Title

A Fluorescent Sex-Sorting Technique for Insects with the Demonstration in Drosophila melanogaster.

Journal

GEN Biotechnology, 3(1)

Authors

Liu, Junru

Rayes, Danny

Akbari, Omar

Publication Date

2024-02-01

DOI

10.1089/genbio.2023.0041

Peer reviewed

GEN Biotechnology
Volume 3, Number 1, 2024

DOI: 10.1089/genbio.2023.0041

GEN Biotechnology

RESEARCH ARTICLE

A Fluorescent Sex-Sorting Technique for Insects with the Demonstration in *Drosophila melanogaster*

Junru Liu, Danny Rayes, and Omar S. Akbari*

Abstract

Recent advances in insect genetic engineering offer alternative genetic biocontrol solutions to control populations of pests and disease vectors. While success has been achieved, sex-sorting remains problematic for scaling many genetic biocontrol interventions. Here, we describe the development of a genetically stable sex-sorting technique for female and male selection with a proof of concept in *Drosophila melanogaster* termed SEPARATOR (Sexing Element Produced by Alternative RNA-splicing of A Transgenic Observable Reporter). This elegant approach utilizes dominantly expressed fluorescent proteins and differentially spliced introns to ensure sex-specific expression. The system has the potential for adaptability to various insect species and application for high-throughput insect sex-sorting.

Genetic biocontrol methods are effective and sustainable alternatives to traditional insecticide-based approaches for suppressing pest and vector populations.[1,2] Sterile insect technique (SIT), for example, is a genetic biocontrol technology that has successfully eradicated screwworms in the United States.[3,4] This technology suppresses populations through the frequent releases of sterile males, as female insects are often responsible for destroying agricultural resources or pathogen transmission.[5] While it is feasible to release sterile females alongside sterile males in specific programs, such as the New World screwworm, this approach often leads to substantial increases in rearing costs and a reduction in overall efficiency. Sterile females may still contribute to damage through oviposition, and there remains the possibility of sterile females mating with sterile males.[6]

On the other hand, although SIT has demonstrated effectiveness in certain species, it demands the exclusive release of sterile males in cases in the Tsetse fly or mosquitoes where females transmit human pathogens.[6–8] This indeed remains a grand challenge for many genetic biocontrol technologies. What is critically needed is a genetically stable technology that can be rapidly developed in many species to enable rapid and accurate sex-sorting.

The primary method for sex-sorting in the lab, which relied on manual sorting based on sexual dimorphisms, is labor-intensive and severely limited by throughput and scalability.[9,10] More recent approaches utilize machine learning to automate sex-sorting.[11,12] However, these still rely on natural sexual dimorphisms, making them challenging to adapt to other species.

Several Genetic Sex Separation (GSS) lines were established, enabling sorting based on pupal color, temperature sensitivity (*tsl*), or chemical sensitivity.[9,13–15] These approaches have demonstrated their effectiveness and scalability in the Mediterranean fruit fly (Medfly). Specifically, using pupal color as a selection marker, generated through Y-translocation, enabled efficient separation of mutant females from wild-type males using comparatively inexpensive rice sorters.[16–18] However, the generation of GSS involves irradiation-induced translocation of a conditional lethal gene, such as the gene conferring resistance to the insecticide dieldrin (Rdl), to the Y chromosome.[19] As a result, only males carrying the translocated gene on their Y chromosome can survive when exposed to the insecticide. This GSS method has success in specific insect species, such as the Mediterranean fruit fly. However, as a whole, the development of this method poses significant challenges.

A scalable sex-sorting method is essential to ensure cost-effective and scalable genetic technologies are available for the control of a variety of insect species. Incorporating fluorescent sex-specific markers facilitates the visual identification and separation of males and females with a Complex Parametric Analyzer and Sorter (COPAS, Union Biometrica) instrument. The COPAS has been used to sort *Drosophila melanogaster* embryos and larvae based on fluorescence as well as transgenic *Aedes aegypti* and *Anopheles gambiae* larvae.[10,20] The COPAS instrument was used to fully automate the sorting process, achieving the efficiency, speed, and accuracy needed to scale genetic technologies.[10]

Department of Cell and Developmental Biology, School of Biological Sciences, University of California, San Diego, La Jolla, California, USA.

*Address correspondence to: *Omar S. Akbari, Department of Cell and Developmental Biology, School of Biological Sciences, University of California, San Diego, La Jolla, CA 92093, USA,* E-mail: oakbari@ucsd.edu

Alternative splicing, a crucial posttranscriptional process in gene expression, enhances protein diversity from a single gene. We leverage sex-specific alternative splicing of the sex-determination gene *transformer* (*tra*) to achieve female-specific expression. *TraF* from four separate species were examined, with two species belonging to the Drosophilidae family (*D. melanogaster* and *Drosophila suzukii*) and the other two belonging to the Tephritidae family (*Ceratitis capitata* and *Anastrepha ludens*). While the alternative splicing of *tra* remains conserved, *tra* regulations use different mechanisms between Drosophilidae and Tephritidae. In Drosophilidae, *tra* is regulated by the presence of the *Sex-lethal (Sxl)* gene. On the other hand, in Tephritidae, *tra* is autoregulated in females and is turned off by the presence of *Maleness-on-the-Y* (Supplementary Fig. S1a).[21–23]

Here, we describe the proof of principle for a sex-sorting technique termed SEPARATOR (Sexing Element Produced by Alternative RNA-splicing of A Transgenic Observable Reporter) that utilizes fluorescent proteins and sex-specific introns of the sex-determination gene *transformer (tra)* to ensure sex-specific expression.[24,25] Female-specific expression is achieved by disrupting the coding sequences (CDS) of a fluorescent reporter with female-specific *transformer* introns (*traF*). When *traF* from *D. melanogaster*, *D. suzukii*, and *C. capitata* is inserted into the CDS of fluorescence protein, we achieved 100% female-specific fluorescence. Notably, *C. capitata traF* permits the selection of females as early as L1 instar larval stage. However, *A. ludens traF* resulted in dsRed expression in both sexes in *D. melanogaster* flies, indicating that *A. ludens traF* is not sex-specific when transcribed in *D. melanogaster*.

This SEPARATOR technique is a valuable method for insect sex-sorting as it (1) exploits highly conserved sex-specific splicing mechanisms, making it widely transferable to different insect species; (2) allows 100% positive selection of either females or males based on fluorescence instead of morphological differences, providing better accuracy; (3) permits sex-sorting during different life stages and as early as L1 instar stage; (4) can be combined with existing genetic control methods and a COPAS machine for precise high-throughput screening.

Results

Development of female-specific expression of the fluorescent protein

To engineer the female-specific expression of a reporter for positive selection of females, we exploited the sex-specific alternative splicing of a conserved sex-determination gene. In *D. melanogaster*, the *transformer (tra)* intron between exons 1 and 2 is spliced out in females, resulting in a functional *tra* protein. In males, alternative *tra* splicing results in a premature stop codon that terminates the *tra* protein.[26] This female-specific alternative splicing mechanism occurs not only in *Drosophila* but also in *Ceratitis* and *Anastrepha*, suggesting that it is highly conserved in Dipterans (Fig. 1a; Supplementary Figs. S1 and S2).[27] Consequently, the *traF* from *D. melanogaster*, *D. suzukii*, *C. capitata*, or *A. ludens* were inserted into the fluorescent protein CDS to test for female-specific fluorescent protein expression (Fig. 1b).

We generated two sets of dual fluorescent marker constructs encoding a fluorescent marker for both sexes and a female-specific fluorescent marker (Fig. 1c). The constructs were cloned into a plasmid containing an attP recombination site and a *piggyBac* (PB) transposable element. Set 1 constructs have an enhanced green fluorescent protein (eGFP) fluorescence expressed under a ubiquitous promoter *Hr5Ie1* (*Hr5Ie1*-eGFP) as the selectable marker for the transgene. To promote constitutive expression of female-specific fluorescent proteins, we used another ubiquitous promoter *Opie2* to express dsRed and inserted *traF* immediately downstream of the ATG translational start codon of dsRed (*Opie2*-ATG-*traF*-dsRed). Constructs in set 2 have the opposite marker configuration, with *traF* inserted downstream of the ATG translational start codon of eGFP under promoter *Hr5Ie1* (*Hr5Ie1*-ATG-*traF*-eGFP) for the female-specific fluorescent expression and *Opie2*-dsRed as the selectable marker for the transgene. In total, nine constructs were created: a control construct (795G) and eight experimental constructs, with four constructs in each set (Fig. 1d).

DmtraF, *DstraF*, and *CctraF* resulted in female-specific fluorescence

The transgene integration site can impact gene expression, so we opted to integrate all nine constructs into the same site through phiC31 *attP* integration on the second chromosome (BDSC #25709). Nine homozygous transgenic strains were established. Six constructs that harbor *DmtraF*, *DstraF*, and *CctraF* resulted in female-specific fluorescence (795H, I, J, L, M, and N; Fig. 2). These results indicate that inserting the *traF* into the CDS of the fluorescence proteins can result in female-specific fluorescence. However, for constructs harboring *AltraF* (795K and O), both females and males exhibited the intended female-specific fluorescence (Fig. 2). This result suggests that *AltraF* is spliced out in both females and males rather than in a female-specific manner.

Adult flies were collected for reverse transcription PCR (RT-PCR) analysis to validate the alternative splicing variants and obtain the fluorescent protein transcripts. Primers were designed to anneal to the 5′ untranslated region at the 3′end of the *Opie2* promoter, and the 3′ end of the dsRed sequence (Supplementary Table S1). Multiple bands were obtained from the RT-PCR samples for *CctraF* males, and both sexes of *DmtraF* and *DstraF* (Fig. 3a, b). Sequencing of these bands indicates that *CctraF*, *DmtraF*, and *DstraF* resulted in functional dsRed expression explicitly in females, while *AltraF* had dsRed expression in both males and females (Fig. 3c). In *Cctra-dsRed* male, a band that is very similar in size from the *Cctra-dsRed* female-specific product was observed. The sequencing result from that particular band indicates that *Cctra-dsRed* male produced a male-specific splicing product with the M1 intron that is only 40 bp larger than the female-specific product. Therefore, the two bands look similar in size on the gel, while the sequencing results indicate otherwise (Fig. 3c; Supplementary Fig. S2).

The molecular results obtained from RT-PCR analysis were consistent with our observations in the flies, confirming that

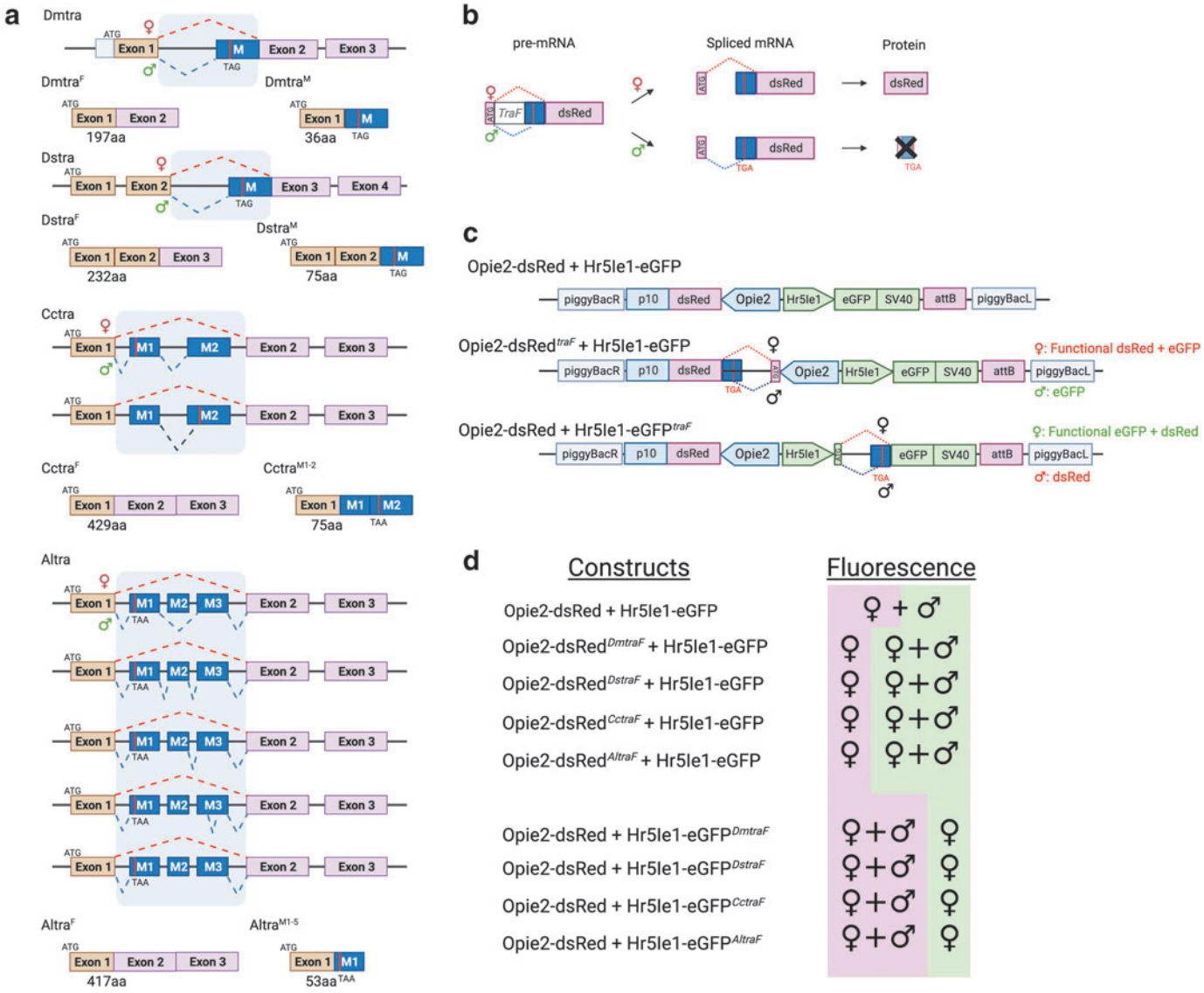

FIG. 1. Sex-sorter cassette in *Drosophila*.

(a) Sex-specific alternative splicing and the resulting protein of the *transformer (tra)* in *D. melanogaster*, *D. suzukii*, *C. capitata*, and *A. ludens*.

(b) Splicing of the female-specific *transformer* (*TraF*) intron should result in functional dsRed protein in females but not in males.

(c) Schematic of the sex-sorter constructs engineered and tested in the study. *TraF* introns from *D. melanogaster*, *D. suzukii*, *C. capitata*, and *A. ludens* are inserted into the coding sequence of either dsRed or eGFP after the ATG translational start codon.

(d) Fluorescence expression of females and males carrying the respective constructs. ATG, translational start codon; eGFP, enhanced green fluorescent protein.

CctraF, *DmtraF*, and *DstraF* exhibited female-specific splicing in *D. melanogaster*. Another interesting observation was that the splicing of *Cctra* male products (*CctraM*) differs in *D. melanogaster* and *C. capitata* (Fig. 3; Supplementary Fig. S1b). The M2 exon is skipped in both *CctraM1* and *CctraM2* in *D. melanogaster* but preserved in *C. capitata*. Nevertheless, the outcome demonstrates the feasibility of this fluorescent sex-sorting approach, as these female-specific splicing events allow for the positive selection of either sex.

Confirmation of sex-specific fluorescence at multiple developmental stages

Next, we evaluated the intensity and sex specificity of the fluorescence over multiple life stages. The six constructs (795H, I, J, L, M, and N) that exhibited female-specific fluorescent expression were evaluated. Female-specific fluorescence was observed as early as in the first instar larvae (L1) life stage in both *CctraF* transgenic lines: 795H and 795L (Figs. 2a and 4; Supplementary

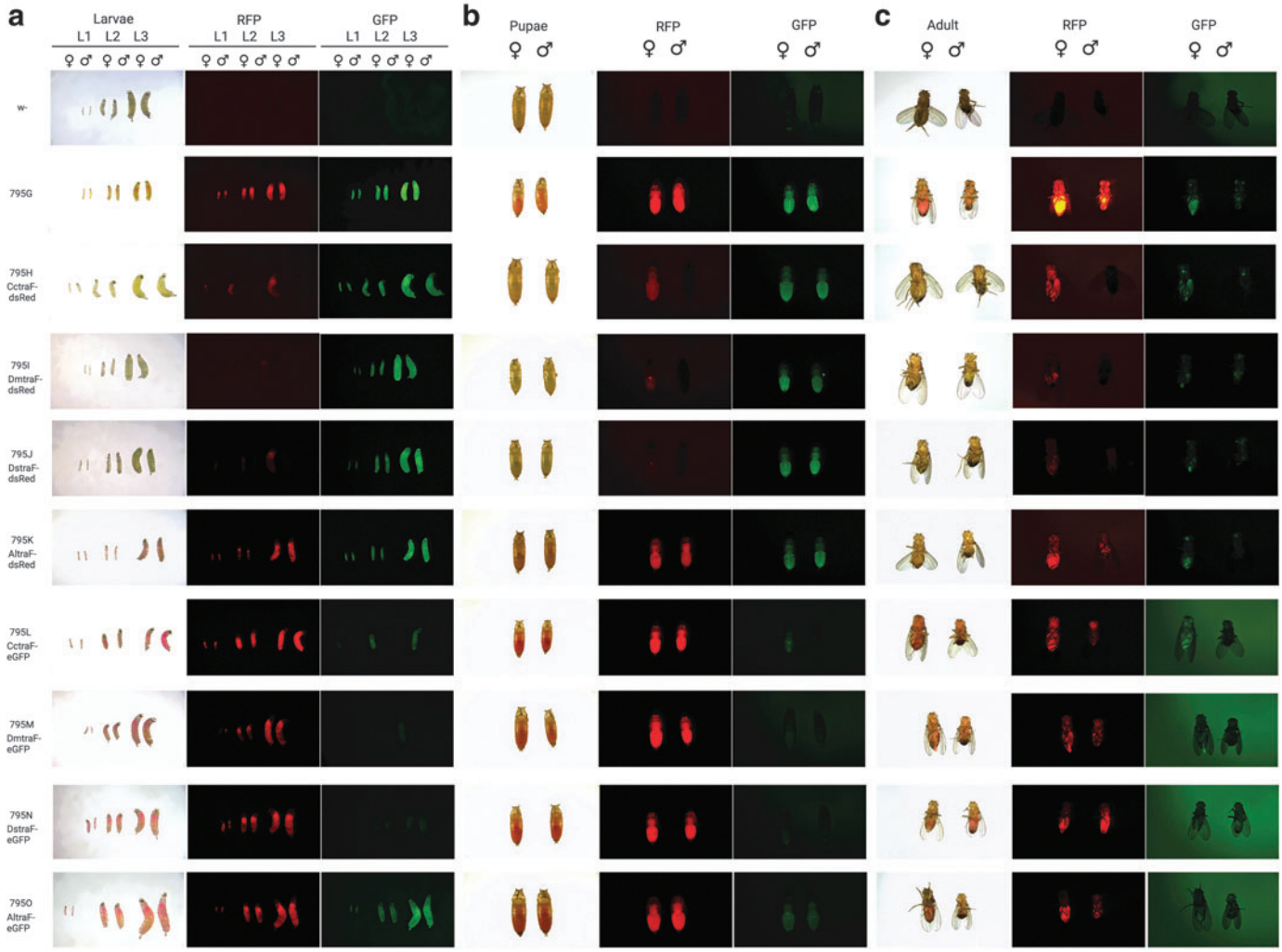

FIG. 2. Expression of Opie2-*TraF*-dsRed or Hr5ie1-*TraF*-eGFP transgenes in *D. melanogaster* can be observed in different developmental stages

(a) L1–L3 larval stage, **(b)** pupal stage, **(c)** adult under white light, RFP and GFP filters. GFP, green fluorescent protein; REP, red fluorescent protein.

Table S2). Female-specific fluorescence was also observed in the third instar larvae (L3) of *DmtraF* 795I and *CctraF* 795L, and the pupal stage for *DmtraF* 795M and *DstraF* 795N. Despite the identical introns in the *DmtraF* 795I and *DmtraF* 795M and the *CctraF* 795L and *DstraF* 795N strains, female-specific fluorescence was detected earlier in strains with the female-specific dsRed marker. This result is presumably due to the deeper tissue penetrance and lower auto-fluorescence of red fluorescent protein.[28] Notably, the intensity of the female-specific fluorescence varies among introns. The *CctraF* exhibits the highest brightness, followed in order of brightness by *DmtraF* and *DstraF* (Fig. 2). This is unexpected as *CctraF* is an exogenous/nonnative intron for *D. melanogaster*, potentially hindering successful intron recognition and splicing efficiency.

Assessing the fitness of the sex-sorting strains

Strain fitness is essential for scalability. Fluorescent proteins have documented fitness costs to genetically engineered organisms, but we expected that including *traF* in their CDS would minimally affect the fitness of the sex-sorting strain. We, therefore, compared the egg hatching and larval to adult survival rate of all eight homozygous sex-sorting strains to a control strain (795G) containing fluorescent reporters lacking *traF* introns. Our finding indicates that there is a significantly higher hatching rate in flies harboring *Opie2-ATG-traF-dsRed* constructs when compared to 795G control (Fig. 5). This effect could possibly be attributed to the fitness cost associated with dsRed functioning as a tetramer.[29] With the inclusion of *traF* introns, the expression of dsRed occurs at a reduced level. As a consequence, the fitness cost is diminished, which, in turn, leads to a higher hatching rate.

We observed lower larvae to adult survival only in the *CctraF-dsRed* 795H strain ($p < 0.05$, Student's *t*-test with equal variance; Fig. 5). These results suggest that the *traF* intron does not impose substantial fitness costs on the strain, making them

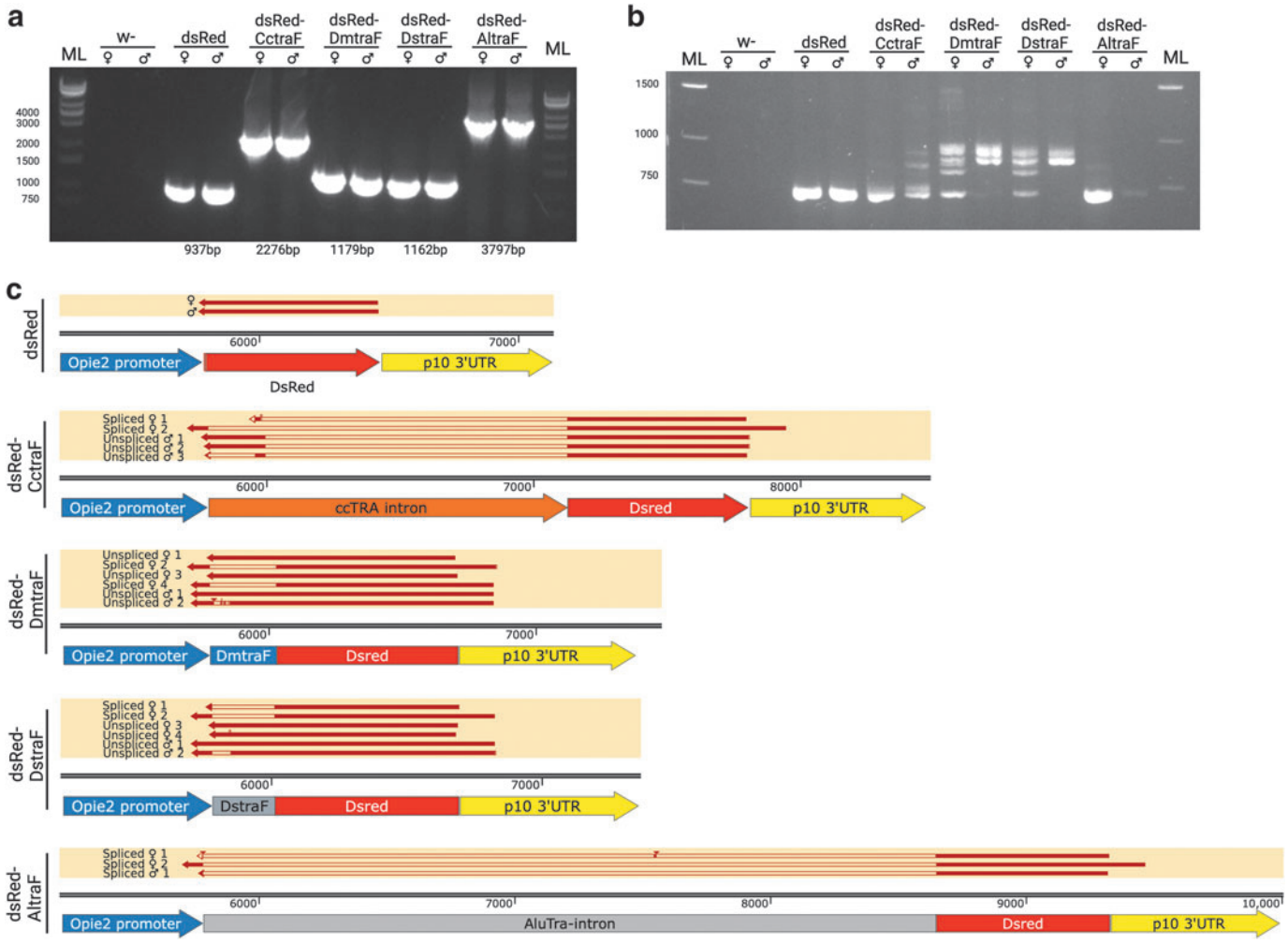

FIG. 3. The *traF* introns splicing patterns in *D. melanogaster*.

(a, b) Gel electrophoresis images show **(a)** the genomic DNA PCR for dsRed-*traF*, **(b)** the cDNA for the dsRed-*traF*.

(c) Sequencing results of the cDNA from each band. cDNA, complementary DNA; ML, molecular ladder.

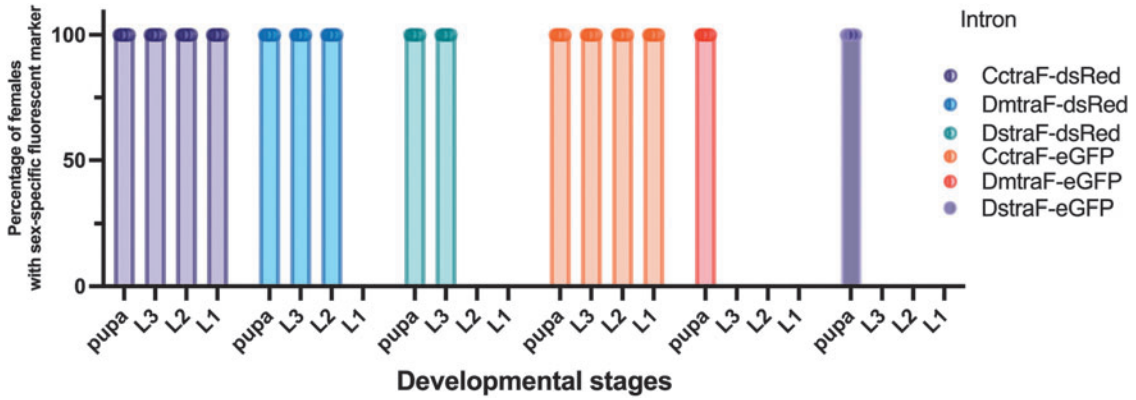

FIG. 4. Female selection efficiency at different life stages in *D. melanogaster* for all six sex-sorter cassettes that exhibited female-specific fluorescence.

The number of scored flies is indicated for each bar. The sex-sorting process achieved 100% accuracy, as no males were detected from either larvae or pupae that were sorted based on sex-specific dsRed or eGFP fluorescence.

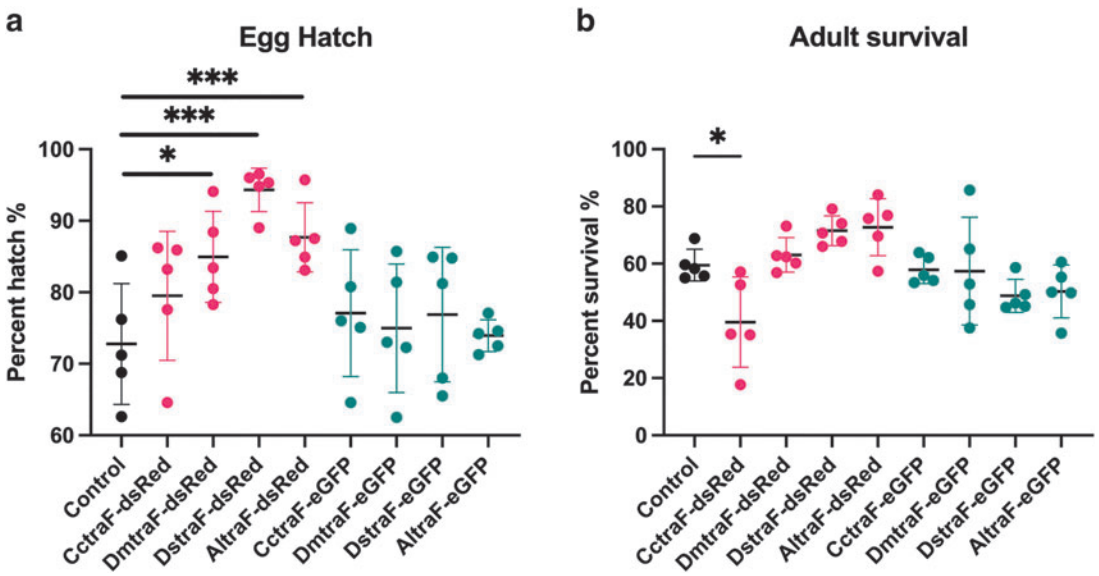

FIG. 5. Fitness cost of all eight sex-sorting cassettes was accessed through two parameters:
(a) Egg-hatching rate and **(b)** survival rate to adulthood.
Fitness cost was observed in *CctraF*-dsRed strain in the parameter of survival rate to adulthood (*$p < 0.05$, ***$p < 0.001$, Student's *t*-test with equal variance.).

suitable for potential large-scale insect population control projects. Similar fitness was observed from egg hatching for constructs with *DmtraF* and *AltraF*, this corresponds to the dsRed expression level.

Discussion

In this study, we engineered a novel sex-sorting method, SEPARATOR, that integrates a female-specific intron into the CDS of a fluorescent reporter to generate an efficient female-specific marker. This innovative technique uses splicing mechanisms conserved across dipteran species and has even been successfully engineered in other dipteran species.[25] We examined the splicing patterns of four *traF* introns, *DmtraF*, *DstraF*, *CctraF*, and *AltraF*, and found that three of them *(DmtraF*, *DstraF*, and *CctraF)* had 100% female-specific splicing. Among the six constructs tested for female-specific fluorescence expression in *D. melanogaster*, those carrying the *CctraF* introns (795H and 795L) exhibited expression at the earliest life stage and the brightest expression. These strains can be used for sex-sorting as early as the L1 stage and throughout the entire life cycle. This observation aligns with previous studies demonstrating the functional conservation of *CctraF* and its applicability in various insect species.[30–32]

It is worth noting the significant size difference between the *traF* introns in *Drosophilidae* (*DmtraF* and *DstraF*) and Tephritidae (*CctraF* and *AltraF*). The *traF* introns in *Drosophilidae* species are ~200 bp in length, whereas in Tephritidae species, they are approximately 10 times longer. Although *AltraF* is not female-specifically spliced, it is spliced out entirely in both females and males, thus resulting in functional fluorescent expression in both sexes. This result could be attributed not only to differences in the sex-determination pathway but also to species-specific variations in splicing signals, given that *AltraF* is an intron foreign to *D. melanogaster*.

According to the RT-PCR result, *D. melanogaster* could recognize the splicing site for *CctraM1*, but not that of *CctraM2*. In *A. ludens*, the splicing of *traM (AltraM)* results in the production of five isoforms (Fig. 1a). It is possible that the splice sites of *AltraM*, or Tephritidae species in general, may not be accurately recognized in *Drosophila*. These size differences may also impact intron splicing efficiency and, consequently, the dual sex expression of the *AltraF* fluorescent reporter. Larger introns, for example, possess more heterogeneous nuclear ribonucleoproteins and serine/arginine-rich protein recognition sites, leading to higher splicing efficiency.[33,34] However, further testing in Tephritidae species like *C. capitata* or *A. ludens* may better elucidate the capabilities of *AltraF* intron for SEPARATOR systems.

The *CctraF* introns, on the other hand, generate efficient and easily screenable female-specific fluorescent phenotypes. This efficiency may be due to the simplified splicing of *CctraF*, which does not depend on the presence of the *sex-lethal (Sxl)* protein required in *Drosophila* for *tra* transcript splicing.[30,35–38] In *C. capitata*, the *tra* gene is autoregulated and continuously expressed from the embryo to adulthood, allowing for the consistent and stable splicing of *CctraF*.[30,39] Therefore, *CctraF* may be a versatile tool for sex-specific expression in a wide range of insect species.

SEPARATOR has several potential advantages over traditional sexing methods. These include its potential *multiple species* adaptability, positive selection based on dominant fluorescence for both sexes, sex differentiation during early stages of

development, genetic stability, and minimal impact on fitness. All of these characteristics improve the potential scalability and timing of sex sorting. The fluorescent sex-sorting cassettes were engineered to be highly transferable to numerous insect species. They utilize a PB transposable element, which has high transgenesis efficiency in many insect species.[40] The two constitutive baculovirus promoters for fluorescent protein expression, *Opie2* and *Hr5Ie1*, have been shown to facilitate high expression in various insects throughout development.[41–43] Furthermore, dsRed and eGFP are the two most commonly used fluorescent proteins and can penetrate most insect tissues.[44] Female-specific alternative splicing of *tra* intron is also highly conserved across insect species.[38,45] Given these adaptable features, we have recently developed SEPARATOR strains in both mosquitoes and Tephritid fruit flies.[24,25]

The SEPARATOR technology enables the positive selection of females or males in all post-embryonic developmental stages.[25] Sex sorting at earlier developmental stages would be advantageous for large-scale insect population control projects, as it would extend the sex-sorting timeline, facilitate the release of earlier life stages, and may reduce the costs of large-scale releases.[46] Early-stage sex sorting in the larval phase can indeed offer significant advantages in the context of SIT programs. This is primarily because the larval diet constitutes a substantial cost factor in such programs. By efficiently eliminating females at an early developmental stage, substantial cost savings can be achieved.

Our approach simplifies manual sex-sorting, but the primary benefit is to large-scale sex-sorting applications. The SEPARATOR can be combined with existing genetic control methods and a COPAS machine for precise and high-throughput screening. Although the COPAS machine has demonstrated its ability to sort first instar *D. melanogaster* larvae, it remains uncertain whether the female-specific fluorescence will be sufficiently robust for detection.[47] This uncertainty arises because autofluorescence can be strong in many species. A point worth emphasizing is that *DmtraF* or *DstraF* should primarily be applied in species that utilize *Sxl* as the master switch gene for somatic sex determination. This is because the alternative splicing of *DmtraF* or *DstraF* relies on *Sxl* to splice out properly in females. Considering all these factors, *CctraF* appears to be a more suitable option as it produces the strongest female-specific fluorescence and the earliest expression. Additionally, it is essential to highlight that the splicing of *CctraF* may vary across different species. Therefore, examining the sequencing results of *CctraF* splicing in the specific species is crucial.

Furthermore, enhancing the accuracy of COPAS sorting can be achieved by incorporating distinct fluorescence markers for both females and males. We have previously demonstrated that the male-specific intron in *double-sex (dsxM)* in *D. melanogaster* can be inserted into the CDS of an antibiotic resistance gene to confer male-specific antibiotic resistance.[48] Given the conservation of male-specific *dsx* introns across many insects, a similar strategy can be employed to achieve male-specific fluorescence as we have done to develop SEPARATOR in mosquitoes.[25,38,45] Alternative methods that have been proposed for achieving male-specific fluorescence include the use of a male-specific promoter (e.g., ß2-tubulin promoter) or the use of a Y-linked transgene.[16,17,49–51] Nevertheless, it's essential to choose a promoter or intron that can be expressed throughout most of the life cycle and, ideally, throughout the entire body for the technology to be effective.

Our system may potentially be vulnerable to undesired genetic events, including loss-of-function, gain-of-function, or genetic exchanges like chromosomal translocation and recombination, ultimately resulting in the loss of fluorescence. Fluorescence can potentially serve as a reliable indicator, as any mutation affecting fluorescence could result in its loss and be subject to selection. Nevertheless, mutations affecting fluorescence or splicing specificity should be further investigated in a large-scale rearing setting.

The GSS used for many current SIT applications relies on reciprocal chromosomal translocations between the Y-chromosome and a region of the autosome containing a selectable marker. These GSS methods have been designed for male-only release programs, where females possess distinct phenotypes or markers that facilitate easy identification and separation.[52] Incorporating the D53 inversion has been an effective method for increasing the GSS stability. This D53 inversion, located on autosome 5 and carrying the *wp* and *tsl* markers, helps maintain strain stability by preventing recombination within the inverted region.[23,53,54] Recombinations in this area result in unbalanced gametes and, consequently, lethality. While having reciprocal chromosomes is the same as having a balancer for *D. melanogaster*, which increases the stability of GSS. Traditional GSS methods were historically discovered by chance, focusing on one species at a time, making them tailored to specific species. Studies on the multigeneration of GSS lines have revealed that chromosomal recombination can still occur at a frequency of $\sim$0.07%, leading to the breakdown of the sexing system.[55] Therefore, it is essential always to utilize a safeguard system

The Bigger Picture

The need for highly accurate and rapid sex sorting is nearly universal for genetic-based biocontrol methods. The demonstrated sex-sorting technique in *D. melanogaster* can be adapted for use in various insect species and high-throughput insect sex-sorting applications because of the ease with which females and males can be distinguished microscopically by fluorescence. Our study tested the *transformer (tra)* intron of four different species (*D. melanogaster*, *D. suzukii*, *C. capitata*, and *A. ludens*) and demonstrated their female-specificity in *D. melanogaster* for all but *A. ludens*. Further testing with *A. ludens traF* intron in its native species should be conducted to test its female-specificity. Different sex-determination genes can be substituted if needed to achieve female or male specificity. Nevertheless, *C. capitata traF* intron was female-specific in a wide range of species, highlighting the versatility of our technique and its potential to be utilized in pest or disease vector control technologies. In a broader context, this technique may enable the study of sex-specific developmental processes.

similar to the Filter Rearing System (FRS). The FRS was explicitly designed to prevent genetic issues, such as genetic recombination, within the Medfly GSS program.[56,57] The FRS is accomplished by maintaining a small colony and regularly sorting to ensure that no recombination occurs. Additionally, to enhance stability, another potential solution is to generate new translocations with a breakpoint located in close proximity to the sexing genes.[55]

Although the main emphasis of this work has sex-sorting applications, SEPARATOR can be used to study fly development. Differentiation between female and male adult flies is easily accomplished under the microscope, but accurate larvae sex-sorting can be challenging, particularly when they are embedded within the food medium. The major distinguishing factor between female and male larvae is the presence or absence of gonads, which is not easily visible.[58] With the *CctraF* 795H or 795L sex-sorter cassettes, the fluorescent protein markers can be easily observed as early as first instar larvae, even in the food medium and with minimal autofluorescence. The ability to accurately sex-sort larvae at early developmental stages provides a valuable tool for tracking the sex of flies and could potentially facilitate the study of sex-specific developmental processes in flies.

Materials and Methods

Molecular cloning

All genetic constructs were produced utilizing the Gibson enzymatic assembly. The construct 795G was created using a preexisting plasmid containing PB, attB-docking sites, and an *Opie2* promoter regulating dsRed. This plasmid was subsequently linearized with XhoI and NotI enzymes. The *Hr5Ie1* promoters, along with eGFP, were cloned into the linearized plasmid to make 795G, which serves as the control plasmid. To generate female-specific dsRed (795H-K), the plasmid 795G was linearized with *Avr*II and BamHI to allow insertion of introns into dsRed. Alternatively, to generate female-specific eGFP (795L-O), 795G was linearized using MluI and BsrGI to insert introns into eGFP. The *traF* introns from *D. melanogaster*, *D. suzukii*, *C. capitata*, or *A. ludens* were amplified from their respective genomic DNA (gDNA) using the primers listed in the Supplementary Table S1.

RT-PCR of the female-specific splicing transcripts

To access the splicing transcripts of four *traF* introns, we screened for female- and male-specific dsRed mRNA. Total RNA of 10 virgin females or males from w-, 795G, H, I, J, and K were extracted using the miRNeasy Tissue/Cells Advanced Kits (Qiagen). DNase treatment is done using the TURBO™ DNA-free (Invitrogen), and followed by the complementary DNA (cDNA) synthesis using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific™). The gDNA was amplified using primers 795.s2F and 795.s2R, and the cDNA was amplified using primers 795.s3F and 795.s1R (Supplementary Table S1). The gDNA samples were run on 1% tris-acetate-EDTA (TAE) agarose gel, and the cDNA samples were run on a 2% TAE agarose gel.

Rearing and fly transgenesis

Transgenic flies were maintained under standard conditions at 25°C with a 12H/12H light/dark cycle and fed on the Old Bloomington Molasses Recipe. Embryonic injections were performed in the lab following the standard injection protocol. Plasmids diluted to 300–350 ng/μL in water were inserted at P{CaryP}at P40 on the 2nd chromosome (Bloomington #25709). Recovered transgenic lines were balanced on the 2nd chromosome using a single chromosome balancer line w1118; CyO/sna[Sco]. Multiple independent lines were obtained for each plasmid and tested for sex-specific fluorescence. We used homozygous transgenic lines containing two copies of the transgene to assess the sex-sorting efficiency. Sex-sorting lines with *CctraF* introns 795H and 795L are deposited at the Bloomington Drosophila Stock Center (BDSC# pending).

Genetics and sex selection

To assess the fluorescent sex selection efficiency, we crossed 10 virgin females to 10 males in a fly vial. The parental flies were flipped into a fresh vial every 12 h, and the numbers of the laid embryos were scored. After hatching, the larvae or pupa were scored and transferred to different vials based on their fluorescent markers. The sex and the fluorescent markers of the adult offsprings were recorded after eclosion. Flies were scored using a Leica M165FC fluorescent stereomicroscope. Images were taken using a View4K camera. Each genetic cross was set up five times using different parental flies.

Fitness estimation

The fitness of the sex-sorting strains is assessed based on two parameters: the rate of egg-hatching (from embryos to larvae) and the rate of adult survival (from larvae to adult). To evaluate the egg-hatching rate, flies are allowed to lay embryos in fly vials for a duration of 24 h, and the number of eggs laid in each vial is recorded. After 24 h of egg laying, the number of larvae is recorded. To assess the adult survival rate, the number of both female and male adult flies that successfully enclosed is recorded.

Statistical analysis

Statistical analysis was performed in Prism9 by GraphPad Software, LLC. Three to five biological replicates were used to generate statistical means for comparisons.

Data availability

Complete sequence maps and plasmids are deposited at Addgene.org (#205481-205489). Transgenic lines 795H and 795L have been made available for order from Bloomington Drosophila stock center. The other transgenic lines are available upon request to O.S.A.

Acknowledgments

The views, opinions, and/or findings expressed are those of the authors and should not be interpreted as representing the official views or policies of the U.S. government. Figures were created with BioRender.com.

Authors' Contributions

O.S.A conceived and designed the experiments. J.L. and D.R. performed molecular and genetic experiments. All authors contributed to the writing, analyzed the data, and approved the final manuscript.

Author Disclosure Statement

O.S.A is a founder of Agragene, Inc. and Synvect, Inc. with equity interest. The terms of this arrangement have been reviewed and

approved by the University of California, San Diego, in accordance with its conflict of interest policies. All other authors declare no competing interests.

Funding Information

This work was funded by the U. S. Department of Agriculture (USDA)—Animal and Plant Health Inspection Service (APHIS)—Plant Protection and Quarantine (PPQ) (AP19PPQS&T00C237 and AP22PPQS&T00C188) and funding from an NIH award (R01AI151004) awarded to O.S.A.

Supplementary Material

Supplementary Table S1
Supplementary Table S2
Supplementary Figure S1
Supplementary Figure S2

References

1. Alphey N, Bonsall MB. Genetics-based methods for agricultural insect pest management. Agric For Entomol 2018;20(2):131–140; doi: 10.1111/afe.12241.
2. Leftwich PT, Spurgin LG, Harvey-Samuel T, et al. Genetic pest management and the background genetics of release strains. Philos Trans R Soc Lond B Biol Sci 2021;376(1818):20190805; doi: 10.1098/rstb.2019.0805.
3. Bushland RC, Lindquist AW, Knipling EF. Eradication of Screw-Worms through release of sterilized males. Science 1955;122(3163):287–288; doi: 10.1126/science.122.3163.287.
4. Scott MJ, Concha C, Welch JB. Review of research advances in the screwworm eradication program over the past 25 years. Entomologia 2017;164(3):226–236.
5. Knipling EF. Possibilities of insect control or eradication through the use of sexually sterile males. J Econ Entomol 1955;48(4):459–462; doi: 10.1093/jee/48.4.459.
6. Dumont Y, Yatat-Djeumen IV. Sterile insect technique with accidental releases of sterile females. Impact on mosquito-borne diseases control when viruses are circulating. Math Biosci 2022;343:108724; doi: 10.1016/j.mbs.2021.108724.
7. Mirieri CK, Mutika GN, Bruno J, et al. A new automated chilled adult release system for the aerial distribution of sterile male tsetse flies. PLoS One 2020;15(9):e0232306; doi: 10.1371/journal.pone.0232306.
8. Li M, Yang T, Bui M, et al. Suppressing mosquito populations with precision guided sterile males. Nat Commun 2021;12(1):5374; doi: 10.1038/s41467-021-25421-w.
9. Papathanos PA, Bourtzis K, Tripet F, et al. A perspective on the need and current status of efficient sex separation methods for mosquito genetic control. Parasit Vectors 2018;11(Suppl. 2):654; doi: 10.1186/s13071-018-3222-9.
10. Lutrat C, Giesbrecht D, Marois E, et al. Sex sorting for pest control: It's raining men! Trends Parasitol 2019;35(8):649–662; doi: 10.1016/j.pt.2019.06.001.
11. Crawford JE, Clarke DW, Criswell V, et al. Efficient production of male Wolbachia-infected *Aedes aegypti* mosquitoes enables large-scale suppression of wild populations. Nat Biotechnol 2020;38(4):482–492; doi: 10.1038/s41587-020-0471-x.
12. Zheng X, Zhang D, Li Y, et al. Incompatible and sterile insect techniques combined eliminate mosquitoes. Nature 2019;572(7767):56–61; doi: 10.1038/s41586-019-1407-9.
13. Meza JS, Bourtzis K, Zacharopoulou A, et al. Development and characterization of a pupal-colour based genetic sexing strain of *Anastrepha fraterculus* sp. 1 (Diptera: Tephritidae). BMC Genet 2020;21(Suppl. 2):134; doi: 10.1186/s12863-020-00932-5.
14. Franz G, Kerremans P. Requirements and strategies for the development of genetic sex separation systems with special reference to the Mediterranean fruit fly *Ceratitis capitata*. In: Fruit Flies and the Sterile Insect Technique. (Calkins CO, Klassen W, Liedo P. eds.) CRC Press: Florida, USA; 2019; pp. 113–122.
15. Willhoeft U, Franz G, McInnis DO. Towards the application of genetic sexing in Tephritid Fruit Fly SIT Programs. In: Fruit Fly Pests. CRC Press: Boca Raton; 2020; pp. 179–184.
16. Meza JS, Schetelig MF, Zepeda-Cisneros CS, et al. Male-specific Y-linked transgene markers to enhance biologically-based control of the Mexican fruit fly, *Anastrepha ludens* (Diptera: Tephritidae). BMC Genomic Data 2014;15(2):1–7; doi: 10.1186/1471-2156-15-S2-S4.
17. Condon KC, Condon GC, Dafa'alla TH, et al. Genetic sexing through the use of Y-linked transgenes. Insect Biochem Mol Biol 2007;37(11):1168–1176; doi: 10.1016/j.ibmb.2007.07.006.
18. Ward CM, Aumann RA, Whitehead MA, et al. White pupae phenotype of tephritids is caused by parallel mutations of a MFS transporter. Nat Commun 2021;12(1):491; doi: 10.1038/s41467-020-20680-5.
19. Focks DA. An improved separator for the developmental stages, sexes, and species of mosquitoes (Diptera: Culicidae). J Med Entomol 1980;17(6):567–568; doi: 10.1093/jmedent/17.6.567.
20. Furlong EE, Profitt D, Scott MP. Automated sorting of live transgenic embryos. Nat Biotechnol 2001;19(2):153–156; doi: 10.1038/84422.
21. Meccariello A, Salvemini M, Primo P, et al. Maleness-on-the-Y (MoY) orchestrates male sex determination in major agricultural fruit fly pests. Science 2019;365(6460):1457–1460; doi: 10.1126/science.aax1318.
22. Primo P, Meccariello A, Inghilterra MG, et al. Targeting the autosomal *Ceratitis capitata* transformer gene using Cas9 or dCas9 to masculinize XX individuals without inducing mutations. BMC Genet 2020;21(Suppl. 2):150; doi: 10.1186/s12863-020-00941-4.
23. Dyck VA, Hendrichs J, Robinson AS. Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management. 2nd ed. (Dyck VA, Hendrichs J, Robinson AS. eds.). CRC Press: London, England; 2021; doi: 10.1201/9781003035572.
24. Davydova S, Liu J, Kandul NP, et al. Next-generation genetic sexing strain establishment in the agricultural pest *Ceratitis capitata*. Sci Rep 2023;13(1):1–8; doi: 10.1038/s41598-023-47276-5.
25. Weng S-C, Antoshechkin I, Marois E, et al. Efficient sex separation by exploiting differential alternative splicing of a dominant marker in *Aedes aegypti*. bioRxiv 2023;2023.06.16.545348; doi: 10.1101/2023.06.16.545348.
26. Boggs RT, Gregor P, Idriss S, et al. Regulation of sexual differentiation in *D. melanogaster* via alternative splicing of RNA from the transformer gene. Cell 1987;50(5):739–747; doi: 10.1016/0092-8674(87)90332-1.
27. Li F, Vensko SP II, Belikoff EJ, et al. Conservation and sex-specific splicing of the transformer gene in the Calliphorids *Cochliomyia hominivorax*, *Cochliomyia macellaria* and *Lucilia sericata*. PLoS One 2013;8(2):e56303; doi: 10.1371/journal.pone.0056303.
28. Shcherbakova DM, Subach OM, Verkhusha VV. Red fluorescent proteins: Advanced imaging applications and future design. Angew Chem Int Ed Engl 2012;51(43):10724; doi: 10.1002/anie.201200408.
29. Matz MV, Fradkov AF, Labas YA, et al. Fluorescent proteins from nonbioluminescent Anthozoa species. Nat Biotechnol 1999;17(10):969–973; doi: 10.1038/13657.
30. Pane A, Salvemini M, Delli Bovi P, et al. The transformer gene in *Ceratitis capitata* provides a genetic basis for selecting and remembering the sexual fate. Development 2002;129(15):3715–3725; doi: 10.1242/dev.129.15.3715.
31. Pane A, De Simone A, Saccone G, et al. Evolutionary conservation of *Ceratitis capitata* transformer gene function. Genetics 2005;171(2):615–624; doi: 10.1534/genetics.105.041004.
32. Perrotta MM, Lucibelli F, Mazzucchiello SM, et al. Female sex determination factors in *Ceratitis capitata*: Molecular and structural basis of TRA and TRA2 recognition. Insects 2023;14(7):605; doi: 10.3390/insects14070605.
33. Blanchette M, Green RE, MacArthur S, et al. Genome-wide analysis of alternative pre-mRNA splicing and RNA-binding specificities of the Drosophila hnRNP A/B family members. Mol Cell 2009;33(4):438–449; doi: 10.1016/j.molcel.2009.01.022.
34. Shaul O. How introns enhance gene expression. Int J Biochem Cell Biol 2017;91(Pt B):145–155; doi: 10.1016/j.biocel.2017.06.016.
35. Inoue K, Hoshijima K, Sakamoto H, et al. Binding of the Drosophila sex-lethal gene product to the alternative splice site of transformer primary transcript. Nature 1990;344(6265):461–463; doi: 10.1038/344461a0.
36. Valcárcel J, Singh R, Zamore PD, et al. The protein Sex-lethal antagonizes the splicing factor U2AF to regulate alternative splicing of transformer pre-mRNA. Nature 1993;362(6416):171–175; doi: 10.1038/362171a0.
37. Saccone G, Peluso I, Artiaco D, et al. The *Ceratitis capitata* homologue of the Drosophila sex-determining gene sex-lethal is structurally conserved, but

not sex-specifically regulated. Development 1998;125(8):1495–1500; doi: 10.1242/dev.125.8.1495.
38. Bopp D, Saccone G, Beye M. Sex determination in insects: Variations on a common theme. Sex Dev 2014;8(1–3):20–28; doi: 10.1159/000356458.
39. Ruiz MF, Milano A, Salvemini M, et al. The gene transformer of anastrepha fruit flies (Diptera, tephritidae) and its evolution in insects. PLoS One 2007; 2(11):e1239; doi: 10.1371/journal.pone.0001239.
40. Yusa K. piggyBac Transposon. Microbiol Spectr 3. MDNA3-0028-2014; 2015.
41. Masumoto M, Ohde T, Shiomi K, et al. A Baculovirus immediate-early gene, ie1, promoter drives efficient expression of a transgene in both *Drosophila melanogaster* and Bombyx mori. PLoS One 2012;7(11):e49323; doi: 10.1371/journal.pone.0049323.
42. Theilmann DA, Stewart S. Molecular analysis of the trans-activating IE-2 gene of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. Virology 1992;187(1):84–96; doi: 10.1016/0042-6822(92)90297-3.
43. Jarvis DL, Weinkauf C, Guarino LA. Immediate-early baculovirus vectors for foreign gene expression in transformed or infected insect cells. Protein Expr Purif 1996;8(2):191–203; doi: 10.1006/prep.1996.0092.
44. Chudakov DM, Matz MV, Lukyanov S, et al. Fluorescent proteins and their applications in imaging living cells and tissues. Physiol Rev 2010;90(3): 1103–1163; doi: 10.1152/physrev.00038.2009.
45. Verhulst EC, van de Zande L, Beukeboom LW. Insect sex determination: It all evolves around transformer. Curr Opin Genet Dev 2010;20(4):376–383; doi: 10.1016/j.gde.2010.05.001.
46. Gendron W, Raban R, Mondal A, et al. Cost-effectiveness of precision guided SIT for control of *Anopheles gambiae* in the Upper River Region, The Gambia. bioRxiv 2023;2023.07.20.549762; doi: 10.1101/2023.07.20.549762.
47. Gasque G, Conway S, Huang J, et al. Small molecule drug screening in Drosophila identifies the 5HT2A receptor as a feeding modulation target. Sci Rep 2013;3:srep02120; doi: 10.1038/srep02120.
48. Kandul NP, Liu J, Hsu AD, et al. A drug-inducible sex-separation technique for insects. Nat Commun 2020;11(1):2106; doi: 10.1038/s41467-020-16020-2.
49. Kandul NP, Liu J, Sanchez C HM, et al. Transforming insect population control with precision guided sterile males with demonstration in flies. Nat Commun 2019;10(1):84; doi: 10.1038/s41467-018-07964-7.
50. Smith RC, Walter MF, Hice RH, et al. Testis-specific expression of the beta2 tubulin promoter of *Aedes aegypti* and its application as a genetic sex-separation marker. Insect Mol Biol 2007;16(1):61–71; doi: 10.1111/j.1365-2583.2006.00701.x.
51. Gamez S, Chaverra-Rodriguez D, Buchman A, et al. Exploiting a Y chromosome-linked Cas9 for sex selection and gene drive. Nat Commun 2021;12(1):7202; doi: 10.1038/s41467-021-27333-1.
52. Porras MF, Meza JS, Rajotte EG, et al. Improving the phenotypic properties of the *Ceratitis capitata* (Diptera: Tephritidae) temperature-sensitive lethal genetic sexing strain in support of sterile insect technique applications. J Econ Entomol 2020;113(6):2688–2694; doi: 10.1093/jee/toaa220.
53. Augustinos AA, Misbah-Ul-Haq M, Carvalho DO, et al. Irradiation induced inversions suppress recombination between the M locus and morphological markers in *Aedes aegypti*. BMC Genet 2020;21(Suppl. 2):142; doi: 10.1186/s12863-020-00949-w.
54. Zacharopoulou A, Augustinos AA, Drosopoulou E, et al. A review of more than 30 years of cytogenetic studies of Tephritidae in support of sterile insect technique and global trade. Entomol Exp Appl 2017;164(3):204–225; doi: 10.1111/eea.12616.
55. Franz G. Genetic sexing strains in Mediterranean fruit fly, an example for other species amenable to large-scale rearing for the sterile insect technique. In: Sterile Insect Technique: Principles and Practice in Area-Wide Integrated Pest Management. (Dyck VA, Hendrichs J, Robinson AS. eds.) Springer Netherlands: Dordrecht; 2005; pp. 427–451; doi: 10.1007/1-4020-4051-2_16.
56. Fisher K, Caceres C. A FILTER REARING SYSTEM FOR MASS REARED MEDFLY K. Fisher. n.d. Available from: https://www.osti.gov/etdeweb/servlets/purl/683040 [Last accessed: November 15, 2023].
57. Caceres C. Filter rearing system for sterile insect technology. In: Encyclopedia of Entomology. Springer Netherlands: Dordrecht; 2005; p. 838; doi: 10.1007/0-306-48380-7_1619.
58. Coutelis JB, Petzoldt AG, Spéder P, et al. Left-right asymmetry in Drosophila. Semin Cell Dev Biol 2008;19(3):252–262; doi: 10.1016/j.semcdb.2008.01.006.

Received: August 11, 2023
Accepted: January 09, 2024
Online publication date: February 20, 2024