Efficient Sex Separation by Exploiting Differential Alternative Splicing of a Dominant Marker in *Aedes aegypti*

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Abstract
Only female mosquitoes consume blood and transmit deadly human pathogens. Therefore, it is critical to remove females before conducting releases for genetic biocontrol interventions. Here we describe a robust sex-sorting approach termed SEPARATOR (Sexing Element Produced by Alternative RNA-splicing of A Transgenic Observable Reporter) that exploits sex-specific alternative splicing of an innocuous reporter to ensure exclusive dominant male-specific expression. Using SEPARATOR, we demonstrate reliable sex selection from larval and pupal stages in *Aedes aegypti*, and use a Complex Object Parametric Analyzer and Sorter (COPAS®) machine to demonstrate scalable high-throughput sex-selection of first instar larvae and beyond. SEPARATOR can simplify mass production of males for release approaches and is designed to be cross-species portable for streamlined development in other species and should be instrumental for genetic biocontrol interventions.

Introduction

The mosquito is the most deadly animal on the planet, estimated to have killed roughly 50% of humans since the dawn of humanity[1]. This year alone, roughly 700 million cases of mosquito transmitted diseases worldwide will occur resulting in over a million deaths[2]. Insecticides and larvicides have traditionally been used to control mosquitoes, however mosquitoes have evolved resistance[3–7], and harmful chemicals are unfortunately poisoning our environment and killing non-target species[8–10]. Moreover, a warming global climate is expanding habitable ranges of mosquitoes and their pathogens[11–13]. Consequently, the number of people at risk of contracting mosquito transmitted pathogens continues to rise, indicating innovative technologies are critical to combat this global threat.

To genetically suppress mosquito populations, several biocontrol strategies have been developed including the classical sterile insect technique (SIT)[14], release of insects carrying a dominant lethal (RIDL)[15], female specific RIDL (fsRIDL)[16–20], Wolbachia-based incompatible insect technique (IIT)[21], the precision guided sterile insect technique (pgSIT)[22], and homing-based suppression gene drives[23]. The ultimate goal for each of these approaches is to scalably release modified mosquitoes into the environment to achieve sustained species-specific population suppression. Given that only female mosquitoes consume blood and transmit pathogens, exclusive release of male-only cohorts is really important. While some of these approaches have built-in self-sexing mechanisms (e.g. gene drives[23], fsRIDL[20], pgSIT[22]), others require the manual separation of females prior to release (e.g. classical SIT, Wolbachia-based IIT[21]) significantly reducing scalability. Moreover, for Wolbachia-based IIT even accidental releases of a few females can undermine the intervention [24,25], underscoring the imminent need for reliable sexing.

Sex-sorting mosquitoes can be done manually by hand, however this is labor intensive, error-prone, and not scalable for genetic biocontrol interventions. In *Aedes* and *Culex* mosquitoes, sexual dimorphism of pupa size can aid sex separation [26,27], however pupal size is density-dependent requiring optimized rearing conditions. To overcome this limitation, AI-assisted optical adult sex-sorting systems have been developed for *Aedes aegypti* that can differentiate adults based upon sexual dimorphic features with high accuracy[28]. However these have only been developed in *Aedes aegypti* and require sorting at the adult stage which is not ideal, slow, and less scalable. Alternatively, selectable markers have been genetically linked to sex chromosomes in *Anopheles*[29–33], or to sex-determining loci in *Aedes*[34–36]. However, genetic linkage is often broken by meiotic recombination, or translocations, making these strains less stable when scaled [14,37]. Strains have also been developed using sex specifically expressed promoters in *Aedes*[38,39], and *Anopheles*[19,40], to express fluorescent proteins in the gonads, however given the small size of the gonads the expression is often difficult to see and is stage-specific[41]. There also has been limited efforts to exploit sex-specific alternative splicing (SSAS) to enable red fluorescent protein (RFP) expression in the flight muscles in *Aedes*[17] or to bias green fluorescent protein (GFP) expression in larvae in *Anopheles*[42,43]. However, these sexing strains are engineered using species-specific components and are therefore less portable across species. Additionally, the expression patterns are often not strong enough, or early enough, to allow for reliable sorting in initial larval stages which would be ideal for genetic biocontrol interventions. Taken together, the development of simple, stable, and reliable sex-sorting technologies that can be facilely engineered in multiple mosquito species and enable early larval sex sorting remains to be developed.

To fill this void, we developed an innovative sex-sorting approach that: (i) is based on innocuous bright fluorescent markers; (ii) enables sex-sorting during early larval development and beyond; (iii) is adaptable for high-throughput sorting; (iv) does not rely on sex-chromosome linkage; (v) is genetically stable and not prone to breakage by meiotic recombination or chromosomal rearrangements; (vi) enables positive selection of males to further increase robustness; and (vi) is facilely portable to other species as it utilizes transposable elements and promoters and markers that have been shown to work in many species. We call our approach SEPARATOR (Sexing Element Produced by Alternative RNA-splicing of A Transgenic Observable Reporter). SEPARATOR exploits SSAS of a widely expressed fluorescent reporter to provide positive selection of males that can be easily integrated into genetic biocontrol interventions. Using SEPARATOR, we demonstrate positive identification of male larvae in *Aedes* mosquitoes, as early as embryos and first instar larva and beyond. By harnessing the capabilities of COPAS, we demonstrate sorting of up to 740 larvae/minute with a female contamination rate ranging from 0.01 to 0.03%. Taken together, this method provides a much needed tool to scale and reliably release males for genetic biocontrol interventions.

Results

Engineering SEPARATOR

To generate SEPARATOR a sex-specific alternatively spliced intron derived from the *Ae. aegypti doublesex* (*AaeDsx*) gene was utilized (**Fig. 1A and ig. S1A**). Dsx is a highly conserved transcription factor involved in sex determination of insects. In *Ae. aegypti* the male specific *AaeDsx* intron is ~26.5 kb which is a bit large to work with. Therefore, we truncated this intron by preserving splicing factor binding sites including Tra/Tra-2 and RNA binding protein 1 (RBP1) binding sites to retain the sex-specificity of this intron [44]. This resulted in a smaller *AaeDsx* intron of 4.5 kb in size (**Fig. 1A and fig. S1A**). The reading frame was initiated by adding a start codon with a Kozak sequence, expressed using a constitutive *Hr5IE1 Ac*MNPV baculovirus promoter previously shown to work in many species [45–52]. To open the reading frame, nine stop codons located in endogenous exon 5b were removed (**table S6**). The coding sequences for EGFP and DsRed were strategically designed to overlap, allowing for their expression in a sex-specific manner. The DsRed coding sequence was designed to be in-frame with a female-specific product (exon4, engineered exon5b, and exon6) to control female-specific DsRed expression. In addition, the male-specific splicing product, involving exon 4 and exon 6, was designed to be in-frame with the EGFP coding sequence (**fig. S1**).

The SEPARATOR construct was then introduced into the mosquito genome to generate a genetic sex-sorting strain via the piggyBac transposon. The intended plan was to ensure that all mosquitoes expressing GFP would be male, while those expressing DsRed would be female. Interestingly, the results following microinjection revealed that all 55 EGFP-expressed larvae were male at the pupal stage in G0 (**table S1**). However, no DsRed-expressed larvae were observed in G0. All the pupae from G0 were sexed and resulting adults were crossed with wild-type mosquitoes, and stable transgenic lines were selected by the fluorescence marker (G1). In G1-onward, similar results were observed, where 100% of the EGFP-positive larvae were male.

To validate the sex-specific splicing pattern of the *AaeDsx* splicing module, a comprehensive analysis was carried out utilizing reverse transcription polymerase chain reaction (RT-PCR) and RNA sequencing. A total of fifty EGFP-positive L1 larvae and fifty EGFP-negative L1 larvae were collected for further analysis. To perform RT-PCR, we utilized primers designed to target the 3' end of the *Hr5IE1* promoter and the 5' end of the EGFP sequence (**fig. S1A**). Subsequently, Sanger sequencing was carried out to analyze the obtained PCR products. Our results showed that the utilization of truncated intron 4, engineered exon 5b, intron 6, and exon 6 sequences in RNA splicing exhibited sex-specificity (**fig. S1B**). Both Sanger sequencing and RNA sequencing (RNA-seq) analysis yielded comparable results, aligning with our anticipated splicing patterns (**fig. S1B and fig. S2**). The sex-specific RNA splicing pattern indicated the female splicing product being in-frame with the DsRed coding sequence, while the male splicing product resulted in (-1) frameshift, leading to the DsRed coding sequence being out of the frame and in-frame with the EGFP coding sequence. Interestingly, both the RT-PCR and RNA-seq analyses revealed that the predominant products observed in females comprised exon4, exon5b, and exon6 (**fig. S1B and C and table S3**). Notably, these exons were found to be in-frame with the DsRed coding sequence (**fig. S1B and fig. S2**). Therefore, it can be inferred that the expression level of transcripts specific to females and the splicing pattern of female-specific products do not present any obstacles to DsRed expression. However, DsRed signals were not observed in the transgenic larvae.

We determined transgene integration sites (**fig. S3**) and generated a homozygous line. To do this, larvae were sorted by fluorescence and separated into two groups, EGFP-positive and EGFP-negative, then the sex ratio of these two groups was examined at the pupal/adult stage. The EGFP-positive males were crossed to EGFP-negative females to enrich for homozygotes (**Fig. 1B**). In 15 generations, we manually screened a total of 3635 EGFP+ larvae and confirmed the resulting sex at the pupa and adult stage. Remarkably, 100% of the GFP+ larvae were male (**Fig. 1C and table S1**). In summary, the results demonstrate that the SEPARATOR technology is an efficient and effective way of separating male (EGFP+) and female mosquitoes (EGFP-), which could have important implications for broad adaptation of SIT for mosquito control.

Measuring Efficacy of Sex Sorting at Scale

To evaluate the effectiveness of the sex sorting system, we manually sorted L1 larvae into two groups (EGFP-positive and EGFP-negative) based on GFP fluorescence. The sex ratio of these two groups was then examined at both pupal/ adult stage. Over 15 generations (G0 to G14), it was found that the EGFP-positive larvae were 100% male (**Fig. 1C and table S1**). We next determined the timing of expression of EGFP. We found that strong EGFP signals were expressed from late embryo all the way through to the adult stage of the mosquitoes (**Fig. 1D and E**). In addition, when compared to the wild-type control (Liverpool), the EGFP intensity of SEPARATOR mosquitoes is strong enough to differentiate between EGFP-positive (male) and EGFP-negative (female) in a pooled condition from the early stage of mosquito's life cycle (**Fig. 1D**). Based on these results, it can be concluded that SEPARATOR is a powerful and robust system for mosquito sex sorting.

To evaluate the suitability of SEPARATOR for automated sex sorting, we conducted fluorescence-based flow cytometry sorting on batches of several thousand first instar larvae using

COPAS. This generated a fluorescence diagram where male larvae expressing EGFP formed a distinct cluster, clearly separate from the GFP negative female larvae (**Fig. 2 and fig. S4**). The EGFP-positive larvae were selected and sorted in pure mode at flow rates ranging from 20 to 70 objects per second. Even at this high flow rate, we were able to recover 70-80% of the EGFP-positive larvae, resulting in sorting speeds of 740 larvae/minute **table S2**). In total, 108,570 larvae were sorted using COPAS from generations G7 to G9. After a single sorting, a single instance of 0.1 to 0.45% contamination of EGFP-negative (female) larvae was observed within the male population (**table S2**). While slower flow rates could potentially yield a more complete recovery of males and minimize or eliminate contamination with females, the high sorting speed represents a compromise between recovery rates and the production speed required for mass production[53]. To address the issue of female contamination, additional measures were implemented. Through quality control sorts, the contamination rate of EGFP-negative larvae (females) was successfully reduced to 0.01-0.03% (**table S2**). This was accomplished by subjecting the sorted larvae, a total of 78,861 individuals at G9, to a second round of sorting using the COPAS. This second sorting phase specifically targeted objects that deviated from the EGFP-positive gate, employing the "enrich mode" for enhanced precision. It is worth noting that these experiments were conducted using an older SELECT COPAS instrument model from 2005. Utilizing a more modern COPAS instrument with an upgraded laminar flow and electronic controls, is expected to further reduce these reported female contamination rates. Taken together, this method allowed us to efficiently and effectively sort a large number of larvae, providing valuable insights into the performance of our sex sorting system.

Sex-enriched genes identification

To identify genes that exhibited sex-specific expression patterns, providing insights into the molecular mechanisms underlying sex determination and differentiation in *Ae. aegypti* mosquitoes, we used SEPARATOR to sort male and female L1 larvae and performed RNA sequencing. Using the data we collected, we conducted a comprehensive analysis of differential gene expression (DGE). Our findings revealed that, at the early L1 larvae stage, 1082 genes exhibited male-enriched expression patterns, while 634 genes exhibited female-enriched expression patterns (**fig. S5**). Subsequently, the sex-enriched genes were subjected to enrichment analysis. Among the male-enriched genes, two distinct clusters emerged based on Gene Ontology (GO) terms. The first cluster was related to cilium and microtubule formation (including terms such as cilium, axoneme, microtubule-based process, and cell projection), while the second cluster was associated with cuticle formation (**fig. S5 and table S5**). Moreover, among the female-enriched genes, several distinct clusters emerged based on GO terms, including immune response, metabolic processing, and cuticle formation (**fig. S5 and table S5**).

Discussion

Sex separation is crucial in the process of utilizing insects for genetic biocontrol[14]. To make this approach more sustainable and cost-effective, it is important to improve methods for sex separation. In this study, we present a highly efficient method for selecting male larvae in *Aedes* mosquitoes called SEPARATOR. This approach utilizes a COPAS instrument to positively select male L1 larvae that express a dominant male-specific reporter gene (EGFP). This is a positive-

selection system, meaning that only those larvae expressing the dominant male-specific GFP are precision sorted. Therefore, if EGFP becomes mutated or breaks, those individuals will not be selected. Moreover, the use of a male spliced intron further increases the robustness since females will not have the machinery to properly splice this intron to generate EGFP.

In comparison to existing techniques, SEPARATOR has several advantages, including its exceptional efficiency, precision, and potential for portability across multiple species. This is due to the evolution conserved sex determination gene, Dsx. In the past, sex sorting relied on morphological differences between male and female mosquitoes during their pupal and adult stages to differentiate between the two sexes. While this method is effective, it does require a significant amount of human effort and time. Unfortunately, this makes it challenging to automate the process on a large scale, which could be a hindrance. Therefore, we need new ways to differentiate between male and female mosquitoes which are faster and more efficient.

In *Aedes*, there is a distinct size difference between female and male pupae, with females being larger. This characteristic allows for pupae to be size-sorted through a sieve, which serves as the first step in sex-sorting. While this method is effective in separating the sexes, up to 2-5% of female pupae can still remain in the male-enriched batch. To further reduce the number of female mosquitoes, a secondary sex-sorting step is employed using an AI-assisted optical system[27,28]. This system utilizes visual cues from sexual dimorphic differences in adult mosquito morphology to identify and remove any remaining female mosquitoes. This second step significantly reduces female contamination rates to an incredibly low level of 0.02% to $1.13 \times 10^{-7}\%$[54]. However, it should be noted that this step is done at the adult stage, which is not ideal for sorting as adults are very fragile. Furthermore, the COPAS instrument is capable of high-throughput selection, able to sort up to 36K larvae per hour which really makes this a scalable approach.

In the *Aedes* mosquito, a Nix expression line, which expresses a male determining factor, has been shown to positively select male mosquitoes [53,55,56]. These specific lines facilitate the identification and segregation of male mosquitoes within a population. Despite a slight reduction in mating competitiveness, these lines exhibit promise as robust Genetic Sexing Strains (GSS)[53]. Nevertheless, these transformed males were unable to engage in flight in *Ae. aegypti*, mainly due to the absence of myo-sex, a closely associated gene found in the M-locus that encodes a flight muscle myosin specific to males[57]. This issue will pose challenges in terms of maintenance and transgene delivery. Interestingly, in *Ae. albopictus*, one or more endogenous copies of myo-sex-like genes, which are not linked to the M-locus, are significantly activated in pseudo-males. As a result, pseudo-males of *Ae. albopictus* exhibited proficient flying abilities[53].

Additionally, the use of Nix expression for sexing in *Aedes* mosquitoes is limited by its species specificity. This means that Nix expression can only be used to accurately sex mosquitoes of the *Aedes* species and cannot be applied to other mosquito species. Additionally, due to the fact that the Nix expression mosquito population is composed entirely of males, the production of homozygous individuals using the Nix expression line is unattainable. Consequently, this approach fails to effectively sex the entire mosquito population as it consistently generates wild-type mosquitoes in each generation. Moreover, in the majority of vector control strategies, homozygous mosquitoes possess greater efficiency owing to the presence of two copies of the

transgene. Therefore, using Nix overexpression for sex sorting has limitations and cannot be used in all cases, and researchers should consider these limitations when utilizing this technique.

There were a few unexpected results associated with SEPARATOR. During the 15 generations reported, no DsRed-expressed mosquitoes were observed. Previous research has shown that in *D. melanogaster* and *Ae. aegypti*, the presence of the fruitless (Fru) protein is not detected in females, despite the expression of the corresponding transcripts [58–60]. Furthermore, a study conducted in *D. melanogaster* has revealed that the translation of the *fru* female splicing product is suppressed by the Tra protein[59]. This finding suggests that the transcripts specific to females are not translated. In *D. melanogaster*, both Dsx and Fru function as sex-determining factors and are regulated by the Tra protein. Nevertheless, Proving the inability of a gene to undergo translation presents significant challenges. As a result, uncertainty persists regarding whether Dsx follows a comparable mechanism of translation inhibition, emphasizing the necessity for additional investigation. Fortunately, the male-specific product of *AaeDsx* demonstrates successful functionality (**Fig. 1C-E**). Additionally, our objective is to generate a positively selected population of males to enhance reliability and ensure accurate sex sorting. Hence, considering the next generation of SEPARATOR, utilizing it for positive male selection and incorporating a ubiquitous expression system for a transgenic marker could be a viable approach.

SEPARATOR is robust and can be easily adapted for use in both classical Sterile Insect Technique (SIT) and *Wolbachia*-based Insecticide-Treated Insect Technique (IIT) [21,24,25](**Fig. 3**). The current method for breeding male mosquitoes involves culturing both sexes to the pupal or adult stage for sex sorting, after which the female mosquitoes are removed. However, this method is not as efficient as SEPARATOR. SEPARATOR utilizes less time for male-sorting at the L1 larvae stage, making it a more efficient use of breeding space and feed to raise male mosquitoes. With this system, researchers and breeders can save time and resources by identifying and separating male mosquitoes at an early stage of development. This allows for a more efficient and cost-effective method of breeding and raising male mosquitoes for research and other purposes. Taken together, SEPARATOR provides a valuable tool to help revolutionize the way we sort sexes, making the process more efficient, cost-effective, and reliable and may prove to be a valuable addition to genetic biocontrol programs.

Materials and Methods

Molecular Cloning and Transgenesis

To create the endogenous AaeDsx splicing module construct, we amplified the fragment of endogenous exons and introns from the genomic DNA of *Ae. aegypti* using PCR. We then linearized the previous mCherry and EGFP containing plasmid, 1122I, using the restriction enzyme PacI. The linearized 1122I plasmid and the fragment of endogenous exons and introns were used in a Gibson enzymatic assembly method to build the 1174CX plasmid. To open the reading frame of the female-specific product and allow for in-frame expression of the mCherry coding sequence, we substituted the endogenous exon 5b with an engineered exon 5b that had the stop codons removed. The sequence of the engineered exon 5b was synthesized using the gBlocks® Gene Fragment service. We removed the endogenous exon 5b by cutting it with the restriction enzymes PmlI and SnaBI, and then used Gibson assembly to incorporate the engineered exon 5b containing fragment into the cut 1174CX plasmid, resulting in the 1174C plasmid. To generate a plasmid for sex-specific expression of DsRed and EGFP, we amplified the fragment of the engineered exon 5b and DsRed coding sequences from the 1174C plasmid and the previous 874Y plasmid. We then fused these two fragments together using overlapping PCR. Finally, we used omega PCR to substitute the sequence from the mCherry coding sequence to the 3xP3 promoter in the 1174C plasmid with the DsRed coding sequence, resulting in the 1174D plasmid. During each cloning step, we selected single colonies and cultured them in LB medium with ampicillin. We then extracted the plasmids (using the Zymo Research Zyppy plasmid miniprep kit) and performed Sanger sequencing. The final plasmids were maxi-prepped (using the Zymo Research ZymoPURE II Plasmid Maxiprep kit) and fully sequenced by Primordium. All primers are listed in **table S6**. The complete annotated plasmid sequences and plasmid DNA are available at Addgene (ID: 200012). Transgenic lines were created by microinjecting preblastoderm stage embryos with a mixture of the piggyBac plasmid and a transposase helper plasmid. Four days after microinjection, the G0 embryos were hatched and the surviving pupae were separated and sexed. The pupae were placed in separate cages for males and females, along with wild-type male pupae in the female cages and wild-type female pupae in the male cages, in a 5:1 ratio. After several days to allow for development and mating, a blood meal was provided and eggs were collected, aged, and hatched. The larvae with positive fluorescent markers were isolated using a fluorescent stereomicroscope. To isolate separate insertion events, male transformants with fluorescent markers were crossed with female transformants without fluorescent markers, and separate lines were established. The individual genetic sexing lines (1174D) were maintained as mixtures of homozygotes and heterozygotes, with periodic elimination of wild-type individuals. The genetic sexing line (1174D) was homozygosed through approximately ten generations of single-pair sibling matings, selecting individuals with the brightest marker expression each generation.

Mosquito Rearing and Maintenance

Ae. aegypti mosquitoes were obtained from the Liverpool strain, which was previously used to generate the reference genome[61]. These mosquitoes were raised in incubators at 30°C with 20-40% humidity and a 12-hour light/dark cycle in cages (Bugdorm, 24.5 x 24.5 x 24.5 cm). Adults were given 10% (m/V) aqueous sucrose ad libitum, and females were given a blood meal by feeding on anesthetized mice for approximately 15 minutes. Oviposition substrates were provided about 3 days after the blood meal. Eggs were collected, aged for about 4 days to allow for

embryonic development, and then hatched in deionized water in a vacuum chamber. Approximately 400 larvae were reared in plastic containers (Sterilite, 34.6 x 21 x 12.4 cm, USA) with about 3 liters of deionized water and fed fish food (TetraMin Tropical Flakes, Tetra Werke, Melle, Germany). For genetic crosses, female virginity was ensured by separating and sexing the pupae under the microscope based on sex-specific morphological differences in the genital lobe shape (at the end of the pupal abdominal segments just below the paddles) before releasing them to eclose in cages. These general rearing procedures were followed unless otherwise noted. In order to increase the number of homozygotes in the 1174D transgenic line, we transferred both the high-intensity GFP pupae and female GFP-negative pupae to a cage and allowed them to mate after eclosion. Female mosquitoes were fed a blood meal, and five adult females were individually transferred to egg tubes for colonization and egg collection. The eggs from each colony were hatched and reared. The colonies with a higher proportion of female EGFP negatives and male EGFP positives were selected for colonization in the next generation. After a few rounds of colonization, the colonies with 100% of males in the strong EGFP positive group and 100% of females in the GFP negative group were selected and propagated for expansion.

COPAS Fluorescent Sorting, Sexing and Imaging

To determine the precise number of larvae in COPAS clusters, the COPAS raw data was filtered based on the optical density and size measurements of the individuals, "log(EXT)" and "log(TOF)," to remove outliers such as egg debris and dust. Then, a filter was applied based on the individuals' fluorescence measurements, "log(first fluorescence)" and "log(second fluorescence)", to further refine the data. Finally, the fluorescence measurements were automatically clustered and denoised using Density-Based Spatial Clustering of Applications with Noise (DBSCAN)[62,63]. The analysis of COPAS data was conducted using the web-based app, Floreada.io (https://floreada.io/). COPAS sorting was performed largely as described for *Anopheles* larvae[43]. *Aedes* eggs stuck to their egg laying paper were briefly rinsed to eliminate dust and debris, immersed in deionized water in a small container, and their hatching was stimulated under partial vacuum (25% of atmospheric pressure) in a vacuum chamber for 30-60 minutes. They were then incubated overnight at 28°C to maximize larval hatching. On the next day, resulting unfed neonate larvae were transferred to the reservoir of a large particle flow cytometry COPAS SELECT instrument (Union Biometrica, Holliston, MA, USA) equipped with a multiline argon laser (488, 514 nm) and a diode laser (670 nm). Larvae were analyzed and sorted with the Biosort5281 software using a 488 nm filter and the following acquisition parameters: Green PMT 500, Red PMT 600, Delay 8; Width 6, pure mode with superdrops. Flow rate was kept between 20 and 70 objects per second through adjusting of the concentration of larvae in the sample. Larvae identified as males (GFP positive) were dispensed in a Petri dish. In these conditions, sorting speed ranged from 4000 to 7400 larvae in 10 minutes (+ 6 minutes for system initialization and 6 minutes for system cleaning and shutdown), the total number of sorted larvae being limited by the number of available larvae. Sorted larval counts provided by the COPAS software were recorded on sorting. For quality control of sorted larvae, the reservoir and fluidics of the instrument were carefully rinsed and the sorted larvae analyzed by passing them once more in the machine. In some experiments, objects falling outside the GFP positive gate were collected in “Enrich” mode to remove GFP negative contaminants from the pool of GFP positive larvae, and verified by microscopy. Mosquitoes were examined, scored, and imaged using the Leica M165FC fluorescent stereomicroscope equipped with the Leica DMC2900 camera. For higher-resolution images, we used a Leica DM4B upright microscope equipped with

a VIEW4K camera. To distinguish between male and female pupae in mosquitoes, we used a microscope to observe the sex-specific morphological differences in the genital lobe shape located at the end of the pupal abdominal segments just below the paddles. This allowed us to ensure that we were selecting both male and female pupae for our experiments.

Determination of Genome Integration Sites

To determine the transgene insertion sites, we performed Oxford Nanopore genome DNA sequencing. We extracted genomic DNA using the Blood & Cell Culture DNA Midi Kit (Qiagen, Cat. No. / ID: 13343) from 5 adult males and 5 adult females of SEPARATOR, following the manufacturer's protocol. The sequencing library was prepared using the Oxford Nanopore SQK-LSK110 genomic library kit and sequenced on a single MinION flowcell (R9.4.1) for 72 hrs. Basecalling was performed with ONT Guppy base calling software version 6.4.6 using dna_r9.4.1_450bps_sup model generating 3.03 million reads above the quality threshold of Q≧10 with N50 of 7941 bp and total yield of 11.08 Gb. To identify transgene insertion sites, nanopore reads were mapped to plasmids carrying SEPARATOR (1174D, Addgene as plasmid #200012) using minimap2[64] and further aligned them to the AaegL5.0 genome (GCF_002204515.2). Subsequently, we calculated the average depth of coverage for the three autosomes and the transgene using samtools and visualized the results in R. The coverage depths for chr1, chr2, and chr3 were determined to be 6.31, 6.30, and 6.08, respectively. Interestingly, the coverage depth for SEPARATOR transgene was notably higher at 16.14. Based on the coverage analysis, it appears that the SEPARATOR transgene is present in three copies (**fig. S3**). By examining the read alignments using Interactive Genomics Viewer (IGV), we were able to determine the exact insertion sites. Notably, we identified three copies of the SEPARATOR constructs sequence. The three integration sites are NC_035109.1:92046983, NC_035108.1:444508475 and NC_035107.1:299022928. This finding aligns with the results of the depth of coverage analysis, further supporting the presence of three insertion sites. The second integration site on NC_035108.1 overlaps with the AAEL005024 gene, which is currently classified as an uncharacterized protein. However, the other two integration sites do not overlap with any known genes. The nanopore sequencing data has been deposited to the NCBI SRA.

RNA sequencing (RNA-seq) analysis

To quantify target gene reduction and expression from transgenes as well as to assess global expression patterns, we performed Illumina RNA sequencing. We extracted total RNA using miRNeasy Tissue/Cells Advanced Mini Kit (Qiagen, Cat. No. / ID: 217604) from 50 GFP-positive male mosquitoes and 50 GFP-negative female mosquitoes at L1 larva stage in biological triplicate (6 samples total), following the manufacturer's protocol. Genomic DNA was depleted using the gDNA eliminator column provided by the kit. RNA integrity was assessed using the RNA 6000 Pico Kit for Bioanalyzer (Agilent Technologies, Cat. No. / ID: #5067-1513), and mRNA was isolated from ~1 μg of total RNA using NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB, Cat. No. / ID: E7490). RNA-seq libraries were constructed using the NEBNext Ultra II RNA Library Prep Kit for Illumina (NEB, Cat. No. / ID: E7770) following the manufacturer's protocols. Briefly, mRNA was fragmented to an average size of 200 nt by incubating at 94°C for 15 min in the first strand buffer. cDNA was then synthesized using random primers and ProtoScript II Reverse Transcriptase followed by second strand synthesis using NEB Second Strand Synthesis Enzyme Mix. Resulting DNA fragments were end-repaired, dA tailed, and ligated to NEBNext hairpin adaptors (NEB, Cat. No. / ID: E7335). Following ligation,

adaptors were converted to the "Y" shape by treating with USER enzyme, and DNA fragments were size selected using Agencourt AMPure XP beads (Beckman Coulter #A63880) to generate fragment sizes between 250-350 bp. Adaptor-ligated DNA was PCR amplified followed by AMPure XP bead clean up. Libraries were quantified using a Qubit dsDNA HS Kit (ThermoFisher Scientific, Cat. No. / ID: Q32854), and the size distribution was confirmed using a High Sensitivity DNA Kit for Bioanalyzer (Agilent Technologies, Cat. No. / ID: 5067- 4626). Libraries were sequenced on an Illumina NextSeq2000 in paired end mode with the read length of 50 nt and sequencing depth of 25 million reads per library. Base calls and FASTQ generation were performed with DRAGEN 3.8.4. The reads were mapped to the AaegL5.0 genome (GCF_002204515.2) supplemented with SEPARATOR transgene sequences using STAR. On average, ~97.4% of the reads were mapped. The analysis of RNA-Seq data was performed using an integrated web application called iDEP[65]. TPM values were calculated from counts produced by feature counts and combined (**table S4**). Hierarchical clustering of the data shows that for each genotype, all replicates cluster together, as expected (**fig. S5A and B**). DESeq2 was then used to perform differential expression analyses between male (GFP-positive) and female (GFP-negative) at L1 larvae stage (**fig. S5C**). For each DESeq2 comparison, gene ontology enrichments were performed on significantly differentially expressed genes. (**fig. S5D and E and table S5**). Illumina RNA sequencing data has been deposited to the NCBI-SRA.

Statistical analysis

Statistical analysis was performed in Prism9 for macOS by GraphPad Software, LLC. At least three biological replicates were used to generate statistical means for comparisons.

Data availability

Complete sequence maps and plasmids are deposited at Addgene.org (#200012). All Illumina and Nanopore sequencing data has been deposited to the NCBI-SRA. All data used to generate figures are provided in the Supplementary Materials/Tables. Generated transgenic lines are available upon request to O.S.A.

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Author Contributions

O.S.A and S.C.W. conceptualized and designed experiments; S.C.W. performed molecular analyses, and genetic experiments; I.A. performed bioinformatics: S.C.W, analyzed and compiled the data. E.M. performed the COPAS experiments and analysis. All authors contributed to writing and approved the final manuscript.

Ethical conduct of research

All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals as recommended by the National Institutes of Health and approved by the UCSD Institutional Animal Care and Use Committee (IACUC, Animal Use Protocol #S17187) and UCSD Biological Use Authorization (BUA #R2401).

Disclosures

O.S.A. and S.W. have filed a patent on this technology. 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.

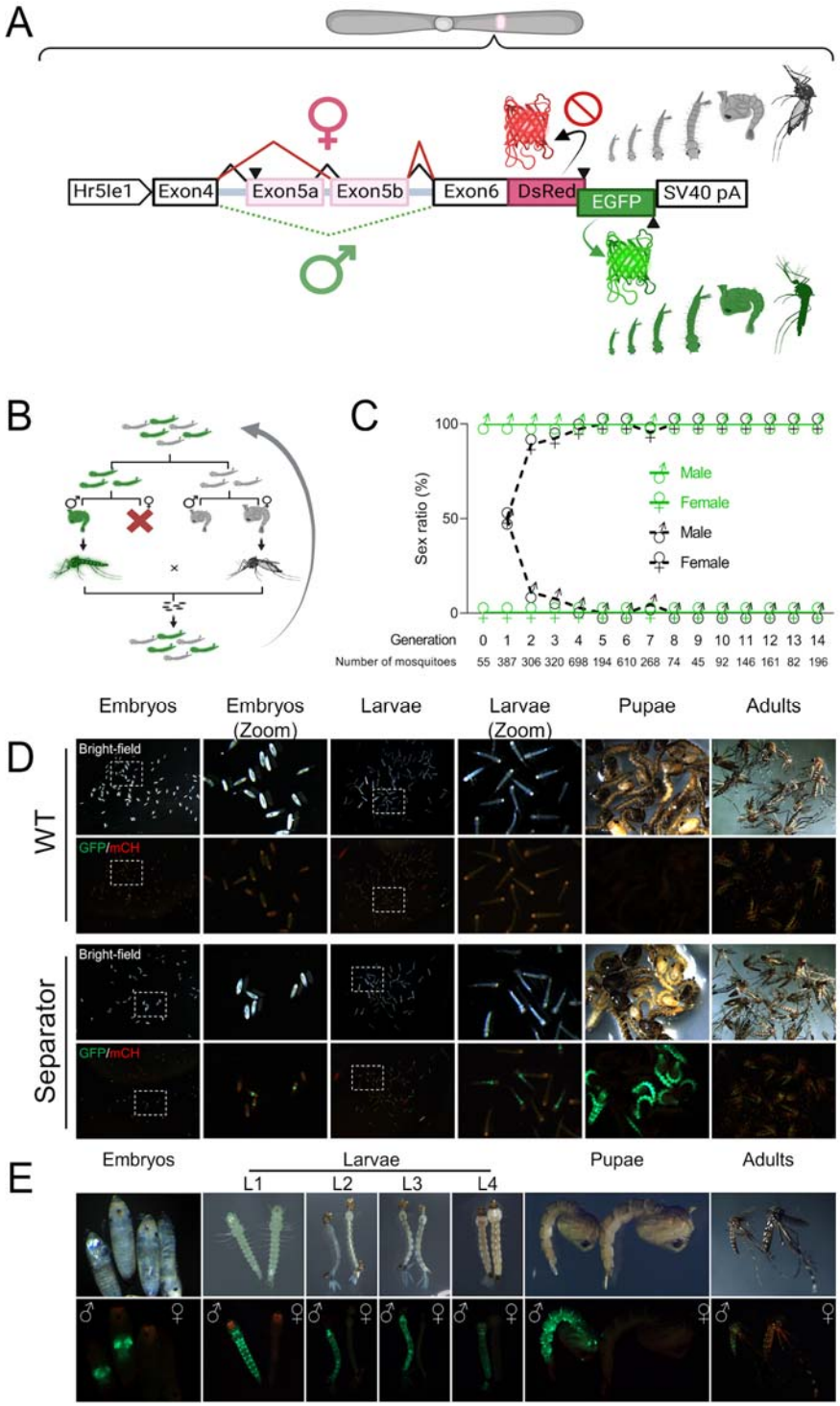

Figure 1 SEPARATOR in *Ae. aegypti.* (A) The sex-specific splicing module of AaDsx was used to construct SEPARATOR in *Ae. aegypti*. The expression of SEPARATOR was driven by the constitutive baculovirus promoter, Hr5Ie1. The male-specific splicing product was in-frame with the EGFP coding sequence, while inclusion of stop codons in exon 5b prevented in-frame expression of the DsRed in females. The SV40 pA served as the polyadenylation signal. The construct is not to scale. (B) Strategy for generating homozygotes. GFP-positive larvae were sorted and the sex of each was determined at the pupal stage. GFP-positive males were then crossed with GFP-negative females in order to produce homozygotes. (C) GFP-positive mosquitoes are male exclusively. Mosquitoes were sorted by their GFP signal at the larval stage and used a microscope to examine the sex ratio based on the morphological differences in genital lobe shape at pupal stage, which are specific to each sex. (D) The embryo, larva, pupa, and adult stages of wild-type (Liverpool) and SEPARATOR mosquitoes were collected and photographed using a fluorescent stereomicroscope (Leica M165FC). Eggs, aged between 24-48 hours after being laid, were subjected to a 15-30 minute treatment with 30% NaOCl solution (containing approximately 3.6% active chlorine at the final concentration) to remove the chorion and enable visualization of the embryo. Eggs were hatched in deionized water within a vacuum chamber, and the resulting hatched larvae were then collected as L1 larvae. (E) The developmental stages of mosquitoes were collected and photographed using a fluorescent stereomicroscope (Leica M165FC). The images consist of two panels: the upper panel displays the bright-field images, while the lower panel showcases the GFP/mCH channel images.

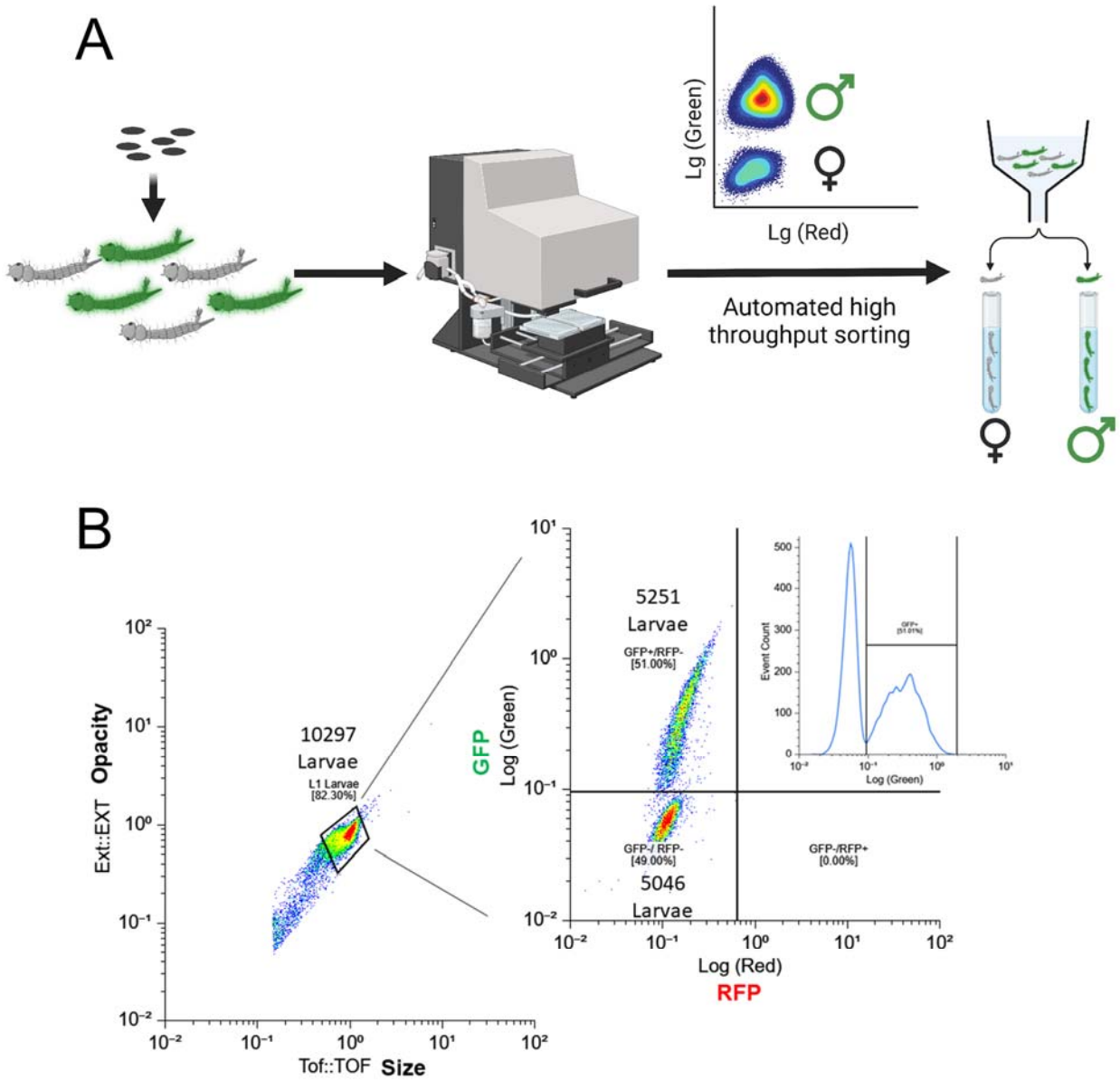

Figure 2 Precise sex-sorting at scale using COPAS. (A) A schematic diagram of large-scale sex-sorting processes using COPAS is shown. The eggs of transgenic mosquitoes, which have been engineered with the SEPARATOR system, were incubated in a vacuum chamber filled with deionized water to hatch them. 24 hours post-egg hatching, the larvae expressing GFP were screened using the COPAS instrument. The GFP-positive larvae were then carefully sorted, and raised in a controlled environment until they reached adulthood. Once the adults have reached maturity, their sexes were verified through various methods. (B) The eggs of transgenic mosquitoes that were genetically engineered to carry the SEPARATOR system were incubated in a vacuum chamber using deionized water. After 24 hours of incubation, the hatched larvae were passed through a COPAS instrument. To ensure accurate sorting, the larvae were selected based on both their opacity and size, and then sorted by the intensity of their GFP expression.

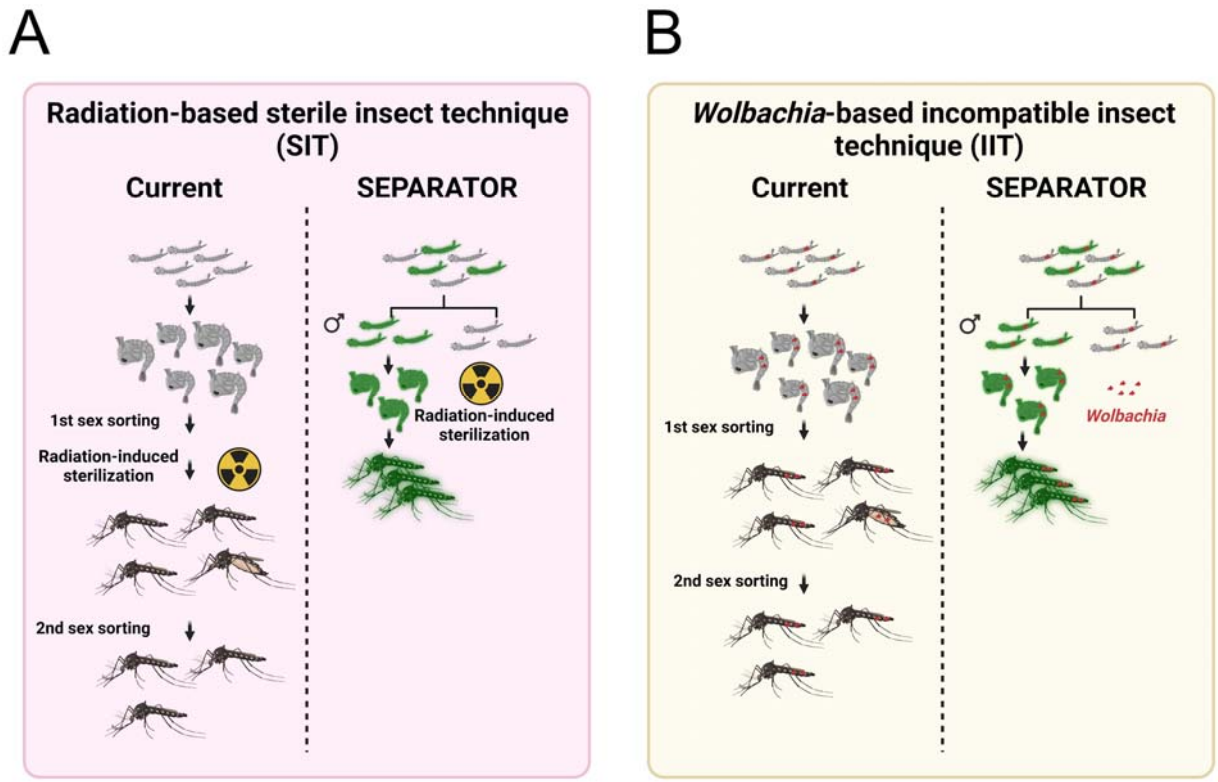

Figure 3 SEPARATOR provides a more simplified and economical sex-sorting of *Ae. aegypti* mosquitoes than the currently available two-step sex-sorting approaches. (A) The process of generating radiation-induced sterile male mosquitoes begins with the cultivation of mosquitoes. The larger female pupae are removed using a sieve. However, it should be noted that some smaller female pupae may still remain after the first sex sorting. Once the mosquitoes have been irradiated, emerging adult mosquitoes are screened out by an image recognition AI that has been trained to discriminate between female and male adult morphologies during the second sex sorting. The SEPARATOR approach utilizes a male-specific reporter (GFP) to positively select male L1 larvae. This can be done using the COPAS instrument, which is capable of high-throughput selection at a speed of up to 10 larvae per second. By removing female larvae early in the development process, SEPARATOR supports a more efficient production of males for SIT application. Furthermore, SEPARATOR allows for the transportation and release of irradiated sex-sorted pupae. This means that adult male mosquitoes can emerge directly into the environment without incurring additional fitness costs from handling and transportation. (B) The *Wolbachia*-based incompatible insect technique (IIT) utilizes a two-step sex-sorting approach for sorting the *Wolbachia*-infected male mosquitoes currently in use. The process of generating *Wolbachia*-infected SEPARATOR mosquitoes is relatively simple, as it involves crossing *Wolbachia*-infected female mosquitoes with SEPARATOR male mosquitoes. The resulting Wolbachia-infected male L1 larvae that express a male-specific reporter (GFP) are then positively selected using the COPAS instrument.

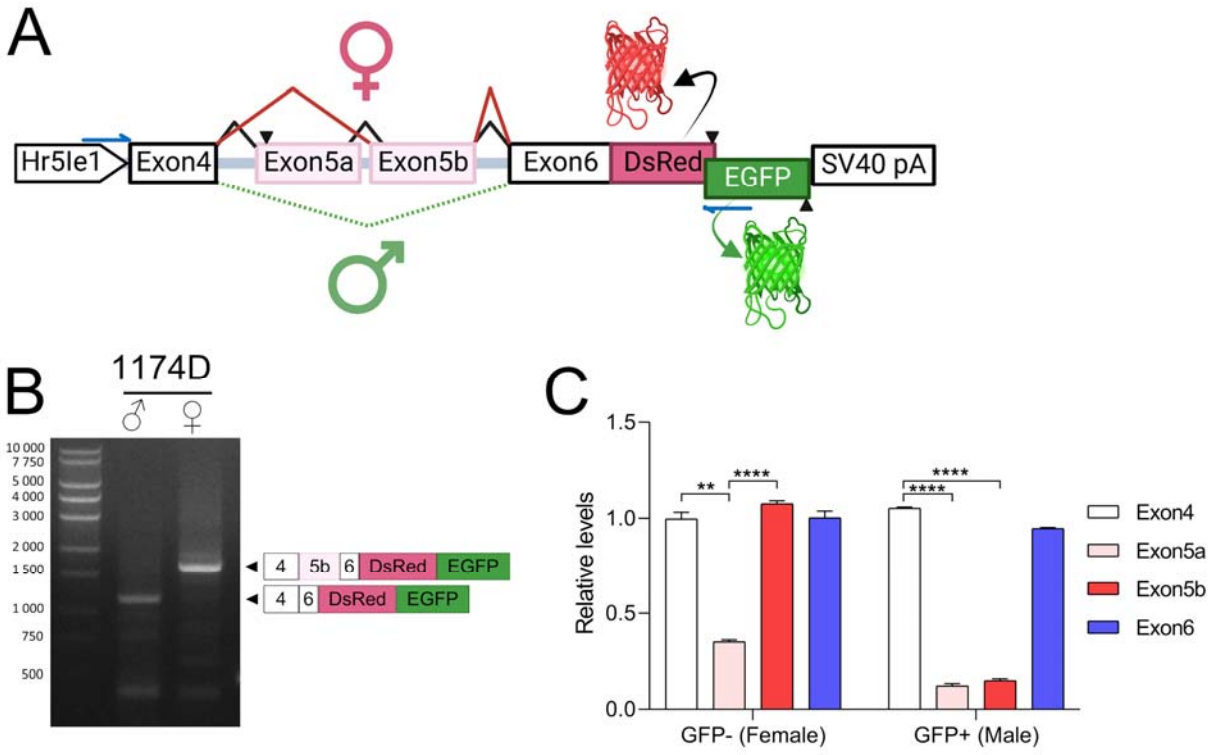

Fig S1. Sex-specific RNA splicing of SEPARATOR. Fifty GFP-positive larvae and fifty GFP-negative larvae at the L1 stage were carefully sorted, and total RNA was extracted from each group. To determine the splicing patterns, RT-PCR was performed using specific primers targeting the 3' end of the Hr5Ie1 promoter sequence and the 5' end of the EGFP coding sequence. (A) The relative locations of the primer target sites are indicated by blue arrows. (B) The PCR products were subsequently purified and subjected to sequencing in order to validate the splicing junctions. The resulting splicing patterns are depicted in the right panel. (C) The relative levels of non-sex-specifically regulated exons (exon4 and exon6) and female-specific exons (exon5a, exon5b) of SEPARATOR were determined through RNA sequencing (RNAseq) analysis. The FPKM (fragments per kilobase per million mapped reads) values of each exon were normalized using the average FPKM of the non-sex-specifically regulated exons (exon4 and exon6). The bar plot displays the means and ± SD (standard deviation) for triple biological replicates. Statistical significance of mean differences was assessed using a Tukey's multiple comparisons test, with p-values denoted as follows: $p < 0.01$** and $p < 0.0001$****.

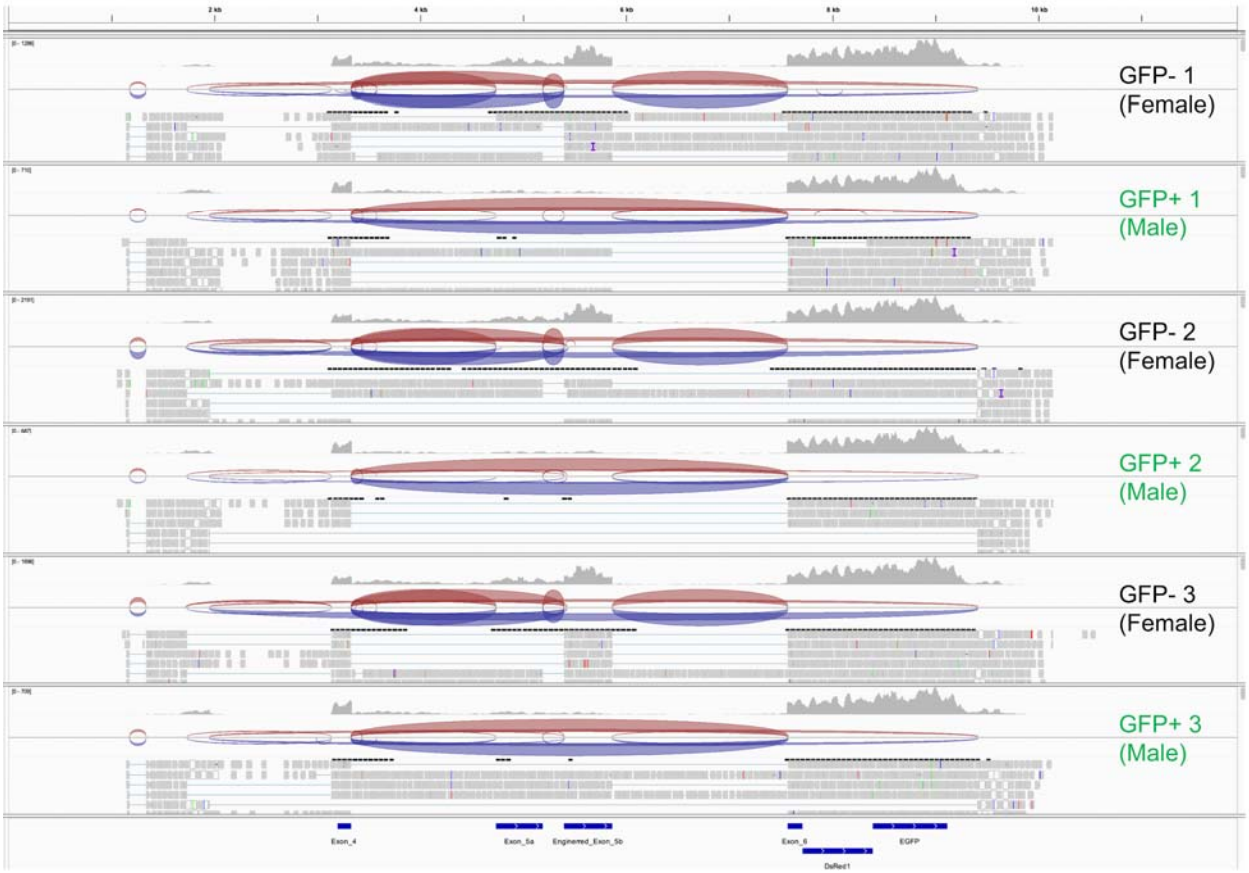

Fig S2. Sex-specific RNA splicing patterns verified through RNA sequencing analysis. The splicing patterns of SEPARATOR were verified through RNAseq analysis in both GFP-positive and GFP-negative mosquitoes, with triple biological replicates for each condition. The RNAseq reads for the different genotypes were aligned, and the location of exons is indicated at the bottom in blue.

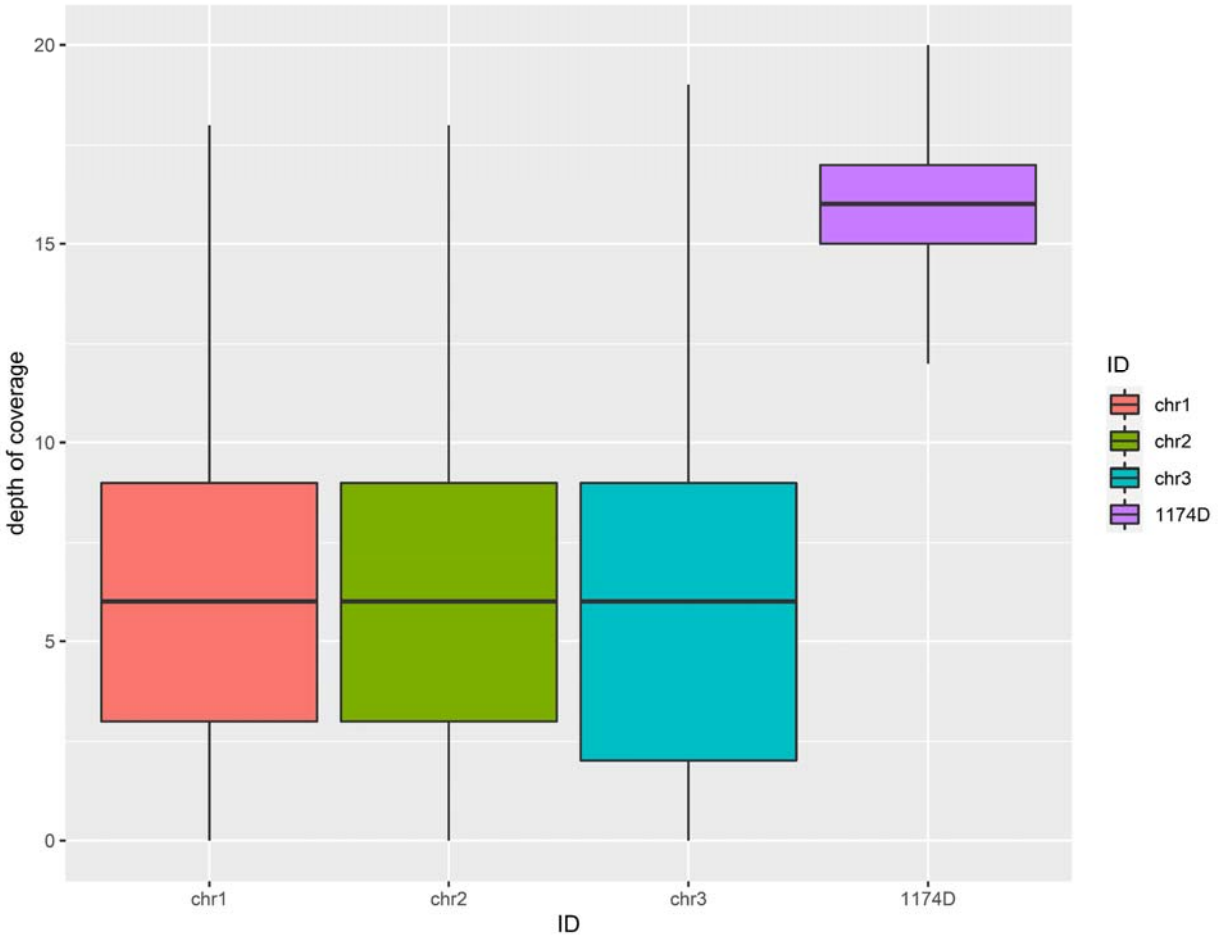

Fig S3. The transgene copy number for SEPARATOR was determined using Oxford Nanopore genome sequencing. A standard box plot is used to illustrate the coverage distributions of three chromosomes (Chr1, Chr2 and Chr3) and the SEPARATOR transgenes (1174D) in SEPARATOR mosquitoes. The center line represents the median, while the first and third quartiles define the boundaries of the box. The upper and lower whiskers extend from the box to the highest and lowest observed values, respectively, but no further than 1.5 times the Interquartile Range (IQR) from the box. Based on the sequencing depths, the coverage for chromosomes 1, 2, and 3 were 6.31, 6.30, and 6.08, respectively, while the coverage for the SEPARATOR transgenes was 16.14. From the coverage analysis, it suggests that the SEPARATOR transgene (1174D) is present in three copies.

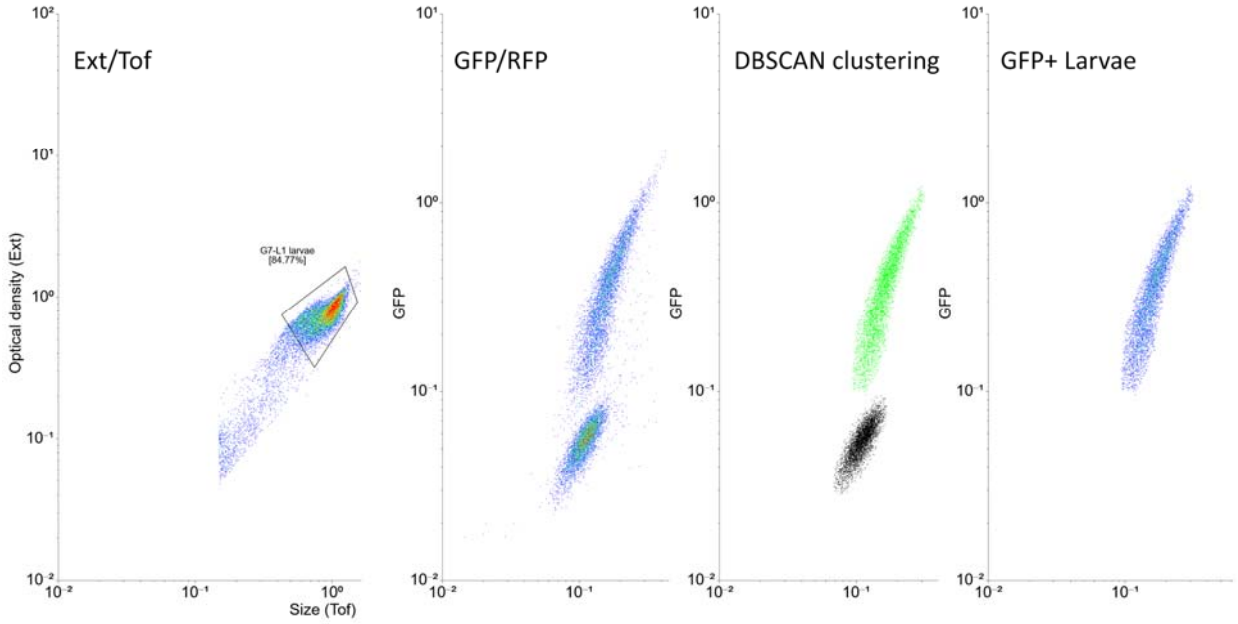

Fig S4. COPAS data processing. The COPAS raw data was initially filtered for the larvae using size and optical density criteria (Ext/Tof). Next, the particles that exhibited fluorescence (GFP/RFP) were gated. Subsequently, DBSCAN clustering was used to automatically cluster and denoise the data. Finally, the larvae that were GFP-positive were selected.

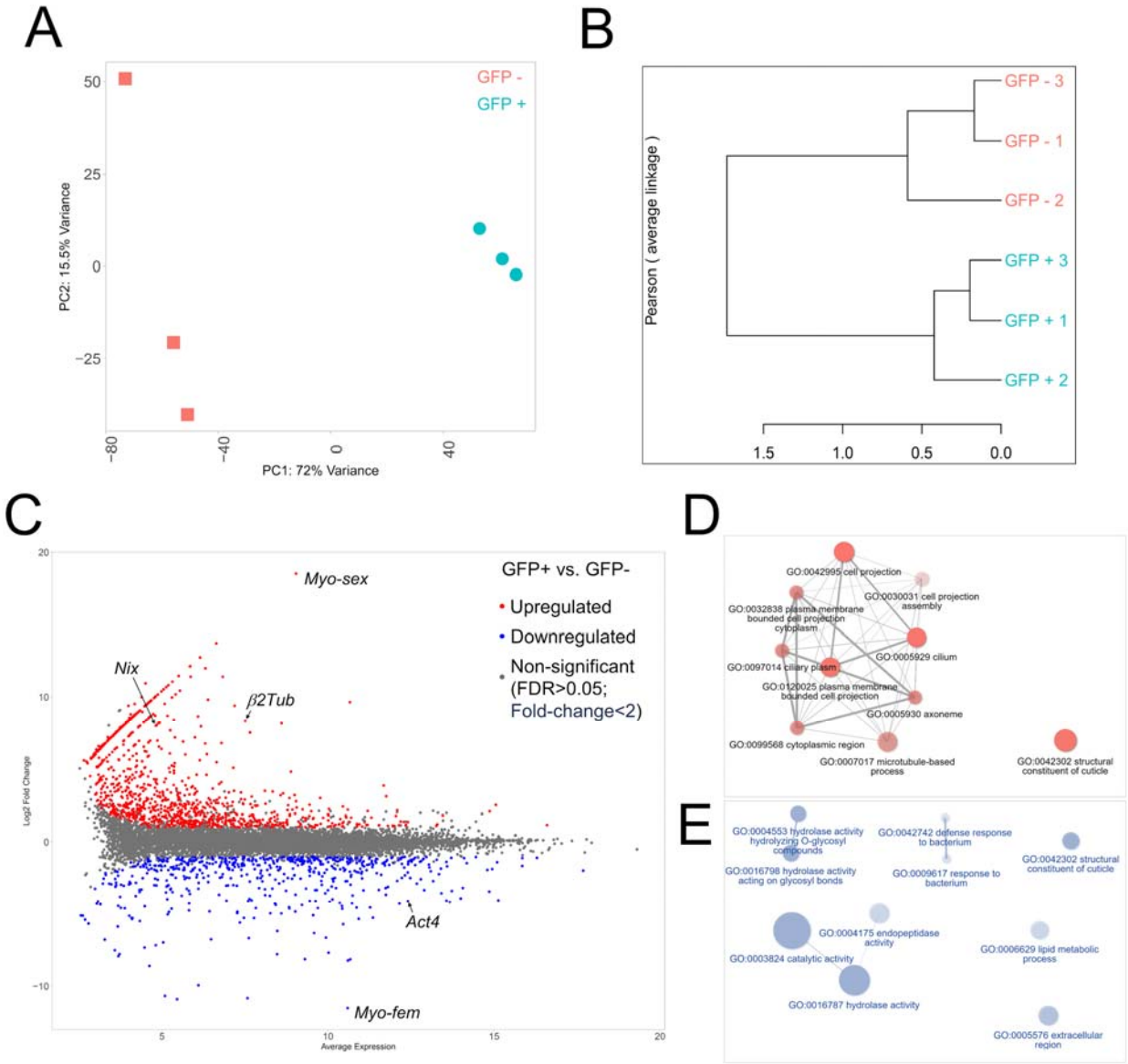

Fig S5. Transcription profiling and expression analysis of GFP-positive and GFP-negative larvae at the L1 stage in SEPARATOR mosquitoes. (A) PCA analysis and (B) hierarchical clustering of six samples used for RNA sequencing.(C) MA-plots were generated to visualize the differential expression patterns between GFP-positive and GFP-negative larvae at the L1 stage in SEPARATOR mosquitoes. In the plot, significantly upregulated (male-enriched) genes (FDR < 0.05 and fold-change > 2) are indicated by red dots, significantly downregulated (female-enriched) genes (FDR < 0.05 and fold-change > 2) are indicated by blue dots, and non-significantly differentially expressed genes are represented by gray dots (FDR > 0.05 or fold-change < 2). Additionally, five well-known sex-enriched genes were marked in the plot. A network visualization was created to illustrate the relationship among enriched Gene Ontology (GO) terms for the upregulated (D) and downregulated (E) genes.

Supplementary Tables

table S1. The sex sorting of SEPARATOR mosquitoes during the initial 15 generations
At the larval stage, the mosquitoes were sorted based on their GFP signal, and a microscope was utilized to analyze the sex ratio by observing the distinct morphological variations in genital lobe shape at pupal stage.

table S2. Summary of results from COPAS sorting experiments

table S3. FPKM of each exons in SEPARATOR mosquitoes

table S4. Combined annotations exons count

table S5. Enriched Gene Ontology (GO) terms for GFP-positive versus GFP-negative larvae in SEPARATOR mosquitoes

table S6. Sequences of primers and gBlock fragment used in this study

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