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MICRO-REVIEW

Developing CRISPR-based sex-ratio distorters for the genetic control of fruit fly pests: A how to manual

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Abstract

Agricultural pest control using genetic-based methods provides a species-specific and environmentally harmless way for population suppression of fruit flies. One way to improve the efficiency of such methods is through self-limiting, female-eliminating approaches that can alter an insect populations' sex ratio toward males. In this microreview, we summarize recent advances in synthetic sex ratio distorters based on X-chromosome shredding that can induce male-biased progeny. We outline the basic principles to guide the efficient design of an X-shredding system in an XY heterogametic fruit fly species of interest using CRISPR/Cas gene editing, newly developed computational tools, and insect genetic engineering. We also discuss technical aspects and challenges associated with the efficient transferability of this technology in fruit fly pest populations, toward the potential use of this new class of genetic control approaches for pest management purposes.

KEYWORDS
CRISPR/Cas9, Redkmer, spermatogenesis, X-chromosome, X-shredding system

1 | GENETIC CONTROL IN PEST MANAGEMENT

Genetic control methods to suppress agricultural pests are alternative approaches that mitigate the negative effects of excessive chemical insecticide use. The sterile insect technique (SIT) was first developed to control insect populations almost 50 years ago and has since been successfully applied in several fruit fly species

(Dyck, Hendrichs, & Robinson, 2006). The SIT relies on the mass-production, sterilization, and release of male insects to induce sterility through infertile matings with wild females, resulting in population suppression. There are several limitations with SIT, however, that have hampered efforts to develop this technology in a broad range of species (Calkins & Parker, 2005). Among the most important limitations is the generation of rigorous strains and the need to release only male insects. The latter requirement is mandatory for most large-scale programs, since co-released females can exacerbate crop damage and agricultural losses. Separating females from released males in early developmental stages is preferred, as this minimizes mass rearing and sterilization costs, which otherwise render the implementation of a SIT program expensive.

Recent advances in insect genetic engineering have provided unprecedented opportunities for overcoming previously intractable limitations using classical genetic approaches and improving the overall efficiency of control programs. One approach that has attracted significant attention is the release of engineered fertile males that produce only viable sons, that is, be daughterless. Eliminating female offspring is appropriate, because in most insect species, population size is determined by the number and productivity of its females. Moreover, in many potential target species, it is only female insects that cause agricultural damage, or transmit diseases. Thus, sons of released fertile males can temporarily maintain and amplify the control trait in the population, when these mate with wild females. Two strategies have been suggested to make daughterless strains, one that eliminates females and one that manipulates sex determination to convert genetic females into phenotypic males. Manipulating the sex determination requires intimate knowledge of the mechanism involved in each species, and, as a result, limited progress has been made to date in generating functional strains using sex conversion (Saccone et al., 2007; Schetelig, Milano, Saccone, & Handler, 2012).

On the other hand, two female elimination strategies have been successfully developed already. The first relies on the conditional, post-zygotic female lethality, which is based on a construct that specifically kills female offspring that inherit it, in the absence of an antibiotic that suppresses lethality in the insect rearing bio-factory (Gong et al. 2005; Heinrich & Scott, 2000; Horn & Wimmer, 2003; Thomas, Donnelly, Wood, & Alphey, 2000). For several fruit flies of economic importance, transgenic pupal lethal fsRIDL (female-specific Release of Insects carrying Dominant Lethal) insects have now been produced (Ant et al., 2012; Fu et al., 2007), as well as insects carrying embryo-specific female lethality systems (Ogaugwu, Schetelig, & Wimmer, 2013; Schetelig & Handler, 2012; Schetelig, Targovska, Meza, Bourtzis, & Handler, 2016).

A second approach, called X-chromosome shredding, is based on constructs that when present in males eliminate sperm containing the X-chromosome. By eliminating X-bearing sperm, offspring sex ratios are biased towards males, since only gametes with a Y-chromosome are produced (Burt, 2003). X-chromosome shredding was developed in *Anopheles gambiae* (Galizi et al., 2014, 2016; Windbichler, Papathanos, & Crisanti, 2008), the main vector of human malaria, as an autosomal self-limiting suppression approach, which has the potential to be converted into a self-sustaining gene drive approach by linking the X-shredder to the Y-chromosome. Mathematical modeling and cage suppression trials have shown that autosomal X-shredding and fsRIDL are both more efficient than SIT, in terms of the number of insects that need to be released (Schliekelman, Ellner, & Gould, 2005). Of the two, autosomal X-shredders are more efficient than fsRIDL, because they operate pre-zygotically to eliminate X-carrying gametes. As a result, heterozygous X-shredder males have, on average, twice the number of male descendants, compared to heterozygous fsRIDL males, increasing the effective number of modified males released.

Below, we summarize recent advances in the development of clustered regularly interspaced short palindromic repeats (CRISPR)-based X-shredding system addressing technical considerations that are required toward transferring this strategy to species other than the malaria mosquito.

2 | FROM NATURAL TO SYNTHETIC SEX-RATIO DISTORTERS

Sex-ratio distorters (SRDs) are naturally occurring elements that modify offspring sex ratio of an individual carrying them, for the purpose of gaining a transmission advantage. SRDs are physically linked to sex chromosomes or

sex-determining loci in X-Y heterogametic systems and have been characterized in several Dipteran species (Hurst & Pomiankowski, 1991). The Y-linked meiotic drivers described in culicine mosquitoes result in the excess of male descendants due to X-chromosome breakage during male meiosis, thus transmitting more functional Y-chromosomes (Gilchrist & Haldane, 1947).

The development of synthetic SRDs is an attractive alternative to overcome complications related to the identification and use of genetically complex traits. Furthermore, because synthetic SRDs are entirely novel, they would not be affected by any naturally occurring suppressors that evolved to counter the detrimental effects of any naturally occurring meiotic drives. In addition, because X-shredding operates at the level of sex chromosome inheritance, the underlying mechanisms of sex determination remain unimportant for developing such sex distorters in other pest species, assuming that sex is determined zygotically by inherited chromosomal factors.

The manipulation of sex ratios with a synthetic X-shredder relies on the expression of a nuclease during male meiosis, when sperm is produced, which recognizes and specifically cleaves tandemly arrayed DNA sequences uniquely located on the X-chromosome (Burt, 2003). Consequently, X-bearing gametes are cut—"shredded"—and therefore distortion of the sex ratio occurs since viable progeny consists only of males. Based on this principle, sex-ratio distorters were first engineered using a synthetic biology approach in *An. gambiae* (Windbichler et al., 2008). The developed SRD expressed a sperm-specific *I-Ppo*I endonuclease that selectively targets a sequence specific to ribosomal DNA repeats, which, in *An. gambiae*, are located exclusively on the X-chromosome. However, this engineered *I-Ppo*I system could not be considered as a universal X-chromosome shredder, because the X-chromosome localization of these targeted rDNA repeats in *An. gambiae* is not conserved among other species.

To establish a similar X-shredding approach in other XY heterogametic species, some specific requirements are needed:

1. Endogenous repetitive X-specific sequences should be identified to shred the X-chromosome in multiple sites.
2. A nuclease is needed to recognize and cleave suitable X-linked sequences.
3. Specific promoters that act during male spermatogenesis should be used to drive the nuclease expression.

Taken together, by targeting exclusively X-linked repeats in the male germline using nucleases driven by a meiotic-specific promoter, it is possible to generate a skewed population sex ratio in any male heterogametic species of interest, if genetic transformation tools are also available. In the following sections, we summarize the basic principles of the above-mentioned requirements to guide the efficient design of an X-shredding system.

3 | NEW TOOLS FOR IDENTIFYING REPEATED X-LINKED SEQUENCES

The first requirement to adapt the current advances in X-chromosome shredding to any XY insect of interest is the availability of repetitive X-linked sequences. However, the heterochromatic nature of sex chromosomes hinders the identification of endogenous tandem X-chromosome sequences suitable for targeting. This feature, coupled with the inadequate sequencing data in many of the potential target species, pose a technical hurdle for the transfer of the X-shredding system to other species.

To overcome this limitation, a bioinformatic pipeline was recently developed to automate the identification of highly abundant X-specific sequences, called Redkmer (Papathanos & Windbichler, 2018). Redkmer requires as input only raw long (e.g., PacBio) and short (e.g., Illumina) whole genome sequencing (WGS) data from the target species avoiding the step of genome assembly, which typically excludes highly repetitive regions. Redkmer identifies and selects repeated X-derived sequences based on (a) the increased coverage of X-chromosome sequences in XX females compared to XY males in WGS data and (b) the relative abundance of the sequences in the same WGS data. The output is a list of high-confidence unique X-linked kmers (sequences of k-length), which are then directly searched for guide RNA (gRNA) selection and off-target prediction depending on the selected RNA-guided endonuclease. These suitable X-linked targets

would be recognized and cleaved by the site-specific endonucleases specifically in the meiotic male germline. The Redkmer pipeline was evaluated in *An. gambiae*, enabling the identification of abundant and X-specific sequences residing in the known ribosomal locus, which has already been experimentally demonstrated as suitable X-shredding target.

4 | ESTABLISHMENT OF CRISPR/CAS9-BASED SYNTHETIC SRDs

The second key success factor for the development of a synthetic sex-ratio distortion trait is the availability of an endonuclease, that will be expressed in vivo. The advent of RNA-guided endonucleases derived from the bacterial CRISPR-Cas system, which facilitates the precise editing of endogenous genomic loci (Mali, Esvelt, & Church, 2013), has accelerated the development of synthetic SRDs. Among the Cas nucleases, Cas9 is the most commonly used. In principle, Cas9 cleaves a target genomic locus, which is specified by a 20-nt targeting sequence within its guide RNA. The only requirement for the selection of Cas9 target sites is the presence of a PAM (protospacer adjacent motif) sequence, consisting of the three-base-pair, NGG directly 3′ of the 20-bp target sequence. In addition, a versatile range of recently discovered RNA-guided endonucleases with distinct properties from Cas9 (e.g., Cpf1), as well as engineered variants of Cas9s with altered and improved PAM specificities and reduced molecular size (Cebrian-Serrano & Davies, 2017), have expanded the existing toolkit, increasing the targeting options and enhancing specificity and efficiency of delivery. Genome-editing methods using the CRISPR/Cas9 system have been successfully applied in several insects (Reid & O'Brochta, 2016) including fruit flies (Aumann, Schetelig, & Häcker, 2018; Bai et al., 2019; Choo, Crisp, Saint, O'Keefe, & Baxter, 2018; Kalajdzic & Schetelig, 2017; Li & Scott, 2016; Meccariello et al., 2017; Sim, Kauwe, Ruano, Rendon, & Geib, 2019; Zhao et al., 2019), indicating its potential use for control approaches as well.

The third important requirement for the establishment of synthetic SRDs is the ability to express the active endonuclease in a tissue-specific manner. This requires the identification and evaluation of suitable native regulatory sequences in each species to drive the testis-specific expression of the endonuclease during spermatogenesis. In X-shredders developed in the malaria mosquito, expression of the endonucleases was driven by the *β-2-tubulin* promoter, which is active during late spermatogenesis in primary spermatocytes (Galizi et al., 2014, 2016; Windbichler et al., 2008). It is currently unclear whether regulatory elements mediating expression during earlier stages of spermatogenesis would result in sex distortion, or whether X-chromosome shredding in earlier stages may be cytotoxic. The *β2-tubulin* promoter is well characterized in several fruit flies (Scolari et al., 2008; Zimowska, Nirmala, & Handler, 2009). In addition, in an engineered construct based on the CRISPR system, the sgRNA that mediate targeting would need to be expressed from a native RNA pol-III promoter such as one of the U6 genes.

In fact, the CRISPR/Cas9 system was recently successfully deployed to act as a synthetic SRD in *An. gambiae* by Galizi et al. (2016). Their approach provided significant opportunities to advance sex-ratio distortion methods by targeting repeated sequences found on the X-chromosome and resulting in their preferential cleavage. Based on their previous I-*Ppo*I work, they constructed a transformation vector that expressed Cas9 ubiquitously under a *β2-tubulin* promoter and a gRNA targeting an X-linked ribosomal repeat under the RNA pol-III promoter *U6:3*. The selected target sequence was different from the original I-*Ppo*I recognition target and was chosen because it is restricted to members of the *An. gambiae* complex. The application of this CRISPR-based SRD demonstrated a high sex ratio distortion of the transgenic male progeny (86.1–94.8%). Meanwhile, these males maintained a fertility level comparable to the wild-type, showing high hatching rates (83.6–93.2%).

5 | BEST PRACTICES AND CHALLENGES FOR SUCCESSFUL DESIGN OF SYNTHETIC SRDs IN FRUIT FLIES

It is clear from the above, that CRISPR-based sex-ratio distorters can be developed for many fruit fly pests. Synthetic and computational biology provides new opportunities for the broad applicability of X-shredding method

in heterogametic species. The flexibility of the CRISPR-Cas9 system, combined with the increasing availability of genomic data for a wide range of these species are substantially contributing toward the generation of the required key elements of this technology. There are several fruit flies that are being considered or already being tested for the application of this sex-ratio distortion system. Among them are species of *Bactrocera* or *Anastrepha* genuses, the Mediterranean fruit fly *Ceratitis capitata*, *Drosophila suzukii*, and others. A typical workflow that should be followed to generate an unbalanced population sex ratio in any male heterogametic species of interest, is summarized in Figure 1.

Cost-efficient next-generation sequencing can provide the genomic resources for the identification of native X-linked repeats that could be further used to generate X-shredder constructs. However, critical analysis is required when selecting the desired target sites to ensure Cas9 efficacy, while minimizing possible off-target effects. Furthermore, challenges associated with CRISPR technology that could reduce the efficacy in field applications, like the development of resistance alleles or the occurrence of natural sequence polymorphism between individuals, can be overcome through the design and use of alternative gRNAs, which will target either a series of genomic loci or multiple sites simultaneously.

However, male mating competition and overall fitness of the engineered flies can be severely compromised by mass rearing protocols, population inbreeding, and transgene expression variability (Reed, Lowe, Briscoe, & Frankham, 2003). The integration of the synthetic SRD traits for population control into vigorous transgenic strains bearing docking sites unsusceptible to mutational and position effects would improve transgene stability and reduce fitness costs upon integration (Horn & Handler, 2005). In addition, carefully selected promoters and target sequences will ensure both the species-specific expression and targeting of the system's key elements (e.g., Cas9 and gRNA), reducing possible risks of horizontal gene transfer among other populations or species.

Another operational concern is that genetic control programs require repeated releases of large numbers of individuals. However, the synthetic X-shredders described so far are autosomal and are constitutively expressed, meaning that the strain can only be maintained by continuous backcrosses to wild types. This characteristic makes these X-shredders in their current form unusable for a large-scale rearing and release operation. Generating a

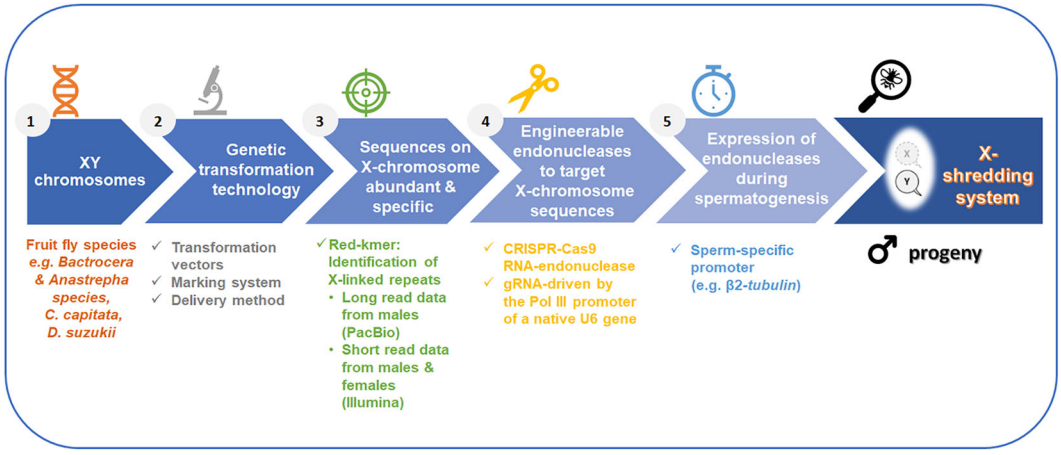

FIGURE 1 A schematic workflow to develop a sex ratio distortion system in a fruit fly species of interest. This innovative technology can be implemented in any species with X-Y heterochromatic system and available transformation tools. The Redkmer computational pipeline uses raw next-generation whole genome sequencing data to identify abundant and specific sequence repeats on the X-chromosomes. These X-linked targets can be recognized and cleaved by the flexible site-specific clustered regularly interspaced short palindromic repeats/Cas9 endonucleases specifically in the meiotic male germline. In the resulting X-shredding system, gametes bearing only the Y-chromosome are formed, that will produce daughterless progeny

conditional X-shredder, as already tested in other genetic control methods (e.g., RIDL), would facilitate the mass rearing conditions of the insects carrying these CRISPR-based SRDs, as it would be suppressed in rearing conditions but active in the field (Scott et al., 2018). For example, a Tet-off system in one construct, or a modified alternative of this, could be generated where a *β2-tubulin* promoter would drive the tetracycline-controlled transactivator (tTA) resulting in the expression of the TRE-regulated (tTA-response element) endonuclease. Therefore, the conditional suppression of a SRD through an appropriate regulatory system would allow the selective increase in the population levels, avoiding continuous and laborious backcrosses for scaling up insect populations in mass rearing before their release. However, a careful consideration should be taken when selecting a strategy to achieve conditional expression to avoid negative fitness effects arising from leaky expression.

Nevertheless, linking the autosomal SRD trait with a drive mechanism such as its transfer to the Y-chromosome, would spread SRD's frequency rate upon release. However, increased considerations for drive's persistence and the risks of permanent establishment, suggest the implementation of controllable, noninvasive methods for pest management. Self-limiting autosomal SRDs meet these criteria since they are not maintained in successive generations, unless continuous releases occur (Oye et al., 2014).

6 | OUTLOOK

This report provides a progress overview in the rapid advances of synthetic X-shredding system, addressing the design principles for its potential use in future control approaches in fruit flies. The prospect of deploying this flexible and precise CRISPR-based intervention system in a pest-management program is challenged by the requirement of risk-assessment evaluations, comprehensive understanding of the target species' biology, population genetics, as well as coordinated efforts between all engaged parties, including scientists, regulatory agencies, and other involved specialists. Taken together, questions that arise out of our need to establish efficient alternative control methods like, "how can knowledge insights of the pest's physiology, behavior or ecology foster practices against it?" or "under which framework will a pest-management strategy implement the genetically modified strain?" should be thoroughly asked to accelerate progress toward the overall goal of effective population suppression of destructive pests.

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