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Differential elimination of marked sex chromosomes enables production of nontransgenic male mosquitoes in a single strain

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Diverse genetic strategies are being pursued to control mosquito-borne infectious diseases. These strategies often rely on the release of nonbiting males to either reduce the target mosquito population or render them resistant to pathogens. Male-only releases are important as any contaminating females can bite and potentially transmit pathogens. Despite significant efforts, it remains a major bottleneck to reliably and efficiently separate males from females, especially when nontransgenic males are preferred. In the yellow fever mosquito *Aedes aegypti*, sex is determined by a pair of homomorphic sex chromosomes, with the dominant male-determining locus (the M locus) and its counterpart (the m locus) embedded in an M-bearing and an m-bearing chromosome 1, respectively. We utilized both naturally occurring and engineered sex-linked recessive lethal alleles (RLAs) to create sex separation strains for *Ae. aegypti* on the basis of differential elimination of marked sex chromosomes (DeMark). DeMark strains are self-sustaining and produce nontransgenic males that are readily separated from individuals carrying RLA- and transgene-marked m chromosomes. For example, the marked m chromosome in the heterozygous mother in some strains was only inherited by her female progeny due to RLA-mediated incompatibility with the M-bearing chromosome in the father, producing nontransgenic males and transgenic females, generation after generation. We further explore strategies to conditionally eliminate females that contain marked sex chromosomes. We also discuss DeMark designs that are applicable for efficient sex separation in organisms with well-differentiated X and Y chromosomes, such as the *Anopheles* mosquitoes.

mosquito control | sex-separation | homomorphic sex chromosome | recessive lethal | sterile insect technique

Significance

Aedes aegypti is a major vector of viruses such as dengue and Zika. This work addresses a bottleneck in all genetic programs that rely on releasing nonbiting male mosquitoes, namely the need to efficiently and cost-effectively produce millions of male mosquitoes that are devoid of contaminations from the biting females. We developed single-strain sex separation lines to efficiently produce nonbiting males that are not genetically modified, providing advantages with regard to the regulatory process and community acceptance. These methods have the potential for broad applications in other pest control programs. Overall, our work contributes to a diverse tool box that is needed to adapt to the biological, geographical, economic, and cultural complexity facing global mosquito-borne infectious disease control programs.

Aedes aegypti is a major vector of arboviruses such as chikungunya, dengue, yellow fever, and Zika. Prevention of infectious diseases caused by these mosquito-borne viruses depends mainly on effective vector control, which is hindered by increasing insecticide resistance. Novel genetic strategies to control mosquito-borne infectious diseases, including both population suppression and population modification, are being actively explored. Population suppression (e.g., Sterile Insect Technique or SIT and *Wolbachia*-based Incompatibility Insect Technique or IIT) aims to achieve disease control by reducing mosquito population density (e.g., refs. 1–5). Population modification aims to use a gene drive mechanism to achieve fixation of an introduced effector gene(s) in the target vector population, rendering the vector refractory to disease transmission (e.g., refs. 6–10). Most of these genetic strategies require that only male mosquitoes are released as any contaminating females in a release population can bite and potentially transmit pathogens. Thus, sex separation (i.e., removing females before release) is a critical but often most costly step for these genetic control programs (reviewed in ref. 11).

Sex separation based on the sex differences in either the pupa size or adult morphology has been used to isolate males in recent releases to suppress *Aedes* mosquitoes in areas of moderate population sizes (2, 3). In addition, a genetic sexing strain with a sex-linked recessive red-eye mutation has been developed, and efforts are made to suppress recombination between the sex locus and the red-eye mutation by irradiation-induced inversion (12, 13). These sex separation methods do not involve genetic modifications of mosquitoes, but further improvements in efficiency and cost-effectiveness are necessary for use in large-scale release programs. In a novel CRISPR/Cas9-mediated method called precision-guided SIT or pgSIT (14, 15), only transgenic sterile males survive to become flying adults. Maintenance and sex separation of two transgenic lines is required as Cas9-expressing females need to mate with sgRNA-expressing males. A temperature-inducible pgSIT was demonstrated in *Drosophila* to negate the need for two lines (16). A sex

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separation method has also been reported which uses a transgenic reporter that is only produced in males (17). Lutrat et al. (18) used sex-linked transgenic markers to produce nontransgenic males, taking advantage of the ease of screening conferred by the transgenic markers while producing nontransgenic males for release. However, two transgenic lines and three independent sex separations are needed to achieve this. Despite these recent successes, efficient sex separation remains a major bottleneck in genetic control programs especially when the release of nontransgenic males is preferred.

We explore two important features of the sex chromosomes of the yellow fever mosquito, *Ae. aegypti*, to develop unique ways to efficiently separate the nonbiting males from the females. One is the homomorphic nature of the sex-determining chromosomes in *Ae. aegypti*. These homomorphic sex chromosomes are difficult to distinguish karyotypically. They consist of an M-bearing and an m-bearing chromosome 1 with M referring to the ~1.3 Mb male-determining locus (M locus) and m being the counterpart to the M locus. The approximately 310 Mb chromosome 1 is largely autosome-like despite differentiations around the M- and m-loci (19–22). The male-only M locus contains at least two protein-coding genes: *Nix,* which is the male determining factor, and *myo-sex*, which is required for male flight (23, 24). However, the vast majority of the genes on chromosome 1 are not hemizygous in the heterogametic males, providing opportunities for naturally occurring (25–27) or engineered sex-linked recessive lethal alleles (RLAs) that may be inherited differently than in organisms with well-differentiated X and Y chromosomes.

The second feature regards the location of the M locus. The M locus in *Ae. aegypti* is near the centromere (21) and surrounded by an approximately 100 Mb region of very low recombination, which overlaps to a large extent with the region that showed molecular differentiation between the M- and m-chromosomes despite homomorphy (19, 28, 29). It is not clear whether the M- and m-bearing chromosomes in *Aedes* mosquitoes represent nascent proto-Y and proto-X, respectively, or whether they represent ancient sex-determining chromosomes that maintained homomorphic status. Regardless, the existence of sex-linked recombination deserts provides opportunities to use tightly sex-linked RLAs to engineer incompatible sex chromosomes. Incompatible sex chromosomes can then be used to achieve efficient production of nontransgenic males using a single transgenic strain, significantly improving upon existing sex separation methods.

In this study, by isolating naturally occurring sex-linked RLAs and by CRISPR/Cas9-mediated knockout of essential yet haplosufficient genes, we generated a set of transgene-marked homomorphic sex chromosomes in *Ae. aegypti* that are incompatible with chromosomes that share the same RLA. We developed multiple self-sustaining single-strain sex separation lines that continuously produce nontransgenic males by differential elimination of marked sex chromosomes (DeMark), taking advantage of these sex-linked RLAs. The DeMark methods can be integrated with existing SIT/IIT programs especially when the release of nontransgenic males is preferred. The DeMark concept can also be adapted to achieve efficient sex separation for organisms with well-differentiated X and Y chromosomes such as the *Anopheles* mosquitoes.

Results

Measuring the Low Rate of Recombination Near the Sex Locus Using Transgenic Markers in *Ae. aegypti*. The *Ae. aegypti* sex locus is in a recombination desert (19, 28–30). To further determine the recombination rate near the sex locus, we used sex-linked markers to enable screening of a large number of progeny to increase the resolution of the measurement of genetic distances between the M locus and the nearby markers. As shown in Fig. 1, C1 is a transgenic insertion that expresses a DsRed fluorescent marker driven by the conserved 3xP3 promoter (*SI Appendix*, Table S1 and Dataset S1) and C1 is inserted at position 167.945 Mb (see *SI Appendix*, Fig. S1 and Dataset S2 for insertion site determination by Oxford Nanopore sequencing). P14 is a recently isolated transgenic line (29) that expresses a GFP fluorescent marker driven by the polyubiquitin (PolyUb) promoter (31) and P14 is inserted at 180.261 Mb. P10 has the same transgene as P14 but the insertion site is at 224.678 Mb (29).

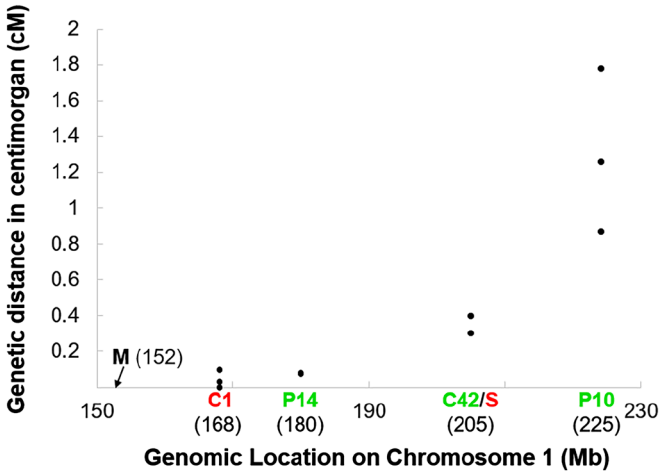


Fig. 1. Genetic distances between the M locus and various markers on the q arm of chromosome 1 (X-axis, in Mb) are shown in centimorgan (cM) on the Y-axis. The locations of the M locus and the markers are shown in brackets. Multiple data points for each marker reflect genetic distances determined from replicate crosses described in this study. Recombination rate data for C1, P14, and P10 can be found in *SI Appendix*, Tables S2–S4. The C42 and S data are from previous publications (27, 30) and these two transgenes are inserted in similar locations (29). The red and green colors indicate DsRed- and GFP-transgenic markers, respectively. We focused on the 1q arm which has a larger region of suppressed recombination than 1p.

Here, we determined the recombination rates between the M locus and C1, P14, and P10, all in the Liverpool (LVP) background, by screening thousands of progenies. The genetic distance between each marker and the M locus is shown in centimorgans (cM) in Fig. 1, and crosses and progeny counts used to calculate the recombination frequencies are shown in *SI Appendix*, Tables S2–S4. In Fig. 1, we also compared these markers with the previously characterized C42 and sensor markers (27, 30). C1 and P14 showed on average 0.043% (or ~4 recombinants per 10,000 progenies) and 0.079% (or less than 8 recombinants per 10,000 progenies) recombination relative to the M locus, respectively. By resolving the extremely low genetic distances, the above results provide an opportunity to revisit the comparison between the physical distance and the genetic distance in the sex-linked recombination desert. For example, the C1 marker is inserted at 167.945 Mb, approximately 16 Mb away from the M locus. Thus, the per Mb recombination rate in this region is 0.043%/16 Mb = 0.00269% per Mb. This is approximately 100-fold less than the average genome-wide recombination rate in *Ae. aegypti* and 1,000-fold less than that in *Drosophila melanogaster* (32). Albeit at an extremely low rate, recombination still occurs between C1 and the M locus. Indeed, there is recombination between the M locus and a recently reported insertion that is 9 Mb away at position 161 Mb (18).

Naturally Occurring Sex-Linked Recessive Lethal Alleles. Recombination events occurring between the M locus and the C1 transgene marker led to DsRed-negative males that lost the

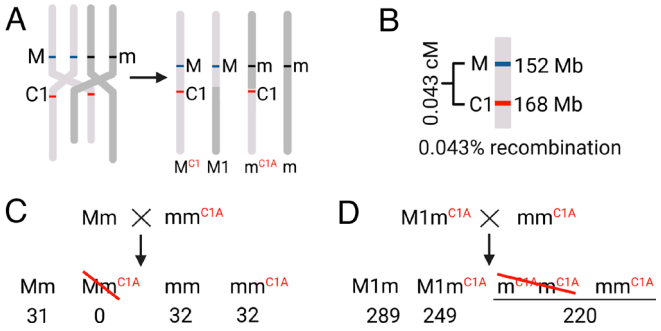


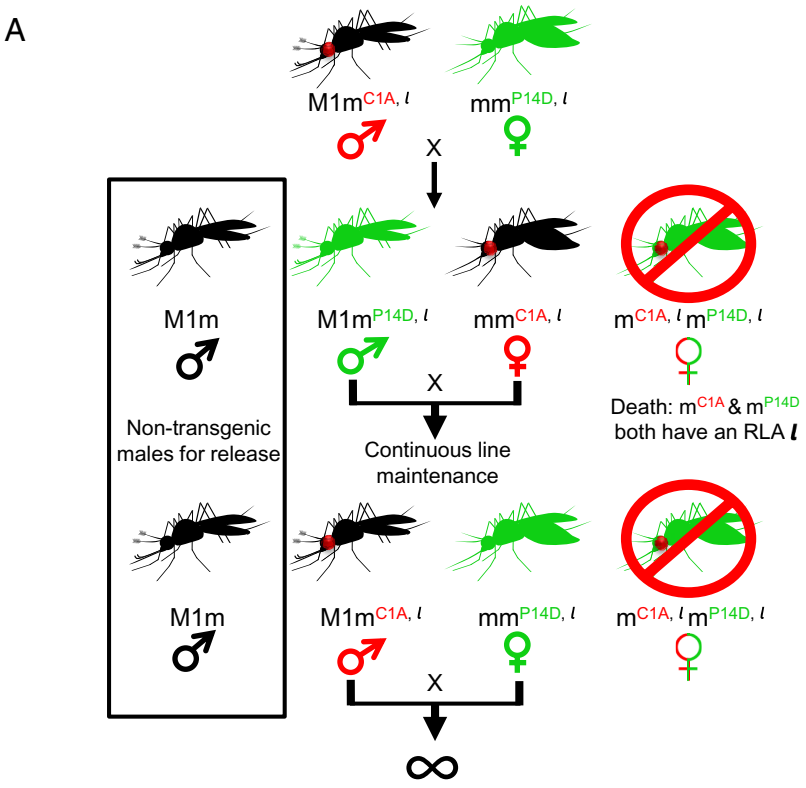
Fig. 2. Sex-linked recessive lethal allele(s). (*A*) Meiotic recombination produces: 1) m^{C1A}, an m-bearing chromosome that acquired the C1 transgene and a chunk of the M-chromosome (light gray); 2) M1, an M-bearing chromosome that acquired a chunk of the m-chromosome (dark gray). (*B*) Despite the 16 Mb distance, the recombination rate between M and C1 is 0.043%. (*C*) Mm^{C1A} males are dead as indicated by the lack of C1(DsRed)-positive males. (*D*) The number of C1-positive females suggests that $m^{C1A}m^{C1A}$ females are dead. Thus, results shown in *C* and *D* are consistent with the hypothesis that the C1-associated RLA(s) is initially M-linked and lethality is likely recessive but not sex-specific.

marker from the M chromosome and DsRed-positive females that gained the marker on the m chromosome (Fig. 2 and *SI Appendix*, Table S2). For ease of description, we will use the following chromosome naming nomenclature. M and m indicate the wild-type M-bearing and m-bearing chromosome 1, respectively, not just the locus. We name the M chromosome with a transgene (T) insertion M^T and the recombinant m chromosome that acquired the transgene m^T. For example, M^{C1} refers to an M chromosome that has a C1 transgene inserted. As different breakpoints will lead to different recombinant m chromosomes, we add a letter to differentiate them (e.g., m^{C1A} in Fig. 2). The same nomenclature applies when we describe M^{P14} and m^{P14D} chromosomes. We use a number after M to indicate a recombinant M chromosome. For example, M1, which was isolated by loss of C1, is still an M chromosome as it has the male-determining M locus.

As shown in Fig. 2*C* and *SI Appendix*, Table S5, crosses between mm^{C1A} females and wild-type Mm males resulted in lethality of Mm^{C1A} male progeny. Sex-specific lethality resulting from crosses with sex-linked recombinants has been previously reported, although the mechanism remains unresolved (25–27). To test whether the lethality of the C1-positive males could be rescued by M1, we crossed M1m males with mm^{C1A} females. Positive male offspring ($M1m^{C1A}$) survived, which is consistent with rescue by M1 (*SI Appendix*, Table S5). To test whether the lethality we observed is sex-specific, we crossed $M1m^{C1A}$ males with mm^{C1A} females (Fig. 2*D* and *SI Appendix*, Table S5). In their progeny, a male:female sex ratio of 2:1 was observed. As either m/m^{C1A} or m^{C1A}/m^{C1A} were positive for DsRed, the lower-than-expected number of DsRed-positive females suggests that the inheritance of two copies of m^{C1A} likely resulted in lethality in females. Thus, the wild-type M chromosome likely bears at least one recessive lethal allele (*l*) which was acquired alongside the C1 transgene markers in m^{C1A} but lost in M1. Similar results were obtained with the P14 line indicating that the m^{P14D} also acquired a recessive lethal allele from the M chromosome (*SI Appendix*, Table S5). Multiple recombinants were acquired with m gaining either C1 or P14 from the M-linked C1 or P14 chromosomes, respectively (*SI Appendix*, Table S6). As described below, we focused on m^{C1A} and m^{P14D} which share a common RLA (*l*) (*SI Appendix*, Table S7). The presence or absence of the RLA (*l*) and other relevant information about various chromosomes investigated in this study are summarized in *SI Appendix*, Table S8.

Nontransgenic Male Mosquito Production by Differential Elimination of 2 Marked Sex Chromosomes (De2Mark). Since M1 rescues the lethality conferred by *l* in both m^{P14D} and m^{C1A} recombinants (Fig. 2 and *SI Appendix*, Table S5), we devised a method that could continuously produce nontransgenic males using a single strain (Fig. 3*A*). We hypothesized that by crossing the surviving $M1/m^{P14D}$ males with m/m^{C1A} females, we could produce three surviving phenotypes: M1/m males, $M1/m^{C1A}$ males, and mm^{P14D} females. The fourth phenotype, double-positive $m^{C1A}m^{P14D}$ females, would not survive if the two marked m chromosomes (m^{P14D} and m^{C1A}) contained the same recessive lethal allele, *l*. Indeed, three expected phenotypes were observed (Fig. 3*B* and *SI Appendix*, Table S7). With these three phenotypes, M1/m males do not have any transgenic marker and can be separated from both the DsRed-positive males ($M1/m^{C1A}$) and GFP-positive females (m/m^{P14D}) by fluorescent screening. As demonstrated in two recent papers, a Complex Object Parametric Analyzer and Sorter (COPAS®) can be used to rapidly separate *Ae. aegypti* larvae based on the absence/presence of fluorescence (17, 18). Since the m^{C1A} and m^{P14D} chromosomes alternate in the two sexes every subsequent generation, the resulting transgene-positive offspring can be used to continue line maintenance and to produce M1/m nontransgenic males every generation (Fig. 3 and *SI Appendix*, Table S7). Additionally, the two different transgenic markers enable the user to track strain stability and to remove recombinants. Out of the 13,186 individuals screened, 11 recombinant individuals were readily identified by the presence of unexpected markers and removed. There were seven recombinant nontransgenic females. However, they were removed by size sorting, resulting in no female contamination. The *l* RLA is tightly linked to the two markers as there is only a single double-positive female, which was also removed.

Engineering Sex-Linked Recessive Lethal Alleles to Expand DeMark Design Options. We took advantage of the homomorphic nature of the sex chromosomes to create additional sex-linked recessive lethal alleles by CRISPR/Cas9-mediated knockout of essential but haplosufficient genes near the sex locus. Targeted knockout of essential genes grants the freedom of constructing custom sex separation strains that do not rely on the discovery of naturally occurring recessive lethal alleles. Among a long list of candidates whose 1:1 *D. melanogaster* orthologs have recessive lethal mutant alleles, we chose to first target *amon* (AAEL014523, an ortholog to *D. melanogaster* gene *amontillado* that encodes a neuroendocrine convertase, only 1 Mb from M/m) and *bag* (AAEL006597, <260 Kb away from the M-linked P14 transgene insertion, encodes a *bagpipe*-like homeobox protein). In short, *amon* was chosen for its tight linkage to the M locus on the p-arm, and *bag* was chosen for its tight linkage to the P14 marker (Fig. 1). Both *amon* and *bag* were knocked out using embryos from the mm^{P14D} females crossed with $M1m^{C1A}$ males (*SI Appendix*, Tables S9–S11). While a *bag* mutant (*bag*-) was generated on m (m^{bag-}, *SI Appendix*, Fig. S2 and Fig. 4*A*), *amon* mutants (*amon*-) were generated on three different chromosomes ($M1^{amon-}$, $m^{P14D,amon-}$, and m^{amon-}, *SI Appendix*, Fig. S3). Next, we tested whether the *bag*- and *amon*- mutants conferred recessive lethality. To test *bag*-/*bag*- lethality, we first crossed $m^{bag-}m^{C1A}$ females to wild-type males to identify heterozygous Mm^{bag-} and mm^{bag-} by PCR. Subsequently, a cross between Mm^{bag-} males and mm^{bag-} females yielded 40 male and 17 female progeny (Fig. 4*B*). Approximately half of the females, which could be either mm^{bag-} or $m^{bag-}m^{bag-}$, died. PCR was performed on the surviving adult females to determine their genotype (Fig. 4*C*).



B

Non-transgenic males (M1/m)	Transgenic males (M1/m$^{C1A, l}$ or M1/m$^{P14D, l}$)	Transgenic females (m/m$^{C1A, l}$ or m/m$^{P14D, l}$)	Double-positive females (m^{C1A*}/m^{P14D*})
4544	4311	4323	1

Fig. 3. Differential elimination of 2 marked sex chromosomes (De2Mark). (*A*) The De2Mark strategy: Continuous production of nontransgenic males using a single-line two-marked-sex-chromosome design. m^{C1A} is marked by DsRed and m^{P14D} by GFP but they share a recessive lethal allele (*l*). Crossing transgenic M1m$^{C1A, l}$ males with transgenic mm$^{P14D, l}$ females results in nontransgenic (M1m) males, M1m$^{P14D, l}$ males, and mm$^{C1A, l}$ females, while females that inherit both m$^{C1A, l}$ and m$^{P14D, l}$ die due to having at least one shared RLA (*l*) linked to the markers. The M1m males, which can be separated from the transgenic males and transgenic females using the fluorescent markers, have no transgene or any other genetic modifications as M1 is derived from recombination and m is wild type. GFP-expressing males (M1m$^{P14D, l}$) and DsRed-expressing females (mm$^{C1A, l}$) can mate to produce more M1m males for release as well as the initial genotypes M1m$^{C1A, l}$ and mm$^{P14D, l}$. The process can be repeated in perpetuity and the markers swap between the two sexes in each subsequent generation, which enables the removal of recombinants and monitoring of stability. (*B*) We screened 13,186 individuals over multiple generations: 4,544 nontransgenic males, 4,311 transgenic males, 4,323 transgenic females, 1 double-positive female recombinant, and 7 nontransgenic female recombinants. As shown in *SI Appendix*, Table S7, the rare recombinants were removed during size separation. *It is unclear which m chromosome lost the RLA (*l*) in this double-positive individual.

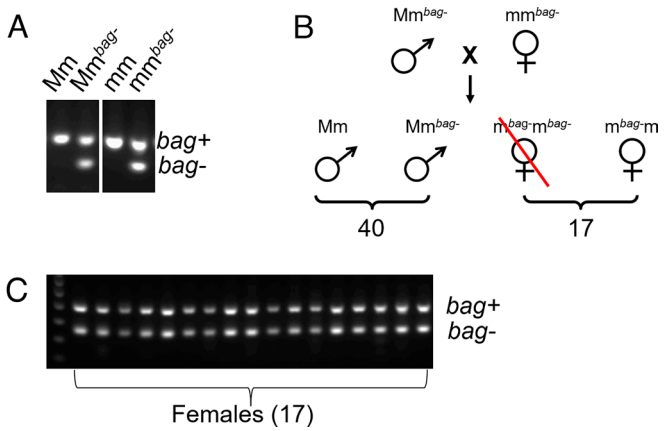
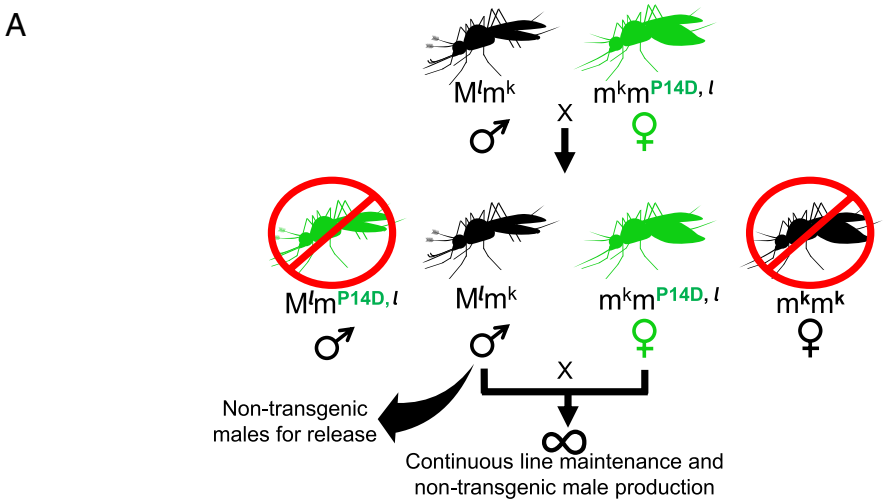


Fig. 4. Knockout an essential but haplosufficient gene to produce a sex-linked RLA. (*A*) Heterozygous *bag-* mutant males (Mm^{bag-}) and females (mm^{bag-}) were identified by PCR amplification followed by a 4% gel electrophoresis. (*B*). Cross of heterozygotes produced progeny showing an approximately 2:1 male to female ratio. (*C*) PCR products amplified from gDNA of legs of the 17 surviving female adults indicate that they are all heterozygous mm^{bag-} and no homozygous m^{bag-}m^{bag-} females were observed, confirming the recessive lethality phenotype for this *bag-* mutation.

All of the 17 females were heterozygous for the *bag-* mutation, indicated by having two bands corresponding to the *bag-* and *bag+* alleles, respectively (Fig. 4*C*), which confirms that *bag-* confers recessive lethality. To test whether or not *amon-* also confers recessive lethality, we performed two crosses between G_1 mutants (*SI Appendix*, Fig. S3*C*). The crosses between M1^{amon-}m males and m^{amon-}m^{C1A} females as well as M1^{amon-}m males and mm$^{P14D,amon-}$ females yielded no homozygous mutant progeny (M1^{amon-}/m^{amon-} and M1^{amon-}/m$^{P14D,amon-}$, respectively), confirming a recessive lethality phenotype for the *amon* knockout.

Differential Elimination of 1 Marked Sex Chromosome (De1Mark) Leads to a Single Strain that Continuously Produces Nontransgenic Males and Transgenic Females. Using a combination of natural (*l*) and engineered recessive lethality (k, representing either *amon-* or *bag-*), we devised a way to produce only nontransgenic male and transgenic female progeny (Fig. 5*A*). By crossing Mm^{k} males with m^{k}m^{P14D} females, with one naturally occurring or engineered recessive lethal allele on each chromosome, we can further limit the number of compatible chromosomes to only the combination of M and m^{k} and the combination of m^{k}



B

	$M^l m^{P14D,\,l}$	$M^l m^k$	$m^k m^{P14D,\,l}$	$m^k m^k$
De1Mark_*amon (k=amon-)*	0	1179	1110	0
De1Mark_*bag (k=bag-)*	0	419	458	0

Fig. 5. Differential elimination of 1 marked sex chromosome (De1Mark). (*A*) The De1Mark strategy: using a combination of natural (*l*) and engineered recessive lethality (*k*), we devised a strategy that enabled the production of only nontransgenic male and transgenic female progeny. The two *k* RLAs resulted from the knockout of the sex-linked genes *amontillado* (*amon*) and *bagpipe* (*bag*), respectively. The nontransgenic males and transgenic females can be separated using a single fluorescent marker (the GFP-expressing P14D) on the marked m chromosome. A fraction of the nontransgenic males can be used for line maintenance while the vast majority of them can be used for nontransgenic male release. (*B*) Only nontransgenic males and transgenic females were produced by both De1Mark strains. Detailed data from both De1Mark_*amon* and De1Mark_*bag* strains are provided in *SI Appendix*, Tables S11 and S12, respectively.

and m^{P14D}. As both the wild-type M and the m^{P14D} contain the RLA (*l*), they are labeled as M^l and $m^{P14D,\,l}$ in Fig. 5. Males that inherit m^{P14D} will die due to lethality conferred by inheriting two copies of *l* (one on the wild-type M and one on m^{P14D}). Likewise, P14-negative females ($m^k m^k$) are homozygous to the engineered RLA and will also die. Thus, the resulting progeny should resemble the paternal phenotypes: Mm^{bag-} males and $m^{bag-}m^{P14D}$ females. Two versions of the De1Mark design were developed using either the *bag-* or *amon-* RLAs. The Mm^{bag-} and $m^{bag-}m^{P14D}$ individuals needed for this De1Mark_*bag* line were produced using the crossing scheme depicted in *SI Appendix*, Fig. S4. A second version, De1Mark_*amon*, was created using *amon-* instead of *bag-* (*SI Appendix*, Fig. S5). Once these lines were established, the cross between Mm^{bag-} males and $m^{bag-}m^{P14D}$ females and the cross between Mm^{amon-} males and $m^{amon-}m^{P14D}$ females yielded only the desired nontransgenic male and transgenic female phenotypes (Fig. 5*B* and *SI Appendix*, Tables S11 and S12) that were easily separated by the GFP marker.

Table 1. Mating competitiveness between LVP and De1Mark_*bag* males

Replicates*	Mated with LVP male†	Mated with De1Mark_*bag* male†
1	9	7
2	7	11
3	8	11

*Twenty F0 females were included in each replicate. However, not all of them produced F1 progeny or provided genotyping results from digital droplet PCR (ddPCR, *SI Appendix*, Table S13).

†Assignments of the genotype of the mate of the F0 females were determined by the copy number of the *bag* deletion mutant relative to a reference gene (23). As shown in *SI Appendix*, Fig. S6, F1 females from a mother that mated with a De1Mark_*bag* male should have one copy of the *bag* mutant allele due to heterozygosity while F1 females from a mother that mated with an LVP male should have zero copy of the *bag* mutant allele. Details such as the number of positive ddPCR droplets, copy number, and mate assignment are provided in *SI Appendix*, Table S13.

De1Mark_*bag* Males and LVP Males Are Not Significantly Different in Mating Competitiveness Under Laboratory Conditions. Mating competitiveness of the nontransgenic males produced by the De1Mark_*bag* strain was evaluated with three replicates. In each replicate, 20 LVP males and 20 De1Mark_*bag* males competed for 20 LVP females (F0) (*SI Appendix*, Fig. S6). Genomic DNA was isolated from a pool of 10 female F1 progeny from each parent F0 female and used to determine the copy number of the mutant *bag* allele by digital droplet PCR (ddPCR, *SI Appendix*, Table S9). F1 females from a mother that mated with a De1Mark_*bag* male should have one copy of the *bag* mutant allele reflecting heterozygosity. In contrast, F1 females from a mother that mated with an LVP male should have zero copies of the *bag* mutant allele. As shown in Table 1 and *SI Appendix*, Table S13, the number of females that mated with LVP versus De1Mark_*bag* males in the three replicates were 9:7, 7:11, and 8:11, respectively. No significant difference was observed in the mean number of matings between LVP and De1Mark_*bag* males (Generalized Linear Mixed Model; $z = 0.686$, $P = 0.493$). The statistical power for this analysis is 81.08% (Cohen's h = 0.347), exceeding the commonly accepted threshold of 80% (33).

Discussion

The Advantages of the Current DeMark Strains. DeMark provides strategies to produce nontransgenic males that are readily separated from transgene-marked individuals in single self-sustaining strains. This was accomplished by using sex-linked RLAs in the recombination desert surrounding the sex locus to create incompatible sex chromosomes, similar to the concept of balancer chromosomes. By establishing one-step sex separation in a single self-sustaining strain, the DeMark strains provide significant operational and cost advantages compared to the recently developed nontransgenic male production method that requires the maintenance of two transgenic lines that involves

three separate sex separations (18). In De1Mark, all females are transgenic and males are not. In addition, each De1Mark strain is self-sustaining, and sex can be separated based on the presence/absence of the transgenic marker. In De2Mark, marked transgenic males and females are used for line maintenance, while the nontransgenic males can be separated from their marked siblings. As the markers (GFP and DsRed) alternate between the two sexes in consecutive generations in the De2Mark strain, these markers can be used to remove the already rare recombinants and monitor stability.

Improving DeMark Stability. Recombination presents a common challenge to the stability of many genetic sexing strains as it could cause the system to break down. In this regard, it is important to note that the C1 and P14 markers exhibited very low rates of recombination relative to the M locus, 0.043% and 0.079%, respectively. Not a single GFP-positive male was detected in the 3,166 offspring screened from the two De1Mark lines (2,289 + 877 = 3,166; *SI Appendix*, Tables S11 and S12), indicating that *l* is also tightly linked to the M locus. As described earlier, *l* is tightly linked to the C1 and P14 markers as there is only one double-positive female among 13,186 individuals screened from the De2Mark crosses. However, the stability of the current DeMark strains remains to be tested under mass rearing conditions. The most straightforward way to mitigate the effect of recombination is to insert the markers as close to the m locus as possible. For example, the *amon* gene is a good insertion target as it is less than 1 Mb away from the sex locus. However, unless we insert the markers into the small m locus, which could be challenging, recombination will still likely occur, albeit at an extremely low rate. The second method to mitigate recombination is to induce chromosomal inversions between the sex locus and the marker(s), as shown recently in *Ae. aegypti* (12). Using inversions to further reduce recombination is a common feature of the balancer chromosomes in *D. melanogaster* (34). Shown in Fig. 6*A* is a third approach, which is a De2Mark design that can further mitigate the impact of the already low recombination. As described earlier, *amon* and *bag* are in a recombination desert and both are tightly linked to the sex locus. *amon* and *bag* are less than 1 Mb (no recombination has been detected) and ~28 Mb (0.079 cM) from the sex locus, respectively. *amon*R indicates an *amon* knockout caused by CRISPR/Cas9-mediated knock-in of cassette R which is a DsRed marker. Similarly, *bag*G indicates a knock-in of the GFP marker cassette that results in the knockout of *bag*. The first advantage of this design is the lack of recombination between the marker and the RLA. The second advantage is that *amon*R and *bag*G are differentially marked recessive lethal alleles that flank the sex locus. Progenies resulting from rare recombination events will either be dead or show the separation of the DsRed and GFP markers, making them very easy to remove.

The Marked m-Chromosome as an "Engineering Playground": All-Male Production Without the Need for Screening. As the transgene-marked m chromosome(s) will not be passed on to the nontransgenic males barring rare recombination events (see above for mitigations), these marked m chromosomes are "playgrounds" for engineering various features for diverse applications without affecting the genotype of the nontransgenic males. The current DeMark strains will require a COPAS-type sorter at the mass-rearing facilities. However, with future incorporations of conditional systems, the production of nontransgenic males may be achieved through chemical or photoinduced selections. For example, if we add a conditional lethal cassette to the R and G cassettes shown in Fig. 6*A*, all transgenic progeny may be selected against by the conditional lethality during the production phase, leaving only nontransgenic males for release. Either the previously described tetracycline-controlled system (e.g., ref. 35) or the recently developed counterselection markers (36) can be inserted with the R and G cassettes to conditionally remove transgenic individuals. This design will negate the need for sex separation for nontransgenic males during the production phase, further improving the efficiency and cost-effectiveness of both De1Mark and De2Mark methods. Effects on the mosquito microbiota should be considered when selecting the most effective counterselection systems. To mitigate the impacts on microbiota, other conditional systems such as novel riboswitches (37) or photoinducible protein inactivation (38, 39) may also be explored.

Other Practical Considerations. Another advantage of the De2Mark system shown in Fig. 6*A* is that completely non–genetically modified males are produced, which should allow full introgression with strains of local genetic background to produce competitive and locally adapted males. On the other hand, the males from the De1Mark system contain a knockout allele of a haplosufficient gene, which may or may not affect their competitiveness depending on the knockout allele. We have shown that De1Mark_*bag* males are not statistically different from the LVP males in mating competitiveness under laboratory conditions.

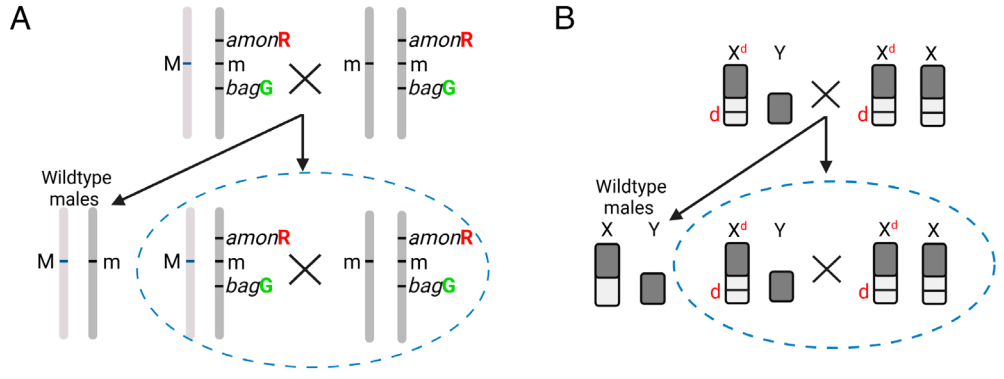


Fig. 6. (*A*) An example of a De2Mark design to further mitigate the impact of the already low recombination. *amon* and *bag* are in a recombination desert and are tightly linked to the m-locus, less than 1 Mb (no recombination has been detected) and ~28 Mb (0.079 cM or 0.079% recombination frequency) from the m locus, respectively. *amon*R indicates a *amon* knockout caused by CRISPR/Cas9-mediated knock-in of cassette R (DsRed marker). *bag*G indicates a knock-in of the G cassette (GFP marker) that results in the knockout of *bag*. Wild-type alleles are not shown. Linking R and G with a conditional lethal cassette will enable all nontransgenic male production without the need for screening as R- and G-containing individuals will die with and only with the provision of the lethal condition. (*B*) Application of De2Mark to organisms with well-differentiated X and Y chromosomes, using *Anopheles stephensi* as an example. Like *amon*R or *bag*G, d is a female-specific recessive lethal allele that resulted from the knock-in of a counterselection marker into an essential but haplosufficient gene.

Before a pilot trial is conducted, the stability of the system and the competitiveness of produced males need to be investigated under mass-rearing conditions following standard protocols.

DeMark Can Also Produce Nontransgenic Males for *Anopheles* Species Which Contain Heteromorphic X and Y Chromosomes. Although we focused on *Ae. aegypti*, which contains homomorphic sex chromosomes, the De2Mark concept developed here can be readily applied to *Anopheles* mosquitoes. As shown in Fig. 6*B*, recessive lethal alleles (d) could be created by knocking out an essential but haplosufficient gene on the X chromosome, which is homologous to the p arm of chromosome 1 of *Ae. aegypti* and contains more than 1,000 protein-coding genes. Like in the case of *amon*R and *bag*G (Fig. 6*A*), d also contains an easily selectable marker such as a fluorescent marker and/or a counterselection marker (36). When XX^d females mate with X^dY males, two male genotypes (XY and X^dY) and two female genotypes (XX^d and X^dX^d) are expected. As d is a recessive lethal allele, X^dX^d females will die. X^dY males and XX^d females can be separated from XY males by either the fluorescent or counterselection marker. XY males are completely devoid of any genetic modifications and can be used in sterile male or other genetic control programs when release of nontransgenic males is preferred. Mating of the two remaining genotypes, the transgene-containing XX^d females and X^dY males, will reiterate the process allowing the continuous production of non–genetically modified XY males for release and XX^d and X^dY individuals for line maintenance. This strategy requires choosing a gene or splice isoform that is essential only in females. Alternatively, a binary system could be used (e.g., ref. 40). For example, a Cas9 cassette is knocked-in to a locus on one X chromosome (X^{Cas9}) while a sgRNA cassette targeting an essential gene is inserted in the same locus of another X chromosome (X^{sgRNA}). Thus, lethality will occur only when X^{Cas9} and X^{sgRNA} are present in the same individual. The general lack of recombination between the X and Y chromosomes will help ensure the stability of this De2Mark design. Although we have not generated transgenic lines to test this method in *Anopheles* mosquitoes, our success in *Ae. aegypti* provides the conceptual foundation as well as conserved genetic reagents for potential applications in *Anopheles*.

Concluding Remarks. Given the biological, geographical, economic, and cultural complexity facing global mosquito-borne infectious disease control programs, diverse measures are needed that can be integrated to address various challenges. Using naturally occurring and engineered sex-linked RLAs, we developed sex separation methods for cost-effective and operation-friendly production of nontransgenic males. These DeMark methods have the potential for broad applications and can be integrated with existing SIT/IIT programs especially when the release of nontransgenic males is preferred.

Materials and Methods

Mosquito Rearing. The *Ae aegypti* Liverpool (LVP) strain was obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI, www.BEI.org) which was used to generate all transgenic and knockout lines described in this work. All mosquito lines were maintained at Virginia Tech at 26 to 28 °C and 60 to 70% relative humidity with a 12/12 h day/night light cycle. Adult mosquitoes were fed a 10% sucrose solution and fed defibrinated sheep's blood (Colorado Serum Company; Denver, CO) using artificial membrane feeders.

Generation and Characterization of the C1 Transgenic Line. A mixture of the *piggyBac* donor plasmid pBac_bZip_albY1_3xP3_DsRed (Full sequence is provided as Dataset S1) and the *piggyBac* mRNA, both at 300 ng/uL, was injected into the *Ae. aegypti* LVP strain embryos. The *piggyBac* mRNA was in vitro transcribed using the mMessage mMachine T7 Ultra kit (Thermofisher) with the *piggyBac*-hsp70-transposase (41) as the template, followed by MEGAclear (Thermofisher) column purification. Surviving G_0 males were mated with G_0 females. G_1 larvae were screened for DsRed using a Leica M165 FC fluorescence microscope. As shown in *SI Appendix*, Table S1, five DsRed-positive G_1 males were identified and outcrossed to Liverpool females at a ratio of 1:5. The iso-male transgenic lines that have M-linked insertions were generated from single males mated with LVP females. DsRed-positive male samples were collected and sequenced using Oxford Nanopore sequencing to determine the insertion site (*SI Appendix*, Fig. S1 and Dataset S2). C1 is inserted at position 167.945 Mb.

High-Resolution Analysis of Genetic Distances Between the Sex Locus (M or m) and Genetic Markers Within the Sex-Linked Recombination Desert. The linkage between the M locus and sex-linked markers including C1 and a few recently reported transgenes (29) was assessed by screening for recombination events among thousands of progenies (*SI Appendix*, Tables S2–S4). During pupal screening of the M-linked C1, P10, and P14 transgenic lines, recombinant negative males and positive females were isolated and recorded. The sex of each potential recombinant was confirmed as adult.

Sex Sorting and Screening. At the pupal stage, male and female pools were separated by size using a mechanical sex sorter (42). The male and female pools were then split into 2 oz cups of smaller groups of 50 to 150 total male or female pupae. The male and female pupae were then screened for the genetic markers present for each genetic sexing strategy. For experiments involving transgenic lines with fluorescent markers, the male and female pools were further separated by screening for either green (GFP) or red (DsRED) fluorescence using a Leica M165 FC fluorescence microscope. Pupa from each sex were placed in separate cartons to emerge and confirm their sex. Data describing recombination frequencies (*SI Appendix*, Tables S2–S4), recessive lethality and its rescue (*SI Appendix*, Table S5), and the DeMark strains (*SI Appendix*, Tables S6, S9, and S10) were all obtained using the above sorting and screening methods.

CRISPR/Cas9-Mediated Knockout of Essential but Haplosufficient Genes. Three sgRNAs were designed to target the predicted coding sequence for genes AAEL014523 located at 150.1 Mbps (p-arm) and AAEL006597 located at 180.1 Mbps (q-arm) on Chromosome 1 (*SI Appendix*, Table S7) using the CHOPCHOP sgRNA design web tool (43–45). DNA templates for each sgRNA were obtained by PCR using previously described protocols (46). Each sgRNA was synthesized by in vitro transcription using the MEGAscript T7 kit (Thermo Fisher Scientific), purified using the MEGAclear kit (Thermo Fisher Scientific), and then aliquoted for embryonic microinjections (47). 0- to 1-h embryos collected from the De2mark sex separation strain (DsRed-positive males × GFP-positive females, Fig. 3) were injected with a mixture containing 300 ng/uL Cas9 mRNA and 100 ng/uL each of the three sgRNAs targeting either AAEL014523 or AAEL006597. G_0 embryos were hatched and reared separately (*SI Appendix*, Table S8). Each of the three G_0 genotypes (nontransgenic male, GFP-positive male, and DsRed-positive female) was screened using a fluorescence microscope and crossed following the scheme in *SI Appendix*, Figs. S2 and S3. Nontransgenic male pool and GFP-positive male pool were mated with nonsibling mm^{C1A} females, and the DsRed-positive female pool was mated with nonsibling $M1m^{P14D}$ males. G_1 eggs were collected from each pool and reared until the pupal stage and then separated by sex and fluorescent phenotype and placed into separate cartons and allowed to emerge as adults. One-to-three-day-old G_1 adults were used to identify possible mutants by removing a single leg from each individual, then performing the high-resolution melting analysis (HRMA) of PCR amplicons of the sgRNA target region as previously described (24) using primers listed in *SI Appendix*, Table S7. PCR products from possible mutant candidates identified by HRMA were purified using the Nucleospin PCR and gel clean-up kit (Macherey-Nagel) and sequenced using Sanger (Genomics Sequencing Center, Virginia Tech).

Mating Competitiveness Assay. The general design of the assay is shown in *SI Appendix*, Fig. S6. Three biological replicates were performed. For each replicate, LVP and De1Mark_*bag* eggs were separately hatched under vacuum. A few days after hatching, one hundred larvae were selected for each strain and reared in 500 mL of water. Two to three days after adult emergence, 20 LVP males and 20 De1Mark_*bag* males were mixed in a 44 oz cage for 2 d before 20 3- to 4-d-old virgin LVP females were added. Three days after blood feeding, the females (F0)

were separated to lay eggs individually. Eggs from each female were hatched and grown to adulthood (F1). Ten F1 female progeny from each F0 mother were pooled for genomic DNA isolation using the Zymo Quick-DNA miniprep kit (Zymo Research, Irvine, CA). Digital droplet PCR (ddPCR) using a TaqMan probe that specifically targets the mutant *bag* allele (*SI Appendix*, Table S9) was performed with the pooled F1 gDNA as the template to determine the copy number of the mutant *bag*. A previously established single-copy gene was used as an internal reference, with the reference copy number set to 2 for diploid alleles [(23), *SI Appendix*, Table S9]. ddPCR was performed with the Bio-Rad QX100 ddPCR machine (Hercules, CA) according to the manufacturer's protocols. As shown in *SI Appendix*, Fig. S6, F1 females from an F0 mother that mated with a De1Mark_*bag* male should have one copy of the *bag* mutant allele reflecting heterozygosity. In contrast, F1 females from an F0 mother that mated with an LVP male should have zero copies of the *bag* mutant allele.

Statistical Analysis. We used a generalized linear mixed model (GLMM) with binomially distributed errors to evaluate binary outcomes, namely mating with either an LVP male or a De1Mark_*bag* male. The GLMM included mating with LVP vs. De1Mark males as the fixed effect and replicate numbers as the random effect to account for variability across experimental groups (48). There is negligible variability between replicates (random effect variance = 0) and no significant effect at the fixed level (fixed effect estimate = 0.189, $z = 0.686$, $P = 0.493$). Model fit metrics, including an AIC value of 77.0, indicate that the model adequately represents the data, balancing goodness-of-fit with model complexity. A power analysis was conducted to determine the study's ability to detect a meaningful difference between the null (proportion = 0.5, or equal mating competitiveness) and alternative hypotheses. Using *Cohen's h* as a measure of effect size ($h = 0.347$) and a sample size of 53, the power was calculated to be 0.811, exceeding the commonly accepted threshold of 80% (33).

Data, Materials, and Software Availability. All study data are included in the article and/or supporting information.

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