been used to increase stability and oral uptake efficiency of dsRNA in mosquitoes (Zhang et al., 2010). Liposomes have also been used as a means to protect nucleic acids in aqueous environments and they were initially tested in various drosophilid species (Whyard et al., 2009). Carrier proteins (Cell-Penetrating Peptides, CPPs) have also been used as delivery systems for dsRNA and have shown to facilitate uptake of dsRNA in the insect gut (Gillet et al., 2017). Furthermore, chemical modifications of siRNAs were shown to improve stability and uptake of these molecules (Joga et al., 2016). Lastly, potato chloroplasts have also been genetically engineered to produce dsRNA, leading to 100% RNAi-induced mortality of Colorado potato beetles that were fed on the modified leaves (Zhang et al., 2015). Given these recent developments, it is conceivable that eRNAi can potentially be used to achieve genetic sexing as part of SIT programs by targeting female-specific transcripts during the developmental stages of the generation to be released (Whyard et al., 2015). Alternatively, eRNAi targeting non-sex specific genes could be useful, if combined with an insect strain expressing male-specifically a recoded, eRNAi insensitive target. This application of eRNAi offers a greater level of control of delivery compared to other eRNAi applications (such as eRNAi pesticides), but unlike these applications it demands near 100% efficacy.

5. Genetic Sexing Strains for SIT applications - validation in the laboratory:

Developing large-scale operational SIT programs, regardless of the target species, depends on solving several common problems. A major problem is the development of suitable methods, ideally genetic sexing strains that will enable the production of large numbers of male insects in mass-rearing facilities. Despite tangible benefits, a 'generic' approach for the development of GSS, one that can be easily transferred to diverse insect species, is not available. The possibility and feasibility of developing such an approach should be the focus of research activities. There are at least two generic strategies that are currently being considered for developing a GSS: 1) the creation of strains that display conditional, female-specific lethal phenotypes, and 2) strains in which the sex determination pathway itself can be conditionally manipulated leading to sex conversion (female to male), or combinations thereof. There are many approaches that have been or could be applied to successfully implement these strategies. Of particular interest are those that are the most widely applicable with respect to the number of target species to which the solution could be implemented with a minimum of research and development efforts. Importantly, the extent of the cross-species transferability of each system will need to be investigated, because gene functions may not be conserved between species, among other things like chromosomal complement. For example, it may be possible to transfer sex determination-based GSS components among tephritid species but not to mosquitoes. Or overexpression a sex determination gene may result in sex conversion in one species but be lethal in another - results that are unknowable a priori. In most cases however, these

'generic' approaches for the development of GSS would reduce research and development time and costs, allowing SIT programs to be more readily developed and implemented.

Approach 1: Exploiting induced or spontaneous mutations and chromosomal rearrangements.
Genetic sexing strains that show conditional sex-specific lethality or sex reversal have been successfully developed using several approaches. The existing Medfly GSS, VIENNA-7 and VIENNA-8, were created by chemical/radiation-induced mutagenesis resulting in strains exhibiting female-specific heat-inducible lethality resulting in male-only survival to adulthood. Females are, therefore, easily eliminated, by submerging bisexual early embryo collections in waterbaths set at 34°C. In other tephritid species, for example, *A. ludens*, selection for spontaneous mutations were exploited for the construction of GSS. Approaches involving mutagenesis and chromosome rearrangement are referred to here as 'classical genetic' approaches. In the Medfly, this approach resulted in highly effective GSS; however, it took many years to develop these strains and recapitulating these efforts in other species using the same 'classical genetic' approaches may not be practical. A novel molecular approach is using transposon-based insertional mutagenesis that creates mutations by vector insertions, thereby 'tagging' mutations that have been selected by a visible or biochemical screen. This allows the straightforward isolation, sequence analysis, and genome mapping of the mutated gene for further use in sex-specific selection, and identification of conserved orthologous genes in other species. This approach also eliminates unintended genomic disruption by chemical or irradiation mutagenesis, and eliminates the need for chromosomal translocations since wild-type alleles can more simply be transposed onto Y-chromosomes for male selection. Temperature-sensitive alleles (e.g. transformer-2-ts2) have also been used to generate a population of CRISPR-mutants in D. suzukii that, when reared at non-permissive temperatures, result in XY males and XX intersexes that are sterile (Li & Handler 2017). These males are capable of mating, while the intersexes neither mate nor oviposit.

Approach 2: CRISPR-induced mutagenesis.
New gene-editing technologies, such as the CRISPR system, will enable the precise and rapid recreation of genetic sexing genotypes. For example, temperature-sensitive lethal alleles, made previously using classical approaches in the VIENNA-7 and VIENNA-8 lines, can now be rationally designed, provided the genetic basis of the phenotype is understood. Furthermore, wild-type rescue alleles can be linked directly to Y chromosomes or M-loci using CRISPR to induce homologous recombination or large chromosomal rearrangements. This strategy, which we call 'neo-classical', essentially replicates the 'classical' genetic efforts and does not necessarily include the introduction of foreign DNA, although initial efforts may include such sequences to aid in strain isolation and validation, for example, using fluorescent markers, which can be omitted in the future. The success of this approach will depend on the identification of genes underlying suitable selectable traits in target species, how generic specific alleles of these genes will be, and to what extent they are applicable across species targeted for GSS strain development. Its feasibility has now been successfully demonstrated by members of this RCM through the identification of the *white pupae* gene in Medfly, *B. dorsalis* and *Z. cucurbitae*, the creation of a novel *white pupae* phenotype strain in *B. tryoni* (Ward et al., 2021) and the successful generation of mini white pupae rescue constructs that completely recover the mutant background. Conditional lethal mutations, including several *tsl* genes, have now been identified in the Medfly as reported from partners in RCM3 to enable sexing at the embryonic stage. These tsl genes are highly conserved in all target insects, so the next step is to induce identical mutations in tsl orthologs in these target species and evaluate how generic the transfer of these specific alleles will be. Additionally, rational engineering of conserved *tsl* genes, including those discovered in model species like Drosophila (*shibire*) or yeast (ubiquitin-conjugating enzymes) are being evaluated by partners as another direction to generate GSS with promising results presented in RCM3.

Approach 3: Oral delivery of sex-specific lethal dsRNAs.
Conditional sex-specific lethality can also be achieved through the transient manipulation of gene expression using orally delivered double-stranded RNA (dsRNA) that induces the silencing of sex-specific genes or sex-specific isoforms of genes (RNAi) leading to lethality. Recent work has shown that diet-mediated delivery of dsRNA designed to specifically silence the expression of the female isoform of *doublesex* (*dsx*) in larvae of *Aedes aegypti* results in sex-specific lethality of female larvae (Whyard et al., 2015). This is the first time that sex-specific lethality has been linked to Dsx function, and as such further investigation is needed to validate the approach for transfer to other SIT target species. This approach is potentially generic assuming that all insects

have an RNAi system and the *dsx* gene is expected to be present and to have the same role in sex determination in all targeted insect species. This would make it a good target for gene silencing. A notable advantage of this approach is that a specially designed GSS may not be required. Diet-mediated delivery of dsRNA could also be a widely applicable mode of delivery although the sensitivity of insects to orally delivered dsRNA is variable (Darrington et al., 2017). Unfortunately, efforts to replicate experimental sex-specific lethality in mosquitoes have failed in most labs that have attempted to implement orally delivered dsRNA-based sex separation. Attempts from different labs to reproduce already published RNAi-based lethal effects lead by silencing of essential genes to *Aedes* mosquitoes through oral delivery of bacterially produced dsRNA, soaking and microinjections of different larval stages have also not been successful. The reasons for this remain unclear and are thought to include low oral uptake of the dsRNA, issues in delivery of the dsRNAs in the relevant cells/tissues, and ultimately the production of high quality/amount of dsRNAs. Although some partners elected to include this approach at the beginning of this CRP program, results and lack of progress have led all groups to abandon this approach for the time being.

Approach 4: Sex-specific splicing factors and effectors.

Genetic sexing strains of a number of tephritid species with genotypes resulting in conditional sex-specific lethality have been successfully created using transgenic technologies. These transgenic approaches are fairly generic in that they rely on sex-specific splicing found in common sex determination genes and effector genes involved in conserved cell-death pathways. While some of these functional elements are known to be functional between species, it is expected that for most species these specific functional elements will need to be re-isolated and assembled. While orthologous genes and regulatory sequences might be found in more distantly related species, identifying, isolating, assembling and integrating new transgenes into new species may be difficult and time-consuming. Nevertheless, conserved elements of the sex-determining splicing-cascade, such as *tra* or *tra-2*, can be targeted to generate artificially designed "M factors" by RNA interference and dCas9 mediated gene knock down (Pane et al. 2002; Primo et al., 2020) or CRIPSR/Cas9 mediated mutagenesis (Aumann et al., 2020; Williamson et al., 2021), which could be made conditional by controlled activation based on site-specific recombination or food supplement-controlled binary expression systems (Eckermann et al., 2014).

Moreover, tetracycline-suppressible female-specific embryonic lethality systems have been shown to be highly efficient in producing male-only populations in *A. ludens*, *A. suspensa*, *Ceratitis capitata*, *L. cuprina*, and *C. hominivorax* using a female-specifically spliced intron from *tra* (Schetelig and Handler, 2012; Ogaugwu et al., 2013; Schetelig et al., 2016; Yan & Scott 2015; Concha et al., 2016). In addition, a highly conserved dominant temperature-sensitive (DTS) mutant allele of a proteasome 20S subunit gene was created and transformed into *A. suspensa*. This resulted in transgenic lines exhibiting 96-100% pupal lethality when reared at 30^{o}C (Nirmala et al., 2009). This conditional lethal mutation can be created for a wide variety of insect species and made female-specific using *tra* intron1-splicing for female-lethality at elevated temperatures. In addition, conditional expression of identified M factors serving as primary signals for suppressing the conserved female-determining *tra* splicing positive autoloop could be tried to establish sexing strains.

Approach 5: Altering expression of sex-determining factors.

The genetics of sex determination is not well characterized in most insect species. However, existing data show that the *doublesex* (*dsx*) and *fruitless* (*fru*) genes play a common role in determining whether an organism develops into a male or a female. It has been shown that silencing the female form of the *dsx* in the larval stage results in female lethality in *Aedes aegypti* (Whyard et al., 2015), and CRISPR/Cas9-based knockout of *dsx* can lead to female lethality that can be used for population suppression in a gene drive approach (Kyrou et al., 2018). Considering the conservation of *dsx* and *fru*, this approach may be generic (Salvemini et al., 2011, 2013). Conditional sex conversion could result in twice the number of male progeny by converting females into males (Saccone et al., 2011). Full masculinization was also achieved in the medfly by transient ectopic expression of *MoY* or injection of MOY recombinant protein in XX embryos (Meccariello et al., 2019). Embryonic transient Cas9 interference of Medfly *tra* (without inducing mutation) is an alternative way to induce full masculinization of XX individuals (Primo et al., 2020). If sex conversion is the goal, manipulation of genes upstream in the sex-determination pathway, either a male determining factor or a *transformer*-like transducer gene, would be needed. One approach is the creation of temperature-sensitive mutant alleles of *transformer-2* using CRISPR/Cas9, such as the *Drosophila suzukii tra-2*^{ts2} allele resulting in sterile XY males and the conversion of XX females to sterile

phenotypic males at non-permissive temperatures (Li and Handler, 2017). A *tra-2*^{ts2} allele for sex conversion was similarly created by CRISPR/Cas9 in *C. capitata* (Aumann et al., 2020), and was used to create *tra-2* knock-outs resulting in XX female to phenotypic male conversion in *A. suspensa* (Li and Handler, 2019). Small RNAs also play a role in sex determination, such as the feminizing factor *Fem* piRNA that acts by suppressing *Masc* in *B. mori* (Kiuchi et al., 2014), and another, albeit converse, role was found for the miRNA-1-3p microRNA that suppresses transformer, resulting in male differentiation, in *B. dorsalis* (Peng et al., 2020). Transient manipulation of *Nix*, the male-determining factor in *Aedes aegypti* (Hall et al., 2015), resulted in partial sex conversion. Transgenic lines that ectopically express *Nix* in both *Ae. aegypti* and *Ae. albopictus* can produce fertile transformed males (Aryan et al., 2020, Lutrat el al 2022; Zhao et al., 2022). In *Ae. aegypti*, masculinized females are unable to fly because they lack a second M-locus gene myo-sex that encodes a male-specific flight muscle myosin. In *Ae. albopictus*, converted males (genetic females) are able to fly but preliminary data indicates that there might be other fitness costs associated with these males. Data presented during RCM3 highlights that masculinization by *Nix* overexpression is significantly affected by position effects. The genomic methods that led to the discovery of *Nix* (Hall et al., 2015) are relatively cost-effective and can be applied to other insect species of agricultural and medical importance. Therefore, efforts to discover male-determination factors in these species may lead to new and efficient ways to produce male-only progeny and facilitate the identification of other key regulators in the sex-determination pathway, which may provide new targets of manipulation.

6. Evaluation guidelines - Quality control of insect strains for SIT applications:

For the successful development and implementation of a SIT project, it is critical to evaluate the quality of a GSS once it is initially developed, as well as to monitor its quality before and after release. The application of quality control analysis as part of SIT programs provides valuable information to improve rearing and release practices for the control of target species populations. Evaluation of strains for use in SIT programmes should be conducted by documenting the two most important parameters: a) rearing performance (production and quality control), and b) field performance (field cage or open field). There is a wealth of available literature on this field in addition to the great experience accumulated over half a century of active SIT projects around the globe. The currently available information and experience in SIT projects to control tephritids has resulted in a manual currently used worldwide (FAO/IAEA/USDA, 2019).

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Sustainable Development Goals (SDG) which are linked to the proposal:

2. End hunger, achieve food security and improved nutrition, and promote sustainable agriculture.
3. Ensure healthy lives and promote well-being for all at all ages.

IAEA.org topic(s):

Nuclear technology and applications; Food and agriculture; Insect Pest Control; Sterile Insect Technique.

Nuclear Component

This CRP aims at the development and / or evaluation of genetic sexing strains for use in SIT programmes. The SIT relies on the use of ionizing radiation to sterilize large numbers of insects. Radiation-induced sterility provides a very high level of biosafety and can be used in combination with genetic sexing strains developed and / or evaluated in this CRP. As radiation induces random dominant mutations, there is no possibility of resistance developing to this physical process, a possibility which cannot be excluded with other methods, for example molecular-based approaches.

Participation of Agency's laboratories

The CRP needs to be supported through adaptive research and development carried out at the IPCL, FAO/IAEA Agriculture and Biotechnology Laboratories, Seibersdorf as part of Projects 2.1.4.1 and 2.1.4.3. This R&D will focus on the isolation and characterization of markers (ideally morphological and / or temperature sensitive lethal), and the evaluation of marker strains and genetic sexing strains for SIT applications developed in the frame of this CRP.

Sequencing and bioinformatic efforts

The CRP project supports the sequencing of *Anastrepha fraterculus, A. ludens, Bactrocera dorsalis, B. zonata, Ceratitis capitata*, and *Zeugodacus cucurbitae* genomes and transcriptomes for: (a) the discovery of genes suitable as selection markers for the construction of genetic sexing strains; (b) studying the volution of tephritid genomes and (c) the characterization of the Y chromosome and its evolution. In addition, RNAseq for gene expression and small RNA discovery in lepidopterans and dipterans is carried out.

Assumptions

Member States continue to recognize the benefits of developing the SIT package and other genetic and environment-friendly methods for sustainable control of insect pests of agricultural, veterinary and medical importance in AW-IPM programmes and continue to request improved technology and high-quality SIT strains in order to maximise benefit/cost projections.

The demand for area-wide integrated insect pest management approaches, including SIT and augmentative biological control as non-polluting suppression/eradication components, continues to increase, mandating expansion and improvement in cost-effectiveness of these environment-friendly, sustainable approaches.

Related TC projects

BGD5035 - Validating the Sterile Insect Technique as a Key Component of an Area-Wide Integrated Pest Management Programme Against *Aedes aegypti* in Dhaka.

BOL5023 - Fruit Fly Control in Bolivia Using Integrated Pest Management Including the Sterile Insect Technique.

BRA5061 - Using the Sterile Insect Technique to Evaluate a Local Strain in the Control of *Aedes aegypti* (Phase II).

BKF5023 - Implementing the Sterile Insect Technique to Reduce Wild Populations of *Aedes aegypti* and Tsetse.

CMR5026 - Supporting the National Fruit Fly Management Programme.

KAM5006 - Implementing Fruit Fly Surveillance and Control Using Area-wide Integrated Pest Management.

CHI5051 - Implementing Pilot Level of Sterile Insect Technique for Control of *Lobesia botrana* in Urban Areas.

CPR5026 - Applying the Sterile Insect Technique as Part of an Area-wide Integrated Pest Management Approach to Control Two Fruit Flies.

CPR5027 - Demonstrating Feasibility of the Sterile Insect Technique in the Control of the Codling Moth, *Cydia pomonella*.

CUB5021 - Demonstrating the Feasibility of the Sterile Insect Technique in the Control of Vectors and Pests.

CYP5020 - Developing a National Rapid Response Strategy for the Prevention of the Establishment of the Asian Tiger Mosquito.

DOM0006 - Building and Strengthening the National Capacities and Providing General Support in Nuclear Science and Technology.

ECU5031 - Enhancing the Application of the Sterile Insect Technique as Part of an Integrated Pest Management Approach to Maintain and Expand Fruit Fly Low Prevalence and Free Areas.

ECU5032 - Building Capacity for Mass Rearing, Sterilization and Pilot Release of *Aedes aegypti* and *Philornis downsi* Males.

ELS5015 - Integrated Management of Fruit Flies using the Sterile Insect Technique to Establish Areas of Low Prevalence of Fruit Flies.

FIJ5003 - Implementing Pesticide-Free Suppression and Management of Fruit Flies for Sustainable Fruit Production.

GUA5021 - Strengthening National Capabilities for the Control of Agricultural Pests Using Nuclear Technologies.

ISR5021 – Assisting in the Development of a Strategy to Counteract *Bactrocera zonata*.

ISR5022 - Establishing the Sterile Insect Technique Methodology for the Management of the False Codling Moth, *Thaumatotibia leucotreta*, and Enhancing Integrated Pest Management Against the Peach Fruit Fly, *Bactrocera zonata*.

LIB5014 – Supporting Control of Fruit Flies by Establishing a Low Fruit Fly Prevalence Zone.

MAR5028 - Enhancing National Capabilities on the Suppression of Aedes Albopictus in an Urban Locality Using the Sterile Insect Technique as Part of an Integrated Vector Management Strategy.

MEX5032 - Scaling up the Sterile Insect Technique to Control Dengue Vectors.

MOR5038 - Strengthening the Sterile Insect Technique.

MYA5029 - Improving Fruit Yield and Quality by Using Sterile Insect Techniques as Part of Area-Wide Integrated Pest Management of Fruit Flies in the Mandalay Region.

PLW5003 – Facilitating Sustainability and Ensuring Continuity of Area-wide Pest Management - Phase III.

POR5006 - Integrating the Sterile Insect Technique in the Control of the Invasive Vector Mosquito *Aedes albopictus.*

SAF5017 - Assessing the Sterile Insect Technique for Malaria Mosquitos - Phase III.

SEY5012 - Establishing Area-wide Integrated Pest Management by Using the Sterile Insect Technique in Combination with Other Control Methods on the Suppression of the Melon Fly.

SUD5038 - Implementing the Sterile Insect Technique for Integrated Control of *Anopheles arabiensis*, Phase II.

TUR5026 - Conducting a Pilot Program on Integrated Management of *Aedes aegypti* Including Sterile Insect Technique.

TUR5027 - Implementation of SIT for Suppression and Eradication of Medfly in Turkey.

URT5035 - Implementing the Sterile Insect Technique as Part of Area-wide Integrated Pest Management for Controlling Invasive Fruit Fly Populations.

RAF5074 - Enhancing Capacity for Detection, Surveillance and Suppression of Exotic and Established Fruit Fly Species through Integration of Sterile Insect Technique with Other Suppression Methods.

RAS5082 - Managing and Controlling Aedes Vector Populations Using the Sterile Insect Technique.

RAS5086 - Assessing the Efficiency of the Sterile Insect Technique for the Control of the Cocoa Pod Borer.

RAS5090 - Advancing and Expanding Area-wide Integrated Management of Invasive Pests, Using Innovative Methodologies Including Atomic Energy Tools.

RAS5095 - Enhancing the Capacity and the Utilization of the Sterile Insect Technique for Aedes Mosquito Control.

RAS5096 - Strengthening and Harmonizing Surveillance and Suppression of Fruit Flies.

RER5026 - Enhancing the Capacity to Integrate Sterile Insect Technique in the Effective Management of Invasive Aedes Mosquitoes.

RLA5082 - Strengthening Food Security through Efficient Pest Management Schemes Implementing the Sterile Insect Technique as a Control Method.

RLA5083 - Enhancing Capacity for the Use of the Sterile Insect Technique as a Component of Mosquito Control Programs.

RLA5084 - Developing Human Resources and Building Capacity of Member States in the Application of Nuclear Technology to Agriculture.

RLA5087 - Validating the Sterile Insect Technique for the Control of the South American Fruit Fly (ARCAL).

LFM-Logical Framework Matrix Input:

Overall Objectives:

The main objective of this CRP is the development and evaluation of generic approaches for the construction of genetic sexing strains (GSS) to be used for sterile insect technique (SIT) applications, as part of AW-IPM programs, to control populations of agricultural pests and disease vectors.

Specific Objectives:

1) To develop generic strategies for the construction of GSS for SIT applications
2) To assess the efficiency, applicability and the range of the species transferability of the generic approaches
3) To evaluate, at small scale, GSS developed through the generic approaches

Outcomes:

1) Generic strategies for the development of GSS for SIT applications developed
2) The efficiency, applicability and the range of species transferability of the generic approaches assessed
3) GSS developed through the generic approaches evaluated at small scale

Outputs:

1) Markers to be used for generic strategies for the development of GSS for SIT applications against targeted agricultural pests identified (at least two markers)
2) Markers to be used for generic strategies for the development of genetic sexing strains for SIT applications against targeted disease vectors identified (at least two markers)
3) Strains carrying selectable markers to be used for the development of genetic sexing strains for SIT applications against targeted agricultural pests evaluated (at least two strains)
4) Strains carrying selectable markers to be used for the development of genetic sexing strains for SIT applications against targeted disease vectors evaluated (at least two strains)
5) GSS based on generic approaches for SIT applications against targeted agricultural pests developed (at least two strains)
6) GSS based on generic approaches for SIT applications against targeted disease vectors developed (at least two strains)
7) GSS developed based on generic approaches for SIT applications against targeted agricultural pests at small scale evaluated (at least two strains)
8) GSS developed based on generic approaches for SIT applications against targeted disease vectors at small scale evaluated (at least two strains)
9) Publication of results in a peer reviewed journal

Activities:

1. Selecting participants and awarding contracts and agreements
2. Organizing the first RCM.
3. Organizing the second RCM.
4. Evaluation of the mid-term CRP.
5. Organizing the third RCM.
6. Organizing the fourth RCM.
7. Final evaluations.
8. Publish the results of the CRP in a special issue of an international journal.

LOGICAL FRAMEWORK:

Narrative Summary	***Objective Verifiable Indicators***	***Means of Verification***	***Important Assumptions***
Overall Objective The main objective of this CRP is the development and evaluation of generic approaches for the construction of genetic sexing strains (GSS) to be used for sterile insect technique (SIT) applications, as part of AW-IPM programs, to control populations of agricultural pests and disease vectors.	N/A	N/A	Requests by Member States in the area of insect pest and disease vector control using the SIT are increasing. To transfer this nuclear technology to Member States, the availability of genetic sexing strains for an efficient, cost-effective, safe and biosecure implementation at large scale is an essential precondition. Biological material is available.

Specific Objectives			
1. To develop generic strategies for the construction of GSS for SIT applications	At least two generic strategies for the construction of GSS developed.	Reports and / or published papers.	Generic strategies for the construction of GSS can be developed.
2. To assess the efficiency, applicability and the range of the species transferability of the generic approaches	The efficiency and the range of the applicability of at least two generic approaches assessed.	Reports and / or published papers.	Assessing the efficiency and the range of the applicability of the generic approaches is possible.
3. To evaluate, at small scale, GSS developed through the generic approaches	At least two GSS developed through the generic approaches evaluated.	Reports and / or published papers.	Protocols for the evaluation of GSS developed through the generic approaches are available or can be developed.
Outcomes			
1. Generic strategies for the development of GSS for SIT applications developed	Protocols and approaches determined	Data collected	Facilities and resources available.
2. The efficiency, applicability and the range of species transferability of the generic approaches assessed	Tools and protocols developed	Data collected	Facilities and resources available.
3. GSS developed through the generic approaches evaluated at small scale	Tools and protocols developed	Data collected	Facilities and resources available.

Outputs			
1. Markers to be used for generic strategies for the development of GSS for SIT applications against targeted agricultural pests identified (at least two markers).	At least two markers identified.	Reports and or published papers.	Biological material is available. Protocols are available or can be developed.
2. Markers to be used for generic strategies for the development of genetic sexing strains for SIT applications against targeted disease vectors identified (at least two markers).	At least two markers identified.	Reports and or published papers.	Biological material is available. Protocols are available or can be developed.
3. Strains carrying selectable markers to be used for the development of genetic sexing strains for SIT applications against targeted agricultural pests evaluated (at least two strains).	At least two strains evaluated.	Reports and or published papers.	Biological material is available. QC protocols are available or can be developed.
4. Strains carrying selectable markers to be used for the development of genetic sexing strains for SIT applications against targeted disease vectors evaluated (at least two strains).	At least two strains evaluated.	Reports and or published papers.	Biological material is available. QC protocols are available or can be developed.
5. GSS based on generic approaches for SIT applications against targeted agricultural pests developed (at least two strains).	At least two strains developed.	Reports and or published papers.	Biological material and tools are available. Protocols are available or can be developed.
6. GSS based on generic approaches for SIT applications against targeted disease vectors developed (at least two strains).	At least two strains developed.	Reports and or published papers.	Biological material and tools are available. Protocols are available or can be developed.
7. GSS developed based on generic approaches for SIT applications against targeted agricultural pests at small scale evaluated (at least two strains).	At least two strains evaluated.	Reports and published papers.	Biological material is available. QC protocols are available or can be developed.
8. GSS developed based on generic approaches for SIT applications against targeted disease vectors at small scale evaluated (at least two strains).	At least two strains evaluated.	Reports and or published papers.	Biological material is available. QC protocols are available or can be developed.

9. Publication of results in a peer reviewed journal.	Papers drafted and submitted.	Journal issue with published scientific papers.	Data for publication available.
Activities			
1. Selecting participants and awarding contracts and agreements.	Proposals evaluated and 9 Research Contracts, 12 Research Agreements and 1 Technical Contract awarded.	Signed contracts and agreements.	Suitable proposals submitted, funding available and approval of Contracts and Agreements by CCRA-NA committee.
2. Organising the first RCM.	1st RCM held 2019.	Participants' activities and logical framework revised.	Contracts and Agreements signed by counterpart organisations.
3. Organising the second 2nd RCM.	2nd RCM to be held 2021.	Participants and RCM Progress Reports.	Progress satisfactory.
4. Evaluation of the mid-term CRP.	Mid-term CRP evaluation presented to CCRA	Mid-CRP report.	Progress satisfactory.
5. Organising the third RCM.	3rd RCM to be held 2023.	Participants and RCM Progress Reports.	Progress satisfactory and mid-CRP evaluation approved by CCRA-NA committee.
6. Organise the fourth RCM.	4th RCM to be held 2024.	Participants and RCM Final Reports.	Final reports are submitted to the Agency.
7. Final evaluations	Final CRP evaluation approved by CCRA	Final CRP evaluation.	Progress satisfying.
6. Special issue published.	Publication	Special issue published.	Each contract and agreement holder contribute with a paper to the Special Issue.

THIRD RESEARCH COORDINATION MEETING

Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture

"Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24 - 28 April 2023

Project Officer: Kostas Bourtzis

Monday, 24 April 2023

09:00 – 09:10 **Kostas Bourtzis** - Opening of the meeting.

09:10 – 09:30 Introduction of participants.

09:30 – 09:45 **Kostas Bourtzis** - "Current status of the CRP and future challenges".

SESSION I: Reviewing individual research workplans

09:45 – 17:00 Reviewing individual research workplans.

Tuesday, 25 April 2023

SESSION II: Presentations by participants (Chairperson: Frantisek Marec)

09:00 – 09.30 **Carlos Caceres** – **"**New generation of genetic sexing strains facilitate colony refreshment for SIT applications: proof-of-principle in medfly".

09.30 – 10:00 **Roswitha A. Aumann, Germano Sollazzo, Maria-Eleni Gregoriou and Lucas Prates –** "Advancements in insect pest control through the identification and characterization of *wp* and *tsl* genes in the medfly for developing novel genetic sexing strains".

10:00 – 10:30 **Ernst A. Wimmer** – "Generation of foreign DNA-free sexing strains based on temperature sensitive lethal mutations and Y-chromosomal rescue by genome editing tools".

10:30 – 11.00 *Coffee Break*

11:00 – 11:30 **Antonios Augustinos** – "Identification and characterization of temperature sensitive lethal genes and response to thermal shock of SIT target species".

11:30 – 12:00 **Angela Meccariello –** A novel Y-linked approach for genetic sex sorting of *Ceratitis capitata*".

12:00 – 12:30 **Kostas D. Mathiopoulos** – "Improving the assemblies of Y chromosomes".

12:30 – 13:30 *Lunch Break*

13:30 – 14:00 **Alistair C. Darby** – "Tsetse flies sex chromosomes"**.**

14:00 – 14:30 **František Marec** – **"**On the development of genetic sex strains and the study of sex determination in the codling moth, *Cydia pomonella*".

14:30 – 15:00 **Alfred M. Handler** – "New target genes for male sterility in *Drosophila suzukii* and *Anastrepha suspensa*".

15:00 – 15:30 *Coffee Break*

15:30 – 16:00 **Silvia B. Lanzavecchia** – "Development of genetic sexing strains for *Anastrepha fraterculus* approaching with generic genomic tools".

16:00 – 16:30 **José S. Meza and Brenda Torres Huerta** – "Induction and evaluation of generic markers for the development of genetic sexing systems in *Anastrepha*".

16:30 – 17:00 **Edwin Ramirez** – "Development and evaluation of genetic sexing strains of fruits flies to be used for sterile insect technique applications".

Wednesday, 26 April 2023

SESSION II (cont'd): Presentations by participants (Chairperson: Kostas Mathiopoulos)

09:00 – 09:30 **Simon W. Baxter** – "A genetic sexing strain of the Queensland fruit fly, *Bactrocera tryoni*".

09:30 – 10:00 **Nidchaya Aketarawong** – "Development of genetic sexing strain(s) for SIT program of agricultural *Bactrocera spp*. using bioinformatics and molecular tools".

10:00 – 10.30 **Wei Peng** – "The development of genetic sexing strains (GSS) for SIT applications in *Drosophila suzukii*".

10:30 – 11:00 *Coffee Break*

11:00 – 11.30 **Giuseppe Saccone** – "Female sex determination factors in *Ceratitis capitata*: molecular and structural basis of TRA and TRA2 recognition".

11:30 – 12:00 **Daniel Bopp** – "Structural and functional analysis of a novel male determiner candidate on chromosome I of the common housefly *Musca domestica*".

12:00 – 12:30 **Jiannis Ragoussis** – "Towards the delineation of temporal transcriptional kinetics in multiplexed and sexed single Medfly embryos by long read sequencing".

12:30 – 13:30 *Lunch Break*

13:30 – 14:00 **Thabo Mashatola** – "Development of temperature sensitive alleles to eliminate females during mosquito mass production: steps towards development of the sterile insect technique against *Anopheles*".

14:00 – 14:30 **Cyrille Ndo** – "Screening for morphological visible markers and additional temperature sensitive strains of *Anopheles arabiensis*".

14:30 – 15:00 **Muhammad Misbah ul Haq** – "Collection and up-scaling of *Aedes aegypti* and *Ae. albopictus* from different climatic and topographic regions of Pakistan for initial screening to hunt natural *tsl* mutation in wild populations".

15:00 – 15:30 *Coffee Break*

15:30 – 16:00 **Philippos Papathanos –** "Development of insect GSSs using male-specific engineered rescues of selectable traits".

16:00 – 16:30 **Jake Tu –** "Chromosome engineering for sex separation of the yellow fever mosquito *Aedes aegypti*".

16:30 – 17:00 General discussion

Thursday, 27 April 2023

SESSION III: Working groups: discussion, planning, and coordination of work plans (Chairperson: Kostas Bourtzis and Group Leaders)

08:30 – 10:00	Open discussion and composition of the working groups
10:00 – 10:30	*Coffee Break*
10:30 – 12:00	Working groups: discussion, planning, and coordination of work plan
12:00 – 13:30	*Lunch*
13:30 – 15:00	Working groups: discussion, planning, and coordination of work plan
15:00 – 15:30	*Coffee Break*
15:30 – 17:00	Revision of the CRP documents (introduction, individual proposals) including planning of the activities to carry out for the next 18 months

Friday, 28 April 2023

SESSION IV: Review of the CRP documents, drafting and compiling the RCM report (Chairperson: Kostas Bourtzis)

08:30 – 10:00	Drafting RCM report and preparation of the list of achievements
10:00 – 10:30	*Coffee Break*
10:30 – 12:00	Compiling RCM report and the list of achievements
12:00 – 13:30	*Lunch*
13:30 – 16:00	Presentation of the RCM report, and decision on the journal for the publication of the Special Issue as well as on the date and place of next RCM.
16:00 – 16:15	***Closing***

ABSTRACTS
THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
New generation of genetic sexing strains facilitate colony refreshment for SIT applications: proof-of-principle in medfly

AUTHOR (S):
Carlos Caceres[1], Marc Vreysen[1], Jose Salvador Meza[2], Maria-Eleni Gregoriou[1], Frantisek Marec[3], Kostas Bourtzis[1]

ORGANIZATION:
1. Insect Pest Control Laboratory, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Friedensstrasse 1, 2444 Seibersdorf, Austria
2. Programa Operativo de Moscas SADER-IICA, Camino a los Cacaotales S/N, Metapa de Domínguez, Chiapas, CP 30860, México
3. Biology Centre CAS, Institute of Entomology, 370 05 České Budějovice, Czech Republic

SHORT SUMMARY OF PAPER

Abstract:

Laboratory domestication triggers significant evolutionary changes in insect populations, including genetic sexing strains (GSS), which may affect their sexual behaviour. Long-established laboratory populations may have gone through several selection processes, for example genetic drift, bottlenecks, and inbreeding phenomena, which may have in turn modified their genetic structure and resulted in the reduction of their genetic diversity. Therefore, refreshment of the colonies of GSS, such as the Ceratitis capitata (medfly) VIENNA 8 strain, with wild material is frequently needed. However, this is a time-consuming and challenging process because wild males do not carry the translocation and wild females are not easily adapted to the artificial oviposition behaviour. To overcome these challenges, we present a new generation of GSS which facilitates the refreshment with wild genetic material, increasing genetic variability and potentially improving sexual performance.

Males from the mutant strain were irradiated to produce the translocation. Irradiated males were mass-crossed with wild-type females. Single pairs were then set up between F1 females and wild-type males. The established families were screened to select those that produced males with the wild-type phenotype and females with the mutant phenotype in subsequent generations. An additional selection step was applied to identify families in which the females could be eliminated thus establishing a novel GSS for further evaluation.

Two lines were isolated and characterized in comparison to the standard T(Y;5) VIENNA 8 GSS. The new GSS has comparable or better quality and production profile than VIENNA 8 GSS under small scale rearing conditions. In field cage tests, males originating from the new GSS and VIENNA 8 GSS competed equally with wild males for mating with wild females. Five consecutive rounds of introgression were performed between females from both novel GSS with wild males from Spain and a follow-up analysis indicated a high degree of genetic refreshment.

Novel medfly GSS were constructed, which have similar characteristics with the T(Y;A)-based VIENNA 8 GSS. Their unique advantage is that they enable the smooth and controlled introduction of fresh genetic material into mass-reared colonies, potentially improving the quality of mass-produced males and the efficacy of medfly SIT action programs.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
Advancements in insect pest control through the identification and characterization of wp and tsl genes in the medfly for developing novel genetic sexing strains

AUTHOR (S):
Roswitha A Aumann[1], Germano Sollazzo[1,2,3], Maria-Eleni Gregoriou[2], Lucas Prates[1], Kostas Bourtzis[2], Marc F. Schetelig[1]

ORGANIZATION:
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2. Insect Pest Control Laboratory, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Department of Nuclear Sciences and Applications, IAEA Laboratories, 2444 Seibersdorf, Austria
3. Current address: Imperial College London, Department of Life Sciences, Sir Alexander Fleming Building, South Kensington Campus, London, UK, SW7 2AZ, United Kingdom

SHORT SUMMARY OF PAPER

Abstract:

The Sterile Insect Technique (SIT) is a powerful method of controlling insect pest populations by releasing sterile insects into the wild. The success of SIT depends on the quality of the sterile insects released. In addition, the availability of genetic sexing strains (GSS) can significantly improve the efficiency and cost-effectiveness of the process. In recent years, there has been growing interest in developing novel GSS in SIT target insect pest species. The identification of the *white pupae* (*wp*) and *temperature-sensitive lethal* (*tsl*) genes could greatly facilitate the achievement of this goal.

In the case of the medfly, research has been ongoing to identify *wp* and *tsl* genes that could be used to develop new GSS. First, a gene associated with white pupae was identified in the medfly, and it was subsequently shown that introducing a mini-wp gene via germline transformation and transposable elements in the medfly could rescue the white pupae phenotype. Additionally, CRISPR knockouts of *wp* gene in other *Bactrocera* species have been successfully conducted.

Second, in the medfly, a pipeline has been developed and published to reduce the candidate genes in the *tsl* region on chromosome 5. Through this approach, two *tsl* candidate genes have been characterized. These genes are potential candidates for further study and could potentially be used in developing new GSS in other Tephritids.

Overall, the identification of *wp* and *tsl* genes in the medfly holds great promise for developing novel GSS that could significantly improve the efficiency and cost-effectiveness of SIT and allow for the development of generic GSS approaches. The ongoing research in this area, including the successful rescue of the white pupae phenotype and the characterization of *tsl* candidate genes, represents a significant step forward in insect pest control.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
Generation of foreign DNA-free sexing strains based on temperature sensitive lethal mutations and Y-chromosomal rescue by genome editing tools

AUTHOR (S):
Hassan M. M. Ahmed & Ernst A. Wimmer

ORGANIZATION:
Georg-August-University Göttingen, Dept. of Developmental Biology, Johann-Friedrich-Blumenbach-Institute for Zoology and Anthropology, GZMB, Ernst-Caspari-Haus, Justus-von-Liebig-Weg 11, 37077 Göttingen, Germany

SHORT SUMMARY OF PAPER

Abstract:

We want to generate genetic sexing strains (GSS) that are based on a temperature-sensitive lethal (tsl) mutation, which represents a selectable marker responsible for female killing, and a rescue of this situation by a wild type allele of that genetic locus translocated to the Y chromosome, which allows only the males to survive at the non-permissive temperature. To reach this aim, we are evaluating tsl mutations of highly conserved proteins that were generated in the baker's yeast *Saccharomyces cerevisiae* by transferring such mutations by genome editing to the agricultural pest, the cherry vinegar fly *Drosophila suzukii*. In this respect the *D. suzukii* homologs of *CkII-alpha, UBC2, UBC3*, and *UBC9* have been isolated and suitable targets for genome editing to generate the tsl mutations D212N or P>S, respectively, have been identified and validated. Genomic regions spanning those genes have been isolated to be used for homology directed repair (HDR). The tsl mutation P>S is currently introduced into *Ds_UBC2* by HDR-mediated genome editing along with a fluorescent marker gene that can afterwards be seamlessly removed by flanking *piggyBac* sequences. To generate visible GSS based on body or pupal color, we isolated the genes *Ds_ebony* (*e*) and *Ds_black* and are currently trying to mutate them by HDR-mediated genome editing introducing a gene-disrupting fluorescent marker.

In collaboration with Dr. Papathanos, Rehovot, Israel, we identify suitable loci on the Y chromosome of *D. suzukii* for gene expression. As a first attempt to insert fluorescent markers onto the Y chromosome, we have isolated genomic regions surrounding the genes *Kl3*, *Kl2*, and *ARY*, which have proven suitable for eye expression in *D. melanogaster*, and sequence validated them. For the gene *ARY*, Y-unique regions were chosen to identify *gRNAs* and generate HDR repair templates to introduce transgenes for evaluating their expression.

Promising loci would then be used to place a wild type allele of a tsl gene or a body/pupal color gene to rescue the mutant phenotype specifically in males and thereby generate a GGS in *D. suzukii*. Current genome editing tools should make it possible to create the tsl allele in a way to resemble a classical mutant, which could also come to existence by mutagenesis, and to introduce a wild-type copy of that gene onto the Y chromosome to resemble a small translocation, which could also come about by chromosomal breaks and rearrangements as induced by classical mutagenesis approaches.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
Identification and characterization of temperature sensitive lethal genes and response to thermal shock of SIT target species

AUTHOR (S):
E. Kotsadam, V. Karathanasi, Ch. Chondrogiannis, A. Augustinos

ORGANIZATION:
Department of Plant Protection Patras, Institute of Industrial and Forage Crops, Hellenic Agricultural Organization - DIMITRA, Patras, Greece

SHORT SUMMARY OF PAPER

Abstract:

For the first two years of this project, we reported advances related to the a) setup of insectaries, b) development of new strains, c) development of quick and accurate protocols for the identification of the typical tsl phenotype, and d) domestication of natural populations. During the last 18 months, project's progress can be summarized in the following:

- *Development of medfly strains through recombination between wp and tsl loci:* Six (6) strains have been developed, two (2) wp tls^+ and four (4) wp^+ tsl. These strains exhibit the expected sensitivity/resistance to thermal stress, verified through repeated tsl tests. In collaboration with IPCL, sequencing analysis is ongoing.
- *Development of medfly strains with two morphological markers of the 5th chromosome and tsl:* Through independent recombination events the following nine strains have been developed: three (3) *we wp tsl* , three (3) *ye wp tsl*, and three (3) *or wp tsl* strains. These strains exhibit the expected sensitivity to thermal stress, verified through repeated tsl tests. In collaboration with IPCL, sequencing analysis is ongoing.
- *Thermal response of recently domesticated medfly populations:* Natural populations have been established and tested. There are differences among them in respect to the response to the thermal stress but not the difference observed between the 'typical' tsl-resistant and tsl-sensitive strains.
- *Aedes albopictus sensitivity to thermal stress:* Thermal response essays with L1 from a laboratory population deriving from Greece, pointed to 39 ^oC – 41 ^oC and time exposure ranging between 8 h – 24 h as the best starting point for the identification of sensitive and resistant genotypes in the tiger mosquito. Optimization is needed in respect to egg hatching synchronization and additional populations are planned to be analysed this year.
- *Interaction between Wolbachia, tsl phenotype, and genetic stability of the medfly VIENNA GSSs:* introducing *Wolbachia* in the medfly VIENNA 8 GSSs does not seem to have a negative effect on genetic stability and/or response to the typical 'tsl' thermal stress. Experiments are now performed in larger scale, to test for putative subtle, previously unidentified, differences.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
A novel Y-linked approach for genetic sex sorting of *Ceratitis capitata*

AUTHORS:
Flavia Krsticevic[1], Serafima Davydova[2], Nikolai Windbichler[2], Angela Meccariello[2], Philippos Papathanos[1]

ORGANIZATION:
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[2] The Hebrew University of Jerusalem, Israel

SHORT SUMMARY OF PAPER

Abstract:

Ceratitis capitata, or the medfly, is among the most economically impactful and widely invasive agricultural pests worldwide. Currently utilised SIT sex sorting strains for the medfly lack stability due to high recombination risk. In this study, we explored a site-specific Y-linked approach to tackle genetic sexing, first by inserting a fluorescence marker to check whether the selected sites are suitable for integration. Two different bioinformatic pipelines, one utilising subtraction and the other male-specific kmers, were used to uncover Y-specific sequences. Altogether we verified 12 separate unique fragments of over 250 bp long as male-specific by using genomic DNA as template. By combining multiple neighbouring Y-specific islands generated through the kmer pipeline together, we reviewed three further targets which, due to greater length, were more favourable for knock-in through homology-directed repair (HDR). Two of the three targets of 0.95 kb and 1.5 kb in length, were shown to be male-specific. They were subjected to guide RNA design, guide RNA efficiency verification *in vivo,* and cloning via Gibson assembly with the GFP marker to verify integration. A third construct was similarly built for a suitable 1.4 kb Y-specific fragment identified through the subtraction pipeline. The three plasmids were microinjected into wild-type medfly embryos and integration success was assessed. For the two kmer pipeline plasmids no integration was confirmed. For the subtraction pipeline plasmid, 10 marker-positive males were discovered, although integration was only confirmed for one of them and no line could be generated thereafter. We, therefore, aim to repeat the same microinjections and to further explre other y-specific sequences as potential targets. Long-term, GFP can be replaced with a copy of an endogenous gene which can be used for sexing, such as *white pupae (wp)* or *temperature sensitive lethal (tsl)*. This will allow for differential development of males and females, whereupon they can be separated by pupae colouration or survival upon heat exposure in the case of *wp* and *tsl* respectively.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
Improving the assemblies of Y chromosomes

AUTHOR (S):
Rallis D[1], Papanicolaou A[2], Mathiopoulos KD[1]

ORGANIZATION:
[1]Department of Biochemistry and Biotechnology, University of Thessaly, Greece
[2]Hawkesbury Institute for the Environment, Western Sydney University, Australia

SHORT SUMMARY OF PAPER

Abstract:

While new insect genome projects are helping resolve most of the genomic content from ecologically and economically important species, reconstructing the Y/W chromosome sequence is challenging. Sex-limited chromosomes are crucial for understanding insect development, sexual conflict, reproduction and speciation-related events which depend on the full ascertainment of genome sequence including the Y. In heteromorphic sex chromosome pairs, the Y typically degenerates into a highly repetitive and heterochromatic sequence which confounds genome project pipelines. As a result, current research often relies on genome sequences where the Y is misassembled or missing. The classical Chromosome Quotient (CQ) method can help identify sex-limited regions but depends on high sequencing depth and computationally demanding whole genome alignments. Heuristics that can overcome these requirements will be needed if we are to reconstruct the Y chromosome in multiple species efficiently. Here we explore the Y chromosome content of Tephritid fruit flies after developing two Y-detection algorithms: a classical CQ and an alignment-free approach. We benchmark these against manually curated and PCR-validated Y contigs of *Bactrocera oleae*, *Ceratitis capitata* and *Drosophila melanogaster*. We find that the kmer method outperformed the CQ approach, could better identify misassembled Y regions, and required significantly fewer computational resources. We further outline why the manual curation of computational data is crucial and required while working with genome projects from non-model species.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
Sex Chromosomes in Glossina: An Update on Genome Progress and Assembly of the XY Chromosomes

AUTHOR (S):
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ORGANIZATION:
Department of Infection Biology and Microbiomes, Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, United Kingdom

SHORT SUMMARY OF PAPER

Abstract:

Glossina, commonly known as the tsetse fly, play a significant role as a vector for trypanosomes, the causative agents of African trypanosomiasis in humans and animals. The tsetse flies as a genus are primarily found in sub-Saharan Africa, where it poses a major threat to public health and agricultural productivity.

Tsetse fly has complex life cycle and exhibits unique biological characteristics. It is viviparous, giving birth to live offspring, with the larvae develop internally within the female's reproductive tract. Glossina requires a blood meal from vertebrate hosts, to sustain its reproductive cycle, making it a potent vector for transmitting trypanosomes during feeding. However, this slow reproductive rate mean that tsetse can be control very effectively with sterile male release programmes.

Therefore, genetic approaches, such as the development of genetic sexing strains (GSS), hold promise for targeted control measures by selectively reducing the population size and limiting disease transmission.

Understanding the sex chromosomes of these species is vital for comprehending the mechanisms governing sex determination. The genome of *Glossina morsitans* is encoded on three chromosome pairs, L1, L2, and sex chromosomes, in addition there are also B or S chromosomes. Notably, the sex chromosomes in Glossina have posed challenges due to the absence of distinguishing markers on the X and Y chromosomes. However, studies have identified a significant genomic insertion of the endosymbiotic bacterium Wolbachia in the Y chromosome, offering avenues for investigation.

This talk will provide an update on the current progress in Glossina genome research and the ongoing efforts towards assembling and comprehending the XY chromosomes. By leveraging PacBio HiFi, Oxford Nanopore and HiC sequencing technologies and bioinformatic tools, we have made significant strides in deciphering the intricate architecture of the Glossina sex chromosomes. However, the identification and characterization of specific markers, including the Wolbachia insertion, have not been straightforward.

Ultimately, a deeper understanding of the Glossina genome and sex chromosome assembly holds promise for developing innovative strategies to mitigate the threat posed by these disease-carrying insects.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
On the development of genetic sex strains and the study of sex determination in the codling moth, *Cydia pomonella*

AUTHOR (S):
Frantisek Marec[1], Kristyna Pospisilova[1,2], Tereza Vajnarova[1,2], Atsuo Yoshido[1], Arjen E. van't Hof[1]

ORGANIZATION:
[1] Biology Centre CAS, Institute of Entomology, Ceske Budejovice, Czech Republic
[2] University of South Bohemia, Faculty of Science, Ceske Budejovice, Czech Republic

SHORT SUMMARY OF PAPER

Abstract:

We have two main objectives in this research. First, we focus on developing genetic sexing strains by inserting a selectable gene into the W chromosome of codling moth (*Cydia pomonella*). Second, we are studying genes involved in codling moth sex determination to find new options for genetic sexing. We want to achieve the first goal by genome editing with the CRISPR/Cas9 method. To this end, in collaboration with Marc Schetelig and Roswitha Aumann (Univ. Giessen, Germany), we have designed a construct, called CpW-DsRedT3-CpNotch, which was synthesised and cloned into the pUC57 vector by GenScript Biotech (NL). The construct contains a fluorescent marker gene (*DsRedT3*) and a truncated allele of the *C. pomonella Notch* gene (*CpNotch*) designed to function as a dominant conditional lethal mutation (DCLM). To ensure embryonic expression, both genes are under the control of the strong IE1 hr5 (Immediate-early gene 1 linked with homologous region) promoter. Using the appropriate restriction enzyme and cloning, we have produced a modified plasmid construct without the mutant *CPNotch* allele, designated CpW-DsRedT3. We are currently injecting this modified construct into codling moth eggs to test the feasibility of CRISPR/Cas9 knock-in to the W chromosome. Regarding the second objective, we have successfully completed the study on the function of the *CpMasc* gene in codling moth sex determination. Silencing of *CpMasc* by RNAi during early embryogenesis resulted in a shift from male- to female-specific splicing of the *doublesex* (*Cpdsx*) gene in male embryos, leading to a strongly female-biased sex ratio in adulthood, apparently due to the high mortality of males at early developmental stages. Our data clearly show that *CpMasc* functions as a masculinising gene in the sex-determining cascade of codling moth. To search for *Fem* piRNA or another upstream feminising factor, we extracted RNAs for sequencing with the mirVana kit and DNAs for sex determination separately from female and male early embryos and obtained sex-specific libraries of small RNA sequences and mRNA sequences. Analysis of these sequence libraries with bioinformatics tools is ongoing.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
New target genes for male sterility in *Drosophila suzukii* and *Anastrepha suspensa*

AUTHOR (S):
A.M. Handler, Q. Xia, R. Furlong and K. Tariq

ORGANIZATION:
USDA-ARS, Center for Agricultural, Medical and Veterinary Entomology, Gainesville, Florida, USA

SHORT SUMMARY OF PAPER

Abstract:

In an effort to identify new genes that can act as targets for male sterility in fruit fly pest species, genes necessary for male fertility in *Drosophila melanogaster* have been isolated in the spotted-wing drosophilid, *Drosophila suzukii*, and the Caribbean fruit fly, *Anastrepha suspensa*. *Dm-wampa* is a recently characterized component of the outer axonemal dynein arm in *D. melanogaster*, that results in male sterility due to loss of flagellar motility in homozygous null mutants, while *Dm-Prosalpha6T*, is a proteasome subunit gene required for spermatid individualization and nuclear maturation that also results in male sterility in homozygous null mutants. In silico Blastn searches identified potential *Ds-wampa* and *Ds-Prosalpha6T* cognates in a California strain of *D. suzukii* with high nucleotide and peptide identity. These cognate coding regions and proximal genomic DNA were isolated by PCR in a Florida *D. suzukii* strain, that revealed 44 synonymous *Ds-wampa* CDS SNP substitutions in the two strains. qPCR studies verified testis-specific expression for both genes in adult males for 10 days after adult eclosion, with no evident expression in females. To generate a null allele for *Ds-wampa*, CRISPR-Cas9 HDR was used to insert an IE1-DsRed marker in its coding region, however heterozygotes were fertile while viable homozygotes (expected to be male sterile) could not be identified in 60 inbred single pair matings, suggesting that a concomitant off-target recessive lethal mutation also occurred. The *A. suspensa* genome is not available as a searchable database, but Blastn searches revealed high identity proteins for potential cognates of both *Dm-wampa* and *Dm-Prosalpha6T* in *Ceratitis capitata* and *Bactrocera dorsalis*. Conserved nucleotide motifs for both genes in these tephritid species were used for PCR that isolated potential high identity cognates in *A. suspensa*. qPCR transcrirpt expression analysis for the putative *A. suspensa* cognates is in progress, as well as creation of CRISPR-Cas9 knock-out mutations for both genes in the two species. These studies will assess their importance to male fertility and their potential use as targets to create sterile males in strategies such as precision-guided SIT, and use of their promoter regulatory DNA in conditional spermatocyte-specific cell death systems.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:

Development of genetic sexing strains for *Anastrepha fraterculus* sp.1 approaching with generic genomic tools

AUTHOR (S):

Rivarola, M.[1]; Conte, C.A.[2]; Giardini M.C.[2]; Scannapieco, A.C.[2]; Milla, F.H.[2]; Wulff, J.P.[3]and Lanzavecchia, S.B.[2]

ORGANIZATION:

[1] Instituto de Biotecnología (IB), INTA, IABIMO-CONICET
[2] Instituto de Genética (IGEAF) gv al IABIMO-CONICET, Instituto Nacional de Tecnología Agropecuaria (INTA), Buenos Aires
[3] North Carolina State University. NCSU, USA

SHORT SUMMARY OF PAPER

Abstract:

The South American fruit fly, *Anastrepha fraterculus* Wiedemann (Diptera: Tephritidae) is considered a quarantine pest in several American countries. Currently, an integrated pest management approach based on the use of toxic baits and trapping is being applied in Argentina to control wild populations of this pest. A species-specific strategy, the sterile insect technique (SIT), based on the use of a classical Genetic Sexing Strain (GSS) has been successfully implemented to control Tephritidae species in the American continent (e.g. *Ceratitis capitata* and *Anastrepha ludens*) and are being tested in the case of *A. fraterculus.* Our main goals of our projects are to provide baseline information of *A. fraterculus* sp. 1 genome and to apply transgenesis/ gene editing technologies to produce GSSs for this pest species. During the last period we performed a purification of an *A. fraterculus* sp. 1 strain carrying *w*AfraCast2 (*Wolbachia* strain) and 100% X_1X_1/X_1Y_5 (sex chromosomes). F8 descendants of this strain were used for genome sequence analysis. We obtained 15.8 and 9.4 M number of reads (for female and male, respectively) based on processing HMW DNA samples from single individuals using Oxford Nanopore sequencing technology. Kmer analysis showed a coverage of ~ 50X and 40X, estimated unique genome size of 508 and 485 Mb (female and male, respectively) and a mean heterozyogisity <1%. First draft genome assembly yielded 4679 contigs (N50: 5125992 bp) for the female and 6035 contigs (N50: 3559397 bp) for the male. Benchmarking Universal Single-Copy Orthologs (BUSCO) analysis showed 98.2 and 98.3 completeness for the female and male, respectively. Mummer comparison between assemblies showed 41 contigs putatively detected only in the male genome while no contigs were unique in the female. Further analysis of these contigs is in progress. Short-term objectives are: 1) To improve *A. fraterculus* sp. 1 genome assemblies with HiCi and Illumina sequencing technologies; 2) To characterize potentially promising genes for editing techniques, and 3) To set up assays of microinjections in *A. fraterculus* early embryos for transgenesis and CRISPR/Cas 9.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
Induction and evaluation of generic markers for the development of genetic sexing systems in *Anastrepha*

AUTHOR (S):
José S. Meza[1], Daisy P. Cárdenas-Enriquez[1], Brenda Torres Huerta[2], Victor García-Martínez[1], Jorge Ibañez-Palacios[1], Martha Roblero-Roblero[1], Obdulia L. Segura León[2]

ORGANIZATION:
1. National Program Fruit Flies SADER/SENASICA
2. Colegio de Postgraduados Montecillo, Texcoco, Estado de México

SHORT SUMMARY OF PAPER

Abstract:

In the past, we developed genetic sexing strains (GSS) for *Anastrepha ludens* and *A. fraterculus* using a *black pupa* (*bp*) recessive marker. Four years ago, the same marker for *A. obliqua* was isolated and determined to be an autosomal recessive mutation. After testing thousands of males with different irradiation doses (10 to 35 Gy) to induce a Y-autosomal translocation, using *bp* mutation, the highest dose (35 Gy) produced for first time a GSS for *A. obliqua*, where males emerge from normal brown pupa and the females from black pupa.

On the other hand, to explore the effect of heat stress on *A. ludens* eggs, we performed a comparative analysis of the egg transcriptome after exposure to high temperature and for that it was analysed the expression levels of genes encoding heat shock proteins (Hsps); a family of proteins produced in response to exposure to stressful conditions. By RNA-Seq, *de novo* assembly of the *A. ludens* egg transcriptome, differential expression analysis (DEA), we identified 641 differentially expressed genes ($p \leq 0.05$) when comparing gene profiles of eggs at 25°C and eggs at 35°C, of which 432 were up-regulated ($\log2FC \geq 1.2$) in eggs exposed to 35°C. Analysis of GO terms and KEGG pathways showed that heat stress significantly affected genes involved in the maintenance of chromatin structure and conformation, DNA repair, DNA binding (chaperone proteins), and oxidoreductases. In addition, we identified an overrepresentation of signaling pathways regulating the physiological response to inflammatory processes, resistance to oxidative stress, apoptosis, the p53 signaling pathway, and inflammatory mediator of TRP channels. Likewise, in the DEA analysis, we identified two genes encoding members of the Hsp70 family with a significant up-regulated differential expression in eggs exposed to 35°C, with 6- and 3-fold increases in the level of expression to eggs at 25°C. This study provides a first look into the gene response to heat stress in *A. ludens* eggs and is the basis for future functional studies.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
Development and evaluation of genetic sexing strains of fruit flies to be used for sterile insect technique applications

AUTHOR (S):
Edwin Ramírez, Pedro Rendón, Cristian Morales

ORGANIZATION:
Medfly Program – Guatemala

SHORT SUMMARY OF PAPER

Abstract:

Currently, in biofactories, mass rearing of males of *Anastrepha ludens* using a colour based genetic sexing strain has been consolidated to support control programs that effectively include the sterile insect technique (SIT) as a control tool for this pest species. However, the mass rearing of sterile males of this fruit fly still presents opportunities for improvement. The efficiency of mass rearing of *A. ludens* males can be increased through the development of genetic sexing strains that allow the separation of males and females early in their life cycle. For example, in *Ceratitis capitata*, the "Vienna 8" *tsl* strain (thermosensitive lethal gene) already allows the elimination of females in the egg stage, avoiding the waste of resources in rearing females throughout their larval stage. In *A. ludens* there is still no "*tsl*" strain available, which would avoid unnecessary mass rearing of females.

To meet the objectives set at the beginning of this CRP and according to our proposed research, during the past year, we had been selecting lines originating from flies that have been treated using the chemical mutagenic (EMS) and exploring the possibility of finding a *tsl* strain for *A. ludens*. As we reported in our previous meeting we produced a line, called family 166, which showed partial lethality (50%) due to heat treatment in the egg stage. However, after many selection processes, we found a line coming from the original 166-10 line that shows a total lethality (100%) when applying heat treatment to the eggs. For the effective heat treatment, was necessary to define: 1) the optimal age of the egg, to avoid the maternal effect, 2) the duration (in hours) of the heat treatment, and 3) the effective temperature. Currently, research is focused on the application of low irradiation doses to males and crosses with the intention of finding a line that shows translocation of thermal sensitivity only in females.

On the other hand, as mentioned before, to find new strains of *A. ludens* through induction of mutations using the original TBP7 females. From GUA10 seven lines were selected (F167, F274, F60, F254, F44, F55, and F121) and from TBP7 three (F10A, F12, and F97). These selected lines have been reared on a scale (medium) that had shown their genetic stability (level of recombination), yield, and dosimetry, and currently is pending to define the mating competitiveness of their males under field cage conditions.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
A genetic sexing strain of the Queensland fruit fly, *Bactrocera tryoni*

AUTHOR (S):
Simon Baxter[1], Zoey Nguyen[1], Elisabeth Fung [2,3], Anzu Okada[3], Ngoc Mai Han Nguyen[4], Peter Crisp[2,3] and Amanda Choo[2]

ORGANIZATION:
[1]University of Melbourne
[2]University of Adelaide
[3]South Australian Research and Development Institute
[4]Macquarie University

SHORT SUMMARY OF PAPER

Abstract:

The Queensland fruit fly, *Bactrocera tryoni*, is a serious polyphagous pest of fruits in Australia. Outbreaks can be managed using sterile insect technique (SIT) biocontrol; however, efficiency of the program would improve if males could be separated from unrequired females prior to mass-release. Here we use CRISPR/Cas9 gene editing technology and chromosomal translocations to develop a *B. tryoni* genetic sexing strain (GSS) based on a pupal colour phenotypic. *Bactrocera tryoni* embryos were previously microinjected with CRISPR/Cas9 mixtures to create gene recessive deletions in a *major facilitator superfamily* gene (MFS), causing "white pupae". A chromosomal translocation was achieved using X-ray radiation and a relatively stable GSS was established. Males carry a wild type MFS gene on the Y-chromosome and develop with wild type brown pupae, and females are homozygous for this mutation and develop with a grey or white puparium. The white pupae locus is linked on the same chromosome as *shibire*, a gene known to cause temperature sensitive phenotypes in *Drosophila* when specific amino acid substitutions are present. A GSS carrying a phenotypic marker plus a temperature dependent mechanism that only kills females is desirable. Three different amino acid substitutions were created in the *B. tryoni* ortholog of *shibire* using CRISPR/Cas9 homology directed repair, and strains were assessed for temperature sensitive phenotypes. Finally, sequencing of the *B. tryoni* Y-chromosome is in progress and this resource may assist in identification of male-only genomic regions for integrating transgenes in the future. This research has created a GSS based on puparium colour plus advanced our genetic and genomic capabilities in *B. tryoni*.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
Development of genetic sexing strain(s) for SIT program of agricultural *Bactrocera* spp. using bioinformatics and molecular tools

AUTHOR (S):
Nidchaya Aketarawong, Kamoltip Laohakieat, Sujinda Thanaphum

ORGANIZATION:
Regional R&D Training Center for Insect Biotechnology (RCIB), Department of Biotechnology, Faculty of Science, Mahidol University, Thailand

SHORT SUMMARY OF PAPER

Abstract:

Prior to the development of a new genetic control insect strain using molecular techniques, a thorough understanding of the sex determination pathway was required. In this regard, the key genes in the pathway of the carambola fruit fly, *Bactrocera carambolae*, which is a destructive pest in Southeast Asia and South America, were studied. Three orthologous genes, *transformer, transformer-2, and doublesex,* were isolated and characterized in *B. carambolae* using knowledge and skills transferred from related species, *B. dorsalis* and *B. correcta*. Gene structures were observed and compared among *Bactrocera* species for all three genes. Additionally, the function of the *transformer* (*Bcartra*) gene in the pathway was confirmed through RNAi knockdown in *B. carambolae*, which was found to be similar to other fruit flies.

CRISPR/Cas9 system was carried out to achieve sequence-specific gene knockout via the non-homologous end joining (NHEJ) pathway. The *MoY* gene, a Y-link gene, was targeted to test the Cas9-mediated disruption of genes involved in the sex-determination pathway. Two independent target sites of the *BdMoY* gene were selected for NHEJ-mediated disruption, and ribonucleoprotein complexes (RNPs) consisting of purified Cas9 protein and each sgRNA were *in vitro* assembled. In this study, we individually injected the RNPs of Cas9-sg_Mo1, Cas9-sg_Mo2, and Cas9-sg_Mo1 + Cas9-sg_Mo2 into the precellular blastoderm embryos of *B. dorsalis* Salaya1. The Salaya1 strain is a genetic sexing strain where the sexual karyotype can be distinguished through pupal color dimorphism. Male adults develop from brown pupae while female adults develop from white pupae. All G0 brown pupae developed into normal males. To screen for possible germline transformation, these G0 brown-pupae males were individually back-crossed to uninjected white-pupae females. Two out of 18 crosses produced G1 brown-pupae females (pseudofemales) that were confirmed to have the Y chromosome and a CRISPR-induced deletion in their *BdMoY* target sequences. This work establishes the baseline data and protocol for CRISPR/Cas9 genome editing in *B. dorsalis* Salaya1 and demonstrates its potential for developing *MoY* minus pseudofemale lines from genetic sexing strain which could be used for further experiments.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
The development of genetic sexing strains (GSS) for SIT applications in *Drosophila suzukii*

AUTHOR (S):
Dan Deng, Shisi Xing, Wen Wen, Zongzhao Zhai, Wei Peng

ORGANIZATIONs:
Hunan Normal University

SHORT SUMMARY OF PAPER

Abstract:

Drosophila suzukii Matsumura (Diptera: Drosophilidae), a recently invasive insect pest commonly known as spotted wing *Drosophila* (SWD), has recently invaded western countries, and it has become an important threat of a wide variety of several commercial soft fruits by causing significant losses in crop yield and quality. To develop the genetically enhanced sterile insect technique (SIT) in controlling the *D. suzukii*, we have identified numerous spermatogenesis genes such as *βTubulin 85D* (*βTub*), *fuzzy onions* (*fzo*), *protamine A* (*ProtA*), *spermatocyte arrest* (*sa*) based on the testes' transcriptome. We have synthesized the gRNAs of *βTub*, *fzo*, *ProtA* and *sa* genes and have done the micro-injection with Cas9 protein into the embryos. The knockout experiments showed that a reducing of sperm activity and male infertility phenotypes, which indicating that these spermless males can be used for SIT applications. To construct the female-specific lethality and masculinization genetic sexing strains (GSS) based on targeting sex determination genes, we identified and synthesised the *Dsvasa* promoter, generating the gene drive *D. suzukii* transgenic strain expressing Cas9 driven under the *Dsvasa* promoter. Meanwhile, we identified the *DsU6* promoter in the NCBI genome database. By counting the proportion of individuals with mosaic eyes in G0 flies under the microscope, we can compare the gene editing efficiency of each *U6* promoter in *D. suzukii*. Our results showed that the promoter *DsU6-3* had the highest gene editing efficiency. Based on the identification of *Dsvasa* and *DsU6-3*, we used the CRISPR/Cas9 gene editing technology established a homing-based gene drive system in *D. suzukii* through targeting the *Sex-lethal* (*Sxl*) gene and *transformer* (*tra*) gene. The *Dsvasa* promoter was used to express Cas9 and the *DsU6-3* was used to express *Sxl* and *tra* gRNAs in *D. suzukii*. In both *Sxl* and *tra* targeted locus, gene drive allele could achieve super mendelian inheritance. In terms of phenotypes, we found that most of the *Sxl* knockout females perished during preadult stages with the majority dying during pupal transition. Variable expressivity of the number of sex comb bristles were observed in *tra* knockout intersexes. Moreover, molecularly the *tra* knockout intersexes expressed both female- and male-specific alternative splice variants of *doublesex* (*dsx*) gene.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:

Female sex determination factors in *Ceratitis capitata*: molecular and structural basis of TRA and TRA2 recognition

AUTHOR (S):

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SHORT SUMMARY OF PAPER

Abstract:

In the model system for genetics, *Drosophila melanogaster*, sexual differentiation, and male courtship behavior are controlled by sex-specific splicing of *doublesex* (*dsx*) and *fruitless* (*fru*). Two hybrid assays have shown that the female-specific Transformer (TRA) and the non-sex-specific Transformer2 (TRA2) splicing factors interact, forming a complex promoting *dsx* and *fru* female-specific splicing. TRA/TRA2 complex binds to 13 nt long repetitive elements in the dsx and fru pre-mRNAs. In the Mediterranean fruitfly *Ceratitis capitata* (Medfly), which shares with Drosophila a ~ 120 million years old ancestor, *Cctra* and *Cctra2* seem to promote female-specific splicing of *Ccdsx*, *Ccfru*, and *Cctra* itself by a conserved 13 nt long repetitive elements. Here we show by a yeast two-hybrid assay that the orthologous CcTRA and CcTRA2 proteins interact with each other, as the orthologous ones in Drosophila, despite the extremely high amino acid divergence of CcTRA when compared with Drosophila TRA. Interestingly, CcTRA2 interacts with itself as also observed for Drosophila TRA2. Using predictive approaches based on Artificial Intelligence, we also generated a three-dimensional model of the complex formed by CcTRA and CcTRA2. This structure also led to identifying an evolutionary and highly conserved TRA2 recognition motif in the TRA sequence. Establishing this Y2H method in the Medfly opens the road for screening other CcTRA- and or CcTRA2 interacting proteins involved in composing the primary sex-determining signal of this species, which involves a male-determining Y-linked gene, *MoY*, encoding a novel very short protein. The Y2H approach, combined with powerful predictive tools of three-dimensional protein structures, could use helpful also in other insect species to understand the potential links between different proteins acting as primary sex-determining signals and the conserved TRA and TRA2 transducers.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
Structural and functional analysis of a novel male determiner candidate on chromosome I of the common housefly *Musca domestica*

AUTHOR (S):
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SHORT SUMMARY OF PAPER

Abstract:

In higher dipteran insects the critical first step in sex determination starts with ON/OFF regulation of the *transformer (tra)* gene. When active, *tra* directs female expression of the downstream effector *doublesex* (*dsx*) and female development is implemented. When *tra* is inactive, *dsx* expresses by default male protein variants which instruct male development. Generally, *tra* is activated in the early zygote by maternally provided TRA gene products engaging a positive feedback loop which maintains *tra* in the active female-promoting mode throughout development. Male development requires that activation of the *tra* loop is prevented either by a dominant *tra* repressor that is active in the early zygote (post-zygotically) or already active in the female germ line (pre-zygotically). The common housefly *Musca domestica* provides an ideal platform to investigate the evolutionary transitions between the use of diverse male determining signals. Apart from the Y chromosome, male-determining loci (*M*) can also be present on the X or any of the five autosomes in natural housefly populations. Most of these *M* loci contain the male determiner *Mdmd*, a neo-functionalized duplication of the spliceosomal factor *CWC22* which translocated to different locations in the genome. The notable exception is the *M* on chromosome I (M^I) which has no *Mdmd* gene suggesting the presence of a novel *M* factor. Differential expression analysis in early embryos recovered 533 transcripts that are significantly enriched in M^I males, of which thus far only one appeared to be specific to M^I males. This candidate transcript was named *Simultaneous Alternative M Factor 1*, *SAM-1*. Interestingly, *SAM-1* is also present in genomic reads of females which contain the maternal *tra* repressor *Ag*. This is consistent with the proposition that, based on the same chromosomal location, *Ag* is a germline-specific derivative of M^I. *SAM-1* lacks a long ORF and is located within intron 4 of the *BuGZ* gene, but seems to be transcribed independently from the same strand in the same orientation. Silencing by dsRNA injections or targeted disruption by CRISPR/Cas9 both result in a significant reduction in M^I male fertility and cause partial feminization of the testes. These results agree with a pivotal role of *SAM-1* in proper male differentiation, but there is no conclusive evidence yet, that *SAM-1* encodes all of M^I functions. Thus, we are also continuing to assess the original 533 candidates. A filtered set of these candidates will be further examined, and the most promising ones will be subjected to functional tests to examine for a potential role in male determination.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:

Towards the delineation of temporal transcriptional kinetics in multiplexed and sexed single Medfly embryos by long read sequencing

AUTHOR (S):
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SHORT SUMMARY OF PAPER

Abstract:

Long-read RNA sequencing has great potential to improve genomic characterization of non-model organisms due to its ability to yield full length genes. Coupled with absolute gene expression quantification, dynamics of development orchestrated at transcript level can be elucidated with high precision. The resolution of this precision can be further improved by studying organisms as close as possible to their basic entities, single cells for example or single embryos. Here, we present preliminary work where we collected developing embryos of the Mediterranean fruit fly (Medfly, *Ceratitis capitata*) at hourly timepoints for the first 15 hours of development. The Medfly is an organism of huge economic importance in agriculture due to its wide host range and destructive capacities of fruits. We show that we can simultaneously isolate total RNA and genomic DNA from single embryos, determine the sex of the embryos, spike embryos with external RNA standards to aid in absolute quantification, then perform cDNA synthesis and sequencing using the Oxford Nanopore long-read technology. We developed a genome-guided transcriptome assembly based on full-length transcripts and identified a total of 44828 transcripts comprising 8320 novel genes, missed in the current NCBI predicted gene models. These novel genes include the recently discovered maleness factor. These are preliminary results and in-depth analysis of this data is ongoing and is hoped to yield precise measurements of dynamics of transcription in the developing embryos and hopefully some sex-specific genes necessary for early embryo development that can be targeted for controlling pest populations.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:

Development of genetic sexing systems for the malaria vector *Anopheles arabiensis*: progress made by the South African sterile insect technique project

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SHORT SUMMARY OF PAPER

Abstract:

The South African mosquito sterile insect technique (SIT) project targeting the major malaria vector *Anopheles arabiensis* is now at an advanced development stage. Numerous technical aspects of the technology have been optimized to support the ongoing small-scale pilot field trial. Having an efficient sex separation mechanism to exclude female mosquitoes from the production line before irradiation and field releases is one of the most critical elements of the technology that continues to present challenges to the South African SIT project. For mosquito SIT program females must be excluded because, unlike males, they are capable of biting, blood feeding and transmitting the *Plasmodium* pathogens that cause the malaria disease. The current methods for separating *An. arabiensis* sexes are proving to be inefficient to support current mass rearing levels. To address this challenge, the development of additional sexing strains using classical genetics and transgenic approaches were proposed and are ongoing. When developing sexing strains (GSSs) it is critical to address two main components: (a) a selectable marker and (b) the linkage of the selectable marker to the one of the sex determining chromosomes. This will allow the distinguishing between sexes based on their phenotypic differences. Our team is investigating use of insecticide resistance and morphological body/colour variations as selectable markers that can be used in parallel with temperature-sensitive lethal (*tsl*) mutations. So far, progress has been made in determining the minimum (permissible) and maximum (restrictive) temperatures at which an *An. arabiensis* laboratory colony survives (i.e., determining the thermal limits/thermosensitive range prior to screening for *tsl* in individuals), (2) using ethyl methanesulfonate (EMS) and gamma radiation (γ) to induce *tsl* and various morphological body differences mutations as selectable markers, (3) investigating the possible association between the resistance to dieldrin (*rdl*) insecticide and temperature sensitivity mutation in *An. arabiensis*, and (4) use of an already established temperature-sensitive strain from Cameroon to develop a *tsl*-based GSS with a South African genetic background. The progress on this work will be presented at the upcoming third coordinated research meeting. Additionally, the next steps which involve a training programme at the International Atomic Energy Agency (IAEA) to: (a) identify and characterize genes suitable as selectable markers for the development of GSS in *An. arabiensis*; (b) obtain expertise in embryonic microinjections in *An. arabiensis* for the induction of mutations that will be used in the development of non-transgenic GSS; (c) genetically and molecularly characterize any developed mutant line(s); and (d) rear and compare the genetic stability, reproductive and physiological fitness of different *An. arabiensis* lines (for example, wild-type vs mutant lines). Furthermore, report on the creation and assessment of two *An. arabiensis* transgenic sexing strains based on a fluorescent marker will be highlighted. The one strain has the fluorescence linked to the X-chromosome while the other is linked to the Y-chromosome. A series of cross-mating of these sexing strains will enable sorting and release of non-transgenic males. This progress has provided the South African SIT with a strong foundation for planning the operational phase and successful completion of the current small-scale pilot trials.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
Screening for morphological visible markers and additional temperature sensitive strains of *Anopheles arabiensis*

AUTHOR (S): Cyrille Ndo and Yacouba Poumachu

ORGANIZATION:
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SHORT SUMMARY OF PAPER

Abstract:

Our general end goal is to develop a temperature sensitive lethal-based Genetic Sexing Strain of the malaria vector *Anopheles arabiensis* that carry a morphological visible marker.

Anopheles arabiensis wild mosquitoes collected in North Cameroon were colonized in the insectary of OCEAC. Males were mutagenized by feeding them with 10% sucrose solution containing ethyl methane sulfonate during 24h. Treated males were subsequently allowed to mate with virgin females and the progenies were reared until F3. Progenies (eggs, larvae, pupae and adults) were screened for a morphological visible marker, over 3 generations. Temperature sensitive phenotypes were screened by assessing mortality at 24h after exposition of L1 larvae to heat at 41°C for 3hours. Life history traits of all TSL strains isolated were further characterized.

In comparison to the control strain (wild type mosquitoes), mutagenesis significantly decreased male fecundity and fertility in all TSL strains. However, longevity and larval developmental time were not affected. Only slight variations were observed between TSL strains. No morphological visible marker was successfully isolated.

Future work will help characterizing the nature and inheritance pattern of genetic variations driving temperature susceptibility in each TSL strain.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
Hunt for naturally existing tsl mutation in *Aedes aegypti* and *Ae. albopictus* for construction of more robust genetic sexing strain (GSS) for SIT

AUTHOR (S):
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ORGANIZATION:
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SHORT SUMMARY OF PAPER

Abstract:

Aedes mosquitoes are known to transmit numerous viral infections globally, and SIT has gained attention as a non-conventional vector control method. However, sex separation at early life stages of *Aedes* mosquitoes in mass rearing facilities has been a major obstacle for successful implementation of SIT programs. To address this issue, our aim is to construct genetic sexing strain based on tsl that could help sex separation. To achieve this, we collected *Ae. albopictus* and *Ae. aegypti* from various topographical and climatic regions in Pakistan in order to identify natural tsl mutation if any in wild populations of these two species. The strains were collected using ovi-traps and larval collections from potential breeding sites in both urban and rural areas. We developed multiple colonies of both species from collected eggs and larvae and then grew them in a laboratory setting for thermal screening in water baths. We subjected developmental stages such as 1st instar larvae and pupae of both species to a range of temperatures (39°C - 41°C) for varying intervals of time (2-24 hours) to observe any difference in their survival rates and to detect any sensitivity or resistance to heat. We shortlisted temperatures of 40°C for 5 hours, and 41°C for 3 hours for thorough screening of all strains. The results indicate that colonies of the same species did not differ significantly, but there were minor differences observed between the two species under the same temperature and duration conditions. We also assessed the biological attributes of the colonies after thermal exposure, such as the percentage of development of L1 up to the L4 stage, the percentage of pupation, and the percentage of adult emergence. We established a colony using individuals that survived the initial heat exposure and subsequently exposed this colony to heat again. We compared the results with those from the first exposure to detect any differences. However, the results showed that there was no significant difference between the colonies that were exposed twice and those that were exposed only once. Additionally, we established several iso-male families, each consisting of one male and five females, from both wild-collected strains. We are currently in the process of scaling up the iso-male families. All the iso-male families and newly collected colonies of both *Aedes* species from more locations will undergo extensive thermal screening to detect naturally occurring tsl mutations.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
Development of insect GSSs using male-specific engineered rescues of selectable traits

AUTHOR (S):
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SHORT SUMMARY OF PAPER

Abstract:

Early female elimination prior to release is a desirable feature in many genetic control systems and a mandatory prerequisite in mosquitoes. Genetic sexing strains are an efficient solution, but their construction in any insect using classical genetics has proven laborious and serendipitous. We will present on our progress towards building a GSS in the Asian Tiger mosquito *Aedes albopictus* and species of the Tephritid genus focusing on the development of improved bioinformatic approaches and genome engineering tools. In *Ae. albopictus* we have developed a first-generation GSS, combining CRISPR-induced *yellow* mutant alleles with an engineered mini-*yellow* rescue construct, which was then linked to maleness leveraging the *Aedes*-specific *Nix* masculinizer. To facilitate the future development of scalable GSSs using high-throughput selectable traits, we have also developed a split-CRISPR toolbox enabling simple maintenance of parental strains and continuous production of hybrid insects harbouring somatic CRISPR-induced edits. We will report on our progress on the use of this system for the purposes of rapidly screening candidate genes for suitability in high-throughput selection, such as conditional lethality. Finally, we will provide an update regarding our efforts to develop bioinformatic methods to characterize the content and evolution of the medfly Y chromosome, for the purposes of identifying landing sites for the targeted knock-in of mini-rescues.

THIRD RESEARCH COORDINATION MEETING
On "Generic approach for the development of genetic sexing strains for SIT applications"

Virtual

24-28 April 2023

TITLE OF WORKING PAPER:
Chromosome engineering for sex separation of the yellow fever mosquito *Aedes aegypti*

AUTHOR (S):
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SHORT SUMMARY OF PAPER

Abstract:

Female *Aedes aegypti* mosquitoes feed on human blood and transmit harmful viruses including dengue, Zika, yellow fever, and chikungunya while males cannot. Efficient and cost-effective sex separation is critical to genetic programs aimed at controlling mosquito-borne infectious diseases. Despite recent progress, methods to separate males from female *A. aegypti* remain a significant bottleneck. It has been shown that sex-linked transgenic markers can be used to produce non-transgenic males. However, two transgenic lines and three independent sex-separations are needed to achieve this. In a novel CRISPR/Cas9-mediated method called pgSIT, only transgenic sterile males survive to become flying adults. Maintenance and sex-separation of two transgenic lines is also required as Cas9-expressing females need to mate with sgRNA-expressing males. Here we report the use of sex-chromosome engineering to develop multiple new sex separation strategies that enable robust sex separation and the production of non-transgenic males for release. These new methods are compatible with diverse applications including releases where non-transgenic males are preferred.

D44003-CR-3
Third Research Coordination Meeting on Generic Approach for the Development of Genetic Sexing Strains for SIT Applications

Virtual
24 to 28 April 2023

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