



# Introgression of the *Aedes aegypti* Red-Eye Genetic Sexing Strains Into Different Genomic Backgrounds for Sterile Insect Technique Applications

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### \*Correspondence:

Antonios A. Augustinos  
antoniosaugustinos@gmail.com

†Present address: Antonios A.  
Augustinos,

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

‡These authors have contributed  
equally to this work

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Antonios A. Augustinos<sup>1\*†‡</sup>, Katerina Nikolouli<sup>1‡</sup>, Lucia Duran de la Fuente<sup>1</sup>,  
Muhammad Misbah-ul-Haq<sup>1,2</sup>, Danilo O. Carvalho<sup>1</sup> and Kostas Bourtzis<sup>1</sup>

<sup>1</sup>Insect Pest Control Laboratory, Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture, Department of Nuclear Sciences and Applications, IAEA Laboratories, Seibersdorf, Austria, <sup>2</sup>Nuclear Institute for Food and Agriculture, Peshawar, Pakistan

*Aedes aegypti* is an invasive mosquito species and major vector of human arboviruses. A wide variety of control methods have been employed to combat mosquito populations. One of them is the sterile insect technique (SIT) that has recently attracted considerable research efforts due to its proven record of success and the absence of harmful environmental footprints. The efficiency and cost-effectiveness of SIT is significantly enhanced by male-only releases. For mosquito SIT, male-only releases are ideally needed since females bite, blood-feed and transmit the pathogens. *Ae. aegypti* genetic sexing strains (GSS) have recently become available and are based on eye colour mutations that were chosen as selectable markers. These genetic sexing strains were developed through classical genetics and it was shown to be subjected to genetic recombination, a phenomenon that is not suppressed in males as is the case in many Diptera. The genetic stability of these GSS was strengthened by the induction and isolation of radiation-induced inversions. In this study, we used the red eye mutation and the inversion Inv35 line of the *Ae. aegypti* red-eye GSS s and introgressed them in six different genomic backgrounds to develop GSS with the respective local genomic backgrounds. Our goal was to assess whether the recombination frequencies in the strains with and without the inversion are affected by the different genomic backgrounds. In all cases the recombination events were suppressed in all Inv35 GSS strains, thus indicating that the genomic background does not negatively affect the inversion result. Absence of any effect that could be ascribed to genetic differences, enables the introgression of the key elements of the GSS into the local genomic background prior to release to the target areas. Maintaining the local background increases the chances for successful matings between released males and wild females and addresses potential regulatory concerns regarding biosafety and biosecurity.

**Keywords:** area wide integrated pest management, insect pest control, vector control, mosquitoes, yellow fever mosquito

## INTRODUCTION

Arthropod-borne viruses or “arboviruses” transmitted by *Aedes* spp. mosquitoes are accountable for the emergence of human epidemic diseases across the globe (Weaver and Reisen 2010; Lucey and Gostin 2016; Siraj et al., 2017; Wilder-Smith et al., 2017). Zika, dengue, yellow fever, and chikungunya viruses infect humans by the bite of an infected *Aedes aegypti* L. (Diptera: Culicidae) mosquito and result in a diverse array of clinical symptoms and implications ranging from systemic febrile illnesses to neurological or cerebrovascular diseases and death (Bhatt et al., 2013; Beckham and Tyler 2015; Paixão et al., 2018). *Ae. aegypti* has successfully spread in tropic and subtropic zones worldwide (Kraemer et al., 2015). It is daylight-active, thrives in urban and peri-urban areas, feeds exclusively on human blood multiple times during a gonotrophic cycle, and shows high susceptibility to arboviruses (Scott and Takken 2012; Wilder-Smith et al., 2017; Ryan et al., 2019). Its ability to breed in human-made breeding settings facilitates the increase of the vector’s population and fuels the spread of the vector-borne diseases. Urbanization of rural areas, increase of travelling activities, globalization, and climate change accelerate the invasion potential of *Ae. aegypti* and enhance the viral transmission (Wilder-Smith and Gubler 2008; Bhatt et al., 2013; Struchiner et al., 2015; Ryan et al., 2019; Iwamura et al., 2020).

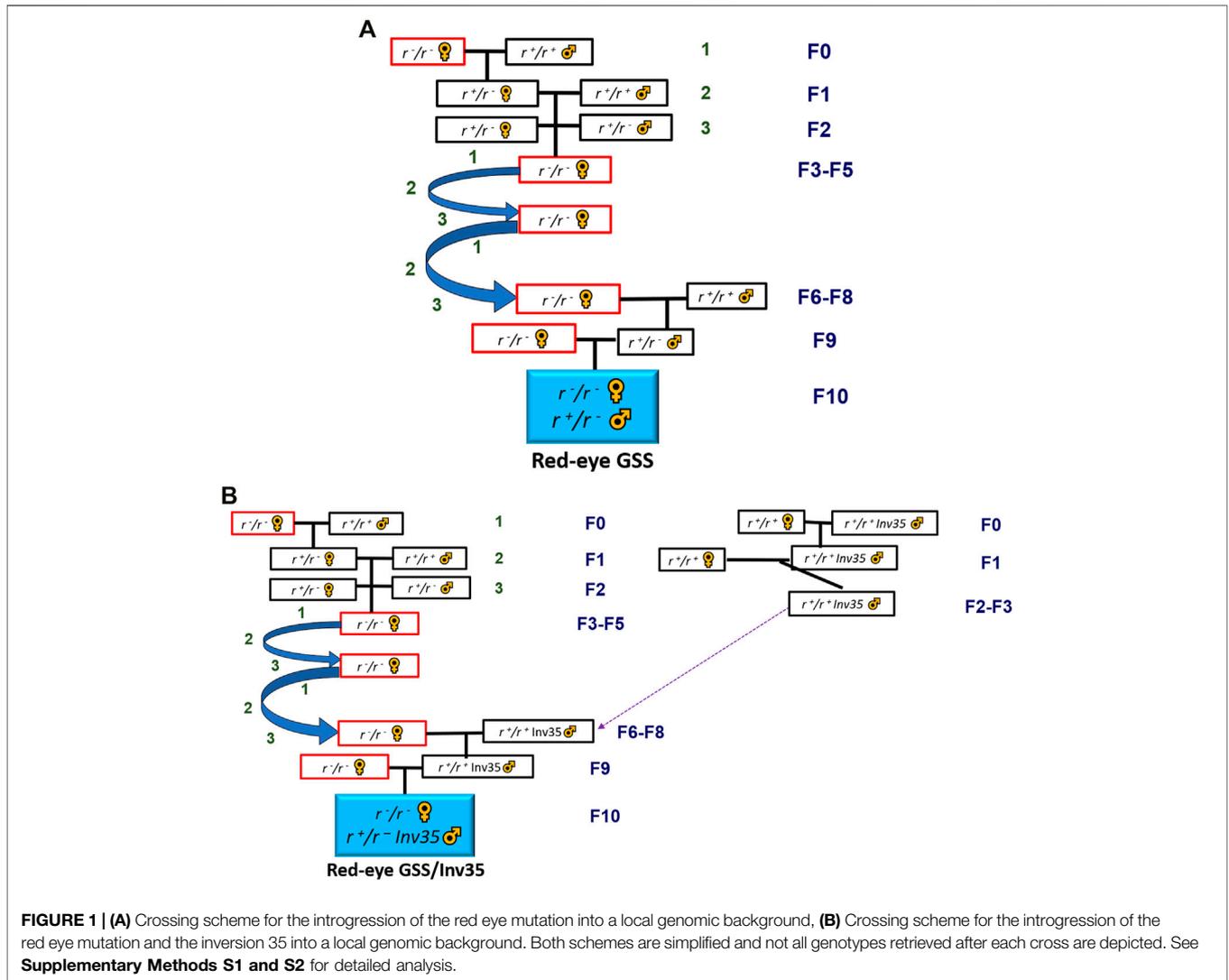
The lack of effective drugs and vaccines against these arboviruses (apart from the yellow fever vaccine) has shifted the spotlight on the vector population control methods (Achee et al., 2015; Lees et al., 2015; Bourtzis et al., 2016; Flores and O’Neill 2018). Current efforts rely on insecticide applications and elimination of breeding sites; however, these methods have been proved both unsustainable and inefficient. The development of insecticide resistance, the rapid expansion of *Ae. aegypti* populations in urban areas and the inadequate control of the cryptic breeding sites led scientists and communities to pursue environmentally-friendly approaches that would control efficiently the vector populations without compromising sustainability (Lima et al., 2011; Achee et al., 2015; Louis et al., 2016; Moyes et al., 2017).

During the recent years, numerous genetically based approaches have been developed aiming either to modify vector populations (i.e., rendering them resistant in pathogen transmission) or to suppress them below the threshold required for disease transmission (Harris et al., 2012; O’Connor et al., 2012; Alphey et al., 2013; Bellini et al., 2013; Carvalho et al., 2015; Mains et al., 2016; Kittayapong et al., 2018; Kyrou et al., 2018; Kandul et al., 2019; Kittayapong et al., 2019; Zheng et al., 2019; Crawford et al., 2020). Some of the population suppression approaches, including the sterile insect technique (SIT), have been tested in the field with encouraging results (O’Connor et al., 2012; Bourtzis et al., 2014; Bourtzis et al., 2016; Kittayapong et al., 2018; Kandul et al., 2019; Kittayapong et al., 2019; Zheng et al., 2019; Crawford et al., 2020). The SIT which relies on the mass production and release of sterile males, has historically been applied for the control and eradication of insect pest populations (Bushland et al., 1955; Knippling 1955; Klassen et al., 2021). When considering the SIT as part of a mosquito control project, one of the greatest challenges to be addressed is the sex separation and elimination of females (Papathanos et al., 2009; Gilles et al., 2014). Unlike agricultural

pests where either bisexual release is the only feasible approach or the accidental release of few females is not considered a major concern, in mosquitoes, release of both males and females is a no go, since adult females create biting nuisance, and are potential disease vectors. Thus, an adequate and robust sex separation system that will reliably separate male and female mosquitoes at a large scale is of critical importance for the implementation of a SIT program (Gilles et al., 2014; Papathanos et al., 2018). In *Ae. aegypti*, sex separation is currently based on the inherent characteristics of the species, i.e., the size dimorphism between male and female pupae and male-specific body parts of adults including genitalia and antennae (Focks 1980; Gunathilaka et al., 2019; Crawford et al., 2020). This approach is rearing-dependent, prone to errors, labor-intensive and appropriate for small-scale operations. Although novel and (semi)-automated methods have been developed, the critical need of a genetic sexing strain (GSS) for *Ae. aegypti* rises as the ideal sex separation method particularly if males and females could potentially be separated at early developmental stages (Gilles et al., 2014; Papathanos et al., 2018). Developing a GSS using classical genetics typically requires a selectable marker (visually detectable or conditionally lethal) and the linkage of the wild type allele of this marker to the Y chromosome or to the sex-determining genetic locus (Franz et al., 2021).

*Aedes* species have homomorphic sex chromosomes and their maleness is defined by a dominant male-determining locus (M locus) of chromosome 1 (Craig and Hickey 1967; Newton et al., 1974; Hall et al., 2015; Aryan et al., 2020; Liu et al., 2020). *Ae. aegypti* males are heterogametic (Mm) while the females are homogametic (mm) for the M-locus (Timoshevskiy et al., 2013). The competence of the selectable marker will in turn determine the robustness of the GSS and in *Ae. aegypti* the ideal marker would reside on chromosome 1, closely linked to the M-locus. In such a strain, male mosquitoes would be heterozygotes and express the “wild-type” phenotype while females would be homozygous for the recessive alleles of the selectable marker expressing the mutated phenotype (Franz et al., 2021). Promising markers that could be used for *Ae. aegypti* GSS development are related to eye colour (Red-eye (*re*) and White-eye (*w*) markers) which are located on chromosome 1 linked to the M-locus and they are fully penetrant and expressive (Bhalla and Craig 1970; Munstermann and Craig 1979). Both markers have been used by our group for the construction of two *Ae. aegypti* GSS, in which males have black eyes and females have either red or white colour eyes (Koskinioti et al., 2020). The *re* and *w* mutant lines were crossed with the wild-type “BRA” strain collected from Brazil and the Red-eye GSS and White-eye GSS were developed. Quality control of both GSSs evidenced no significant differences regarding sex ratio and immature development duration of both sexes. The Red-eye GSS showed outstanding productivity compared to the White-eye GSS and significantly elevated lifespan and flight ability compared to the wild type “BRA” strain (Koskinioti et al., 2020).

The stability of a GSS, in particular under the demanding mass-rearing conditions, is a pivotal factor for its successful implementation in operational population suppression programmes. Instability during mass-rearing conditions is mainly



attributed to genetic recombination events. Unlike other Diptera, in *Aedes* mosquitoes, recombination events occur in males almost as frequently as in females, and these events can compromise the GSS stability and lead in breakdown of the GSS due to accumulation of recombinants. Incorporation of recombination-suppressing factors, such as inversions, can improve the stability of a GSS (Franz et al. 2021; Gilles et al., 2014; Zacharopoulou et al., 2017). In *Ae. aegypti* induction of inversions has been shown to suppress recombination between the M locus and morphological markers of chromosome 1 (Bhalla 1973). Using irradiation our group induced inversions in *Ae. aegypti* and showed that irradiation frequency can be suppressed between *re* and the M locus (line 35), while at least two lines in which recombination is suppressed between *w* and the M locus (lines 5 and 35) were identified (Augustinos et al., 2020). Inversion line 35 was incorporated in the Red-eye GSS and White-eye GSS by crossing wild-type males having the recombination suppressor (from the Inv35 line) with females from the two GSSs. Recombination frequencies were measured for consecutive generations under filtered and non-filtered conditions, i.e., removal or not of

recombinant progeny from each generation, and recombination was consistently reduced for both strains (Koskinioti et al., 2020).

GSSs may still face issues when released in the field that could lead in performance reduction. The genomic background has been shown to be a driving factor when it comes to mosquito performance. Among others, variation in vector competence, reproductive incompatibility, effects on fitness traits and differences in the reproductive effects of *Wolbachia* infections have been shown to stem from variations in the genomic background of mosquito populations (Bennett et al., 2002; Menge et al., 2005; Axford et al., 2016; Dickson et al., 2016; Campbell et al., 2017; Carvalho et al., 2020; Enkerlin 2021). The success of sterile mosquito releases relies massively on the mating performance of the released males. However local mosquito populations might vary significantly in terms of ecology, biology, and behavior and this could in turn lead to mating barriers which would compromise the efficiency of a SIT programme (Krafsur and Ouma 2021). These barriers can be overcome by developing mosquito GSS that will be integrated

into the local genomic background of the release area. In this study the *A. aegypti* red eye mutation and the inversion Inv35, the latter developed previously in the Insect Pest Control Laboratory (IPCL, Seibersdorf, Austria), were introgressed in populations originating from different geographic areas to develop Red-eye GSS and Red-eye GSS/Inv35 strains with local genomic backgrounds and their genetic stability was assessed for several generations (Augustinos et al., 2020; Koskinioti et al., 2020).

## MATERIALS AND METHODS

### *Ae. aegypti* Strains and Rearing Conditions

The Rexville Red Eye strain, which is homozygous for the recessive *re* allele, was used in the present study and was kindly provided by Dr. Margareth Capurro at the Department of Parasitology, University of Sao Paulo, Brazil. In the *re* strain all individuals have red eye color which is evident throughout all developmental stages and it darkens as adults age. Six wild type *Ae. aegypti* strains originated from Brazil (BRA), Indonesia (IDN), Mexico (MEX), Singapore (SGP), Sri Lanka (LKA), and Thailand (THA) were used for the introgression crosses described below and checked for their recombination rates. The *Ae. aegypti* inversion line 35 (Inv35) (Augustinos et al., 2020) was used to incorporate the inversion in all six genomic backgrounds. In all wild-type strains the eye color is dark brown/black and remains stable at all developmental stages. All strains were maintained in the insectary of the Insect Pest Control Laboratory (Joint FAO/IAEA Centre, Seibersdorf, Austria) at  $27 \pm 1^\circ\text{C}$ , 80% relative humidity and a 12/12 h day/night photoperiod.

Adult mosquitoes were kept in standard (30 × 30 × 30 cm) insect plastic rearing cages (BugDorm-41,515 insect cage) and a 10% sucrose solution was constantly provided. Female mosquitoes were blood-fed with porcine blood twice per week. The blood used was collected in Vienna, Austria during routine slaughtering of pigs in a nationally authorized abattoir, conducted at the highest possible standards strictly following EU laws and regulations. Egg collections were initiated 72 h after the last blood feeding using moistened oviposition papers (white germination paper, Sartorius Stedium Biotech, Austria).

### Crosses

#### Development of Red Eye-GSS Strains in Local Genomic Backgrounds

Females of the Red Eye strain and males from IDN, SGP and LKA populations were used to initiate the three introgression crosses while the respective crosses with males from BRA, MEX and THA populations are reported in the study of Chen et al. (2021) (Figure 1A).

The introgression of Inv35 in the local genomic backgrounds was initiated independently and it was continued until a semi-introgressed inversion line had been acquired. At that stage, partially Introgressed Inv35 males were crossed with highly introgressed Red Eye females to create a Red-GSS with Inv35 in a local genomic background (Figure 1B). The genomic backgrounds of the introgressed Red Eye females were from BRA, IDN, MEX, SGP, LKA and THA populations. In all

cases, fifty females and twenty males were crossed in every generation in a 15 × 15 × 15 cm rearing cage (BugDorm-4M1515). The detailed introgression protocols are provided in the **Supplementary Materials S1 and S2**.

### Estimation of Recombination Rate

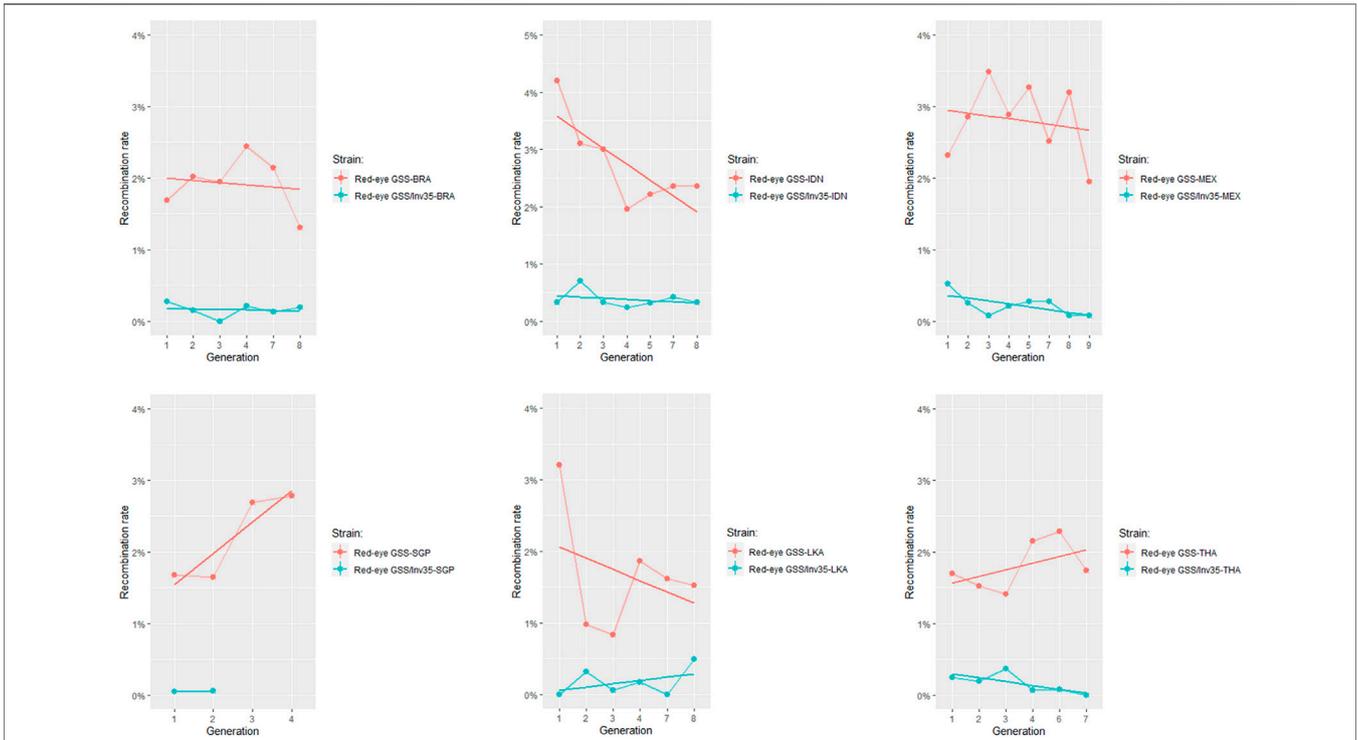
The recombination rate was estimated for all the newly established Red-eye GSS and Red-eye GSS/Inv35. All progeny were screened in every generation and recombinants (males with red eyes and females with black eyes) were recorded and subsequently discarded. At least six generations per strain were screened. Black eye males and red eye females were used to set up the new cages. A minimum number of 1,000 individuals were used to set up the new cages.

### Data Analysis

All statistical analyses were performed using R version 4.0.5 (R Core Team, 2021). The recombination rates between the strains with and without inversion of the same origin and among the different origins represent proportional data and therefore, they were analyzed using a GLM-binomial family and a logit link function (Dunn and Smyth, 2018). In case overdispersion was detected, a Quasi-Binomial model with a logit link function was applied (Demétrio et al., 2014). Analysis of deviance was performed with a Chi-squared test for GLM-Binomial models and with a F-test for GLM-Quasi-Binomial models (Nelder and Wedderburn, 1972). Residuals of the models were checked for normality and homogeneity of variance. Goodness-of-fit of the models was visually inspected with half-normal plots with simulation envelopes (Moral et al., 2017). Emmeans package was used for the pairwise comparisons of the fitted model estimates (Searle et al., 1980).

## RESULTS AND DISCUSSION

The *Ae. aegypti* Red-eye GSS has been developed through classical genetics and is based on the *re* morphological marker that has been mapped to chromosome I (Koskinioti et al., 2020). The red eye mutation presents full penetrance and expressivity and the red eye color is evident throughout all developmental stages. Estimation of the recombination frequency between *re* and M locus confirmed that *re* is a recessive, sex-linked gene. Recombination events in *Aedes* species occur both in males and females and, in the case of a GSS under mass-rearing conditions, they can eventually lead in reduced genetic stability and colony collapse (Augustinos et al., 2017; Franz et al., 2021). Elements that suppress recombination between the M locus and the marker are therefore required to be incorporated in the GSS. In the study by Augustinos et al. (2020) an inversion (Inv35) was induced through irradiation aiming to suppress recombination between *re* and the M locus. Indeed, the recombination frequency was significantly suppressed, and the inversion was incorporated in the Red-eye GSS thus creating the Red-eye GSS/Inv35. These two strains were screened for numerous generations and results demonstrated significantly decreased recombination in the

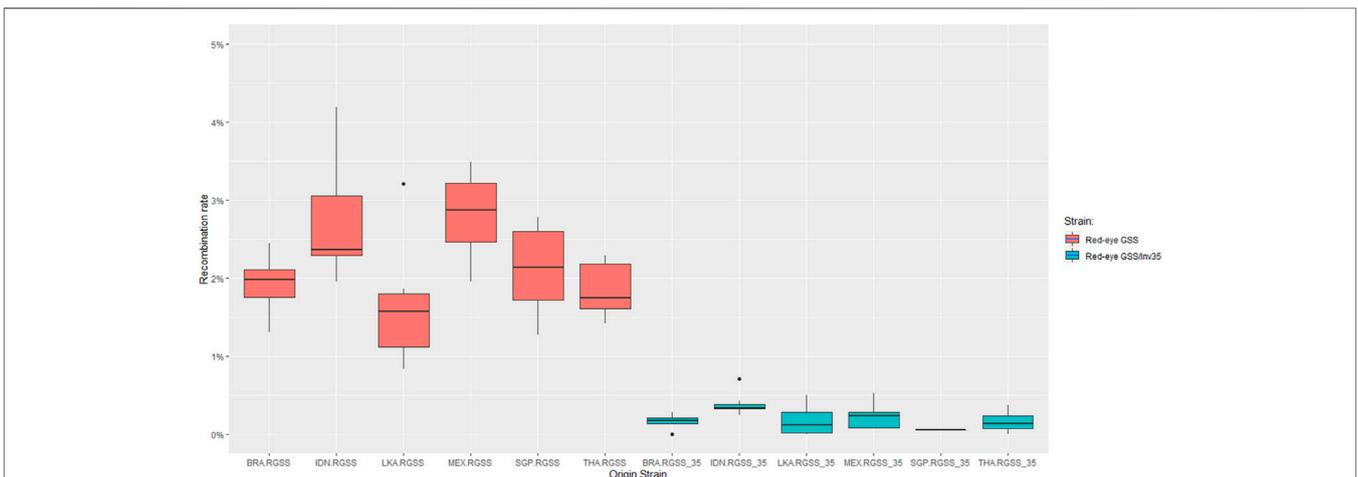


**FIGURE 2 |** Recombination rates of the Red-eye GSS and Red-eye GSS/Inv35, after their introgression in six local genomic backgrounds. Recombinant males and females were recorded in each generation and results were analyzed with a GLM (binomial family). In all genomic backgrounds, the strain incorporating the inversion had significantly lower recombination rates compared to the respective strains without the inversion. The straight line represents the fitted linear model.

Red-eye GSS/Inv35 compared to the original strain (Koskinioti et al., 2020).

Variability in recombination frequencies can be attributed, among other factors, to genomic differences, with chromosomal rearrangements being the most likely reason (Dickson et al., 2016). In the present study, we received six *Ae. aegypti*

populations from countries that could be possible target areas of a future operational SIT programme. The red eye mutation line and the inversion Inv35 were introgressed into the six genomic backgrounds, following a crossing scheme that lasted for eleven generations. Our goal was to assess whether the novel genomic background would affect the recombination frequencies that had



**FIGURE 3 |** Recombination rates of the Red-eye GSS and Red-eye GSS/Inv35 throughout the course of generations. Generations were used as replicates. No significant effect was detected among the Red-eye GSS/Inv35 strains, indicating that Inv35 suppresses recombination irrespectively of the genomic background.

been estimated in the original GSS. As soon as the introgression crossing scheme was completed and the twelve new strains had been established, the recombination frequencies were evaluated for all strains. A total of 110,799 mosquitoes from 73 generations of 12 strains were screened and recombination frequencies were recorded (**Supplementary Materials S3 and S4**).

The recombination frequency was estimated for each genomic background individually and results indicated that the Red-eye GSS/Inv35 presented significantly lower recombination rates compared to the Red-eye GSS throughout the course of generations (**Figure 2**). Except for Singapore for which data availability is limited, the strains with the inversion were more stable and with significantly lower recombination rates compared to the ones without (**Supplementary Material S5**). Recombination frequencies were analyzed to check for any possible effect of the genomic background, using data from different generations as replicates. In all genomic backgrounds the recombination rates were significantly lower for the Red-eye GSS/Inv35 ( $F = 51.375$ ,  $df = 11$ ,  $p < 2.2e-16$ ), thus indicating that the effect of inversion is evident in all genomic backgrounds (**Figure 3 and Supplementary Material S6**). Assessment of the recombination frequencies among the six Red-Eye GSS/Inv35 strains showed no statistically significant differences, thus suggesting that the inversion suppresses recombination similarly, irrespective of the genomic background (**Supplementary Material S6**). Interestingly, the pairwise comparisons of the six Red-Eye GSS strains showed an effect of the genomic background on the recombination rates. Red-eye GSS-BRA was shown to be significantly different from the MEX ( $z = -3.495$ ,  $p = 0.0005$ ) and IDN ( $z = -3.035$ ,  $p = 0.0024$ ) strains, while the same was also true for the IDN-LKA ( $z = 3.422$ ,  $p = 0.0006$ ), IDN-THA ( $z = 2.855$ ,  $p = 0.0043$ ), MEX-LKA ( $z = -3.889$ ,  $p = 0.0001$ ), and MEX-THA ( $z = 3.353$ ,  $p = 0.0008$ ) Red-Eye GSS comparisons. However, in some Red-eye GSS strains *per se* the recombination frequencies varied among generations. Results for Indonesia, Mexico, and Sri Lanka showed that there was a statistically significant difference among the tested generations (**Supplementary Material S7**) which could be attributed to factors such as age, sex and temperature (Augustinos et al., 2020). No variation was detected for the Red-eye GSS from Brazil, Singapore and Thailand. The same conclusion was also reached for the Red-eye GSS/Inv35 strains (**Supplementary Material S7**). The recombination rates were stable through the course of generations per strain and no statistically significant differences were detected for all the genomic backgrounds which confirmed the robustness and effectiveness of the inversion in suppressing recombination regardless of genomic background and generation. This clearly indicated that the genomic background did not negatively affect the genetic stability of the strains and confirmed the robustness and effectiveness of the inversion in suppressing recombination regardless of genomic background and generation. The Red-eye GSS/Inv35-SGP is the only strain for which availability of data is limited and therefore more generations are required to reach a safe conclusion.

The results of the present study are encouraging, in respect to the genetic stability of Red-eye GSS/Inv35 developed in local

genomic backgrounds. However, the biological quality of the newly established GSS needs to be assessed first under laboratory and later in field conditions (Carvalho et al., 2020; Koskinioti et al., 2020). The genomic differences might be proved detrimental to important fitness traits as has been shown in both fruit flies and mosquitoes (Meza et al., 2011; Facchinelli et al., 2013; Rempoulakis et al., 2016; Ramírez-Santos et al., 2017; Ramírez-Santos et al., 2017; Carvalho et al., 2020). An in-depth quality control analysis that will assess important parameters like fecundity, fertility, longevity, flight ability, male mating competitiveness and response to irradiation, prior to upscaling and releasing in the field.

The release of a mosquito GSS as part of an operational SIT programme is ruled in most cases by concerns regarding the biosafety and biosecurity of the released strain, as well as by uncertainties related to the performance of the strain in the wild. A mosquito GSS has been developed and reared in laboratory conditions for several generations and carries its own genomic background. Decision-making bodies could reject the release of a GSS in an area based on the notion that breeding of human disease vectors bearing different genomic backgrounds could result in previously undetected risks related to humans and the environment. To address these issues, it is advisable to use either a local strain or to integrate the mosquito strains into the local genomic background prior to release. That way the potential effects associated with mating incompatibility are minimized and the chances for increased male mating competitiveness are raised since the released males and the wild females will share the same genomic background. In addition, maintaining the local genomic background can resolve any regulatory issues posed by the countries, reaffirm the biosecurity and biosafety of the released strain, and enhance the public acceptance towards the SIT programmes.

The recent discovery of the gene responsible for the red eye phenotype in *Aedes aegypti*, namely *cardinal*, opens the way for a faster and easier transfer of the sexing characters of the red eye GSS in local genomic background and will thus avoid the long (10–11 generations) and tedious genetic crosses described in this manuscript (Chen et al., 2021). This can be achieved by using CRISPR/Cas9 targeted mutagenesis of the *cardinal* gene of the local population to *de novo* develop a red eye mutant line. Next step would be to perform two simple genetic crosses as the ones described for the original construction of the red-eye GSS (Koskinioti et al., 2020). First, mutant females should be crossed with wild type males and second, F1 males should be backcrossed with mutant females to establish a genetic sexing strain with local genomic background.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

AA: conceived, designed, and performed the experiments, interpreted the data, drafted the first draft of the manuscript, and contributed to the preparation of the final version. KN: analyzed the data and wrote the manuscript. LD, MM-u-H, DC performed experiments. KB: conceived and supervised the study, designed the experiments, interpreted the data, critically revised the first draft of the manuscript, and contributed to the preparation of the final version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fbioe.2022.821428/full#supplementary-material>

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