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Methods for the generation of heritable germline mutations in the disease vector *Culex quinquefasciatus* using clustered regularly interspaced short palindrome repeats-associated protein 9

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Abstract

***Culex quinquefasciatus* is a vector of many diseases that adversely impact human and animal health; however, compared to other mosquito vectors limited genome engineering technologies have been characterized for this vector. Clustered regularly interspaced short palindrome repeats-associated protein 9 (CRISPR-Cas9) based technologies are a powerful tool for genome engineering and functional genetics and consequently have transformed genetic studies in many organisms. Our objective was to improve upon the limited technologies available for genome editing in *C. quinquefasciatus* to create a reproducible and straightforward method for CRISPR-Cas9-targeted mutagenesis in this vector. Here we describe methods to achieve high embryo survival and mutagenesis rates and we provide details on the injection supplies and procedures, embryo handling and guide RNA (gRNA) target designs. Through these efforts, we achieved embryo survival rates and germline mutagenesis rates that greatly exceed previously reported rates in this vector. This work is also the first to characterize the *white* gene marker in this species, which is a valuable phenotypic marker for future transgenesis or mutagenesis of this vector. Overall, these tools provide the framework for future functional genetic studies in this important disease vector and may support the development of future gene drive and genetic technologies that can be used to control this vector.**

Keywords: CRISPR, *Culex quinquefasciatus*, germline, mutations, reverse genetics, Cas9.

Introduction

Culex quinquefasciatus is a primary vector of West Nile virus (WNV), eastern equine encephalitis virus, Saint Louis encephalitis virus, lymphatic filariasis and avian malaria (Petersen and Roehrig, 2001; Jones *et al*., 2002; LaPointe *et al*., 2005; Reisen *et al*., 2005). Since the introduction of WNV to the USA in 1999, there have been over 48 000 confirmed cases in this country (United States Centers for Disease Control, 2019). Although this is certainly an underestimate of the overall impact of this disease in the USA, the global impact of lymphatic filariasis is far greater. Current estimates indicate that despite remarkable mass drug administration (MDA) programs, millions of people are currently infected with lymphatic filariasis, which is considered one of the leading global causes of disability (Gyapong *et al*., 2018). Furthermore, there are many areas of the world where MDA is not expected to eradicate this disease, so greater mosquito control efforts may be needed to accomplish this goal (Koudou *et al*., 2018). Moreover, *C. quinquefasciatus* has established itself as the dominant vector of avian malaria on many islands, and in some instances caused the extinction of many rare bird species (LaPointe *et al*., 2012). Thus, technologies to improve *C. quinquefasciatus* control are essential to reduce and potentially eliminate these diseases. However, the widespread development of insecticide resistance in *C. quinquefasciatus*, the primary method for control of this

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vector, has made it imperative that alternative control methods are developed for this vector.

Genome engineering technologies facilitate important functional genetic research as well as the development of tools required to create genetic control strategies for important disease vectors. Recently, the clustered regularly interspaced short palindrome repeats-associated protein 9 (CRISPR-Cas9) system has been used to generate somatic and heritable germline mutations and genetic drive technologies in *Aedes* (Kistler *et al*., 2015; Li *et al*., 2017b, Li *et al*. 2019) and *Anopheles* (Gantz *et al*., 2015; Hammond *et al*., 2016; Dong *et al*., 2018; Kyrou *et al*., 2018; Li *et al*., 2018) disease vectors. In this system, a Cas9 endonuclease and a single guide RNA (sgRNA) complementary to the target site facilitate site directed double-stranded genome breaks, which are repaired by homology directed repair or nonhomologous end joining (NHEJ). Despite this being a useful mutagenesis tool in other vector species, there has been limited emphasis on improving CRISPR genome engineering technologies to study and develop new control tools for *Culex* disease vectors. In 2016, transcription activator-like effector nucleases and CRISPR-Cas9 genome engineering technologies were used to generate frameshift mutations to disrupt the function of an insecticide resistance gene in *C. quinquefasciatus* (Itokawa *et al*., 2016), but no additional CRISPR-Cas9 mutagenesis work has been published for this vector. Furthermore, with the publication of the *C. quinquefasciatus* genome in 2010 (Arensburger *et al*., 2010), there have been few functional genetic studies of this species, which is unfortunate owing to not only its importance as a disease vector, but its unique biological characteristics (Severson and Behura, 2012) including its susceptibility to diverse pathogens (ie viral, nematode and protozoan) (Bartholomay *et al*., 2010) and opportunistic blood-feeding behaviour (ie birds, humans and other mammals), as well as diverse geographical and habitat preferences. Therefore, in order to make CRISPR technologies more accessible to the research community, we aim to meticulously describe the development of a CRISPR-Cas9 mutagenesis system for *C. quinquefasciatus* targeting the *white* gene. This is the first characterization of this gene in *C. quinquefasciatus*, which has been an important phenotypic marker for genome engineering of other mosquito vectors (Coates *et al*., 1997). We hope that this tool will be useful for further functional genetic studies in this important disease vector and may lay the framework for the development of genetic control tools.

Results

Development of an CRISPR-Cas9 embryo microinjection protocol

We established efficient techniques for egg collection, preblastoderm stage embryo microinjection, and subsequent rearing and genetics. In brief, we first evaluated different types of capillary glass injection needles (quartz, aluminosilicate, borosilicate). The needle pulling settings were also evaluated to minimize breakage and clogging during the injection procedures. Table S2 shows the needle pulling parameters for all three needle types. We found that aluminosilicate needles produced the highest embryo survival at the most affordable cost. We then conducted experiments to improve the mosquito handling procedures, which are outlined in the Experimental procedures.

Mutagenesis of the white *gene locus in* C. quinquefasciatus

To test the efficiency of our CRISPR-Cas9 based genome editing platform in *C. quinquefasciatus*, we targeted the recessive *white* (*w*) gene (CPIJ005542), which codes a protein critical for eye pigment transport. In other species, biallelic mutations in the *w* gene disrupt production of dark eye pigmentation and generate an easily screenable unpigmented eye colour (Bassett *et al*., 2014; Ren *et al*., 2014; Li *et al*., 2017a, 2018; Xue *et al*., 2018). Consequently, we designed three sgRNAs targeting three conserved regions of the third exon of the *w* gene (Fig. 2A).

Embryo survival postmicroinjection ranged from 64 to 82% and somatic mutagenesis rates (ie mosaic eyes, with an intermediate wild-type black and white knockout phenotype, Fig. 1B) were 37–57% for sgRNA injections (Table 1). Notably, coinjection with two or more sgRNAs targeting different gene regions of *w* including: (1) *w*sgRNA-1 and *w*sgRNA-2, (2) *w*sgRNA-1 and *w*sgRNA-3, (3) *w*sgRNA-2 and wsgRNA-3 (4) *w*sgRNA-1, *w*sgRNA-2, and *w*sgRNA-3 increased generation zero (G_0) mutagenesis efficiencies to 74, 73, 78 and 86%, respectively. These indicate that with these methods, we can achieve high single and multi-target somatic mutagenesis rates in *C. quinquefasciatus*, which is key to efficient CRISPR-mediated genome engineering in this species.

Heritable mutation rates

After demonstrating efficient somatic editing, we next wanted to test for germline editing as this is required for heritable transmission of the engineered mutations. Germline mutation transmission efficiency from Cas9-directed genome engineering in *C. quinquefasciatus* was determined by intercrossing mosaic G_0 males and females. The percentages of completely white eyed G_1 progeny (Fig. 1C) from single target injections are shown in Table 1. G_1 mutation rates increased to > 79% when co-injected with multiple sgRNAs targeting different regions of the *w* gene. Deletion and insertion mutations in several independent mutant G_2 lines were confirmed by sequencing the genomic DNA fragment containing the *w*sgRNA target sites (Fig. 2B). Some of the gene deletions were large,

Figure 1. Clustered regularly interspaced short palindrome repeats-associated protein 9 (CRISPR-Cas9) efficiently generates heritable, site-specific mutations in *Culex quinquefasciatus*. (A) Representative image of wild-type *C. quinquefasciatus* adult eyes. (B) Representative generation zero (G_0) mosaic white-eyed mutant mosquito postembryonic injection with a mixture of three unique single guide RNAs (sgRNAs) targeting the *white* gene and the Cas9 endonuclease. (C) Representative homozygous white-eyed mutant G_1 mosquito generated by pairwise crossing mosaic G_0 male and female mosquitoes.

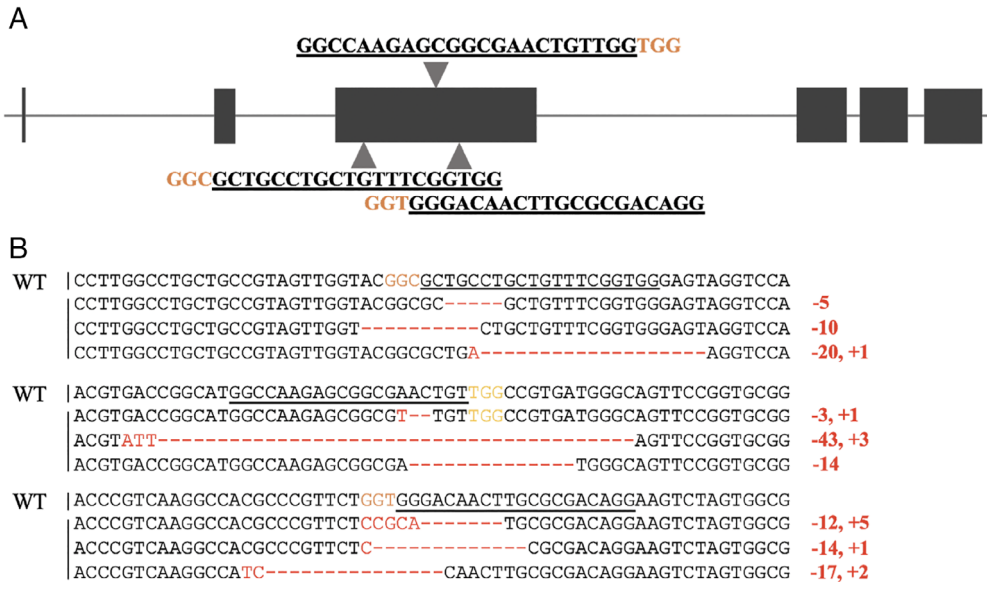

Figure 2. Mutagenesis of the *white* locus. (A) Schematic representation of the *white* locus with exons indicated as black boxes. Locations and sequences of the three single guide RNA (sgRNA) targets are indicated with the protospacer-adjacent motifs (PAM) highlighted in orange. (B) Genomic sequencing analysis of indels from individuals sequenced from the sgRNA injections. Top line represents wild-type (WT) sequence; PAM sequences (NGG) are indicated in yellow; and *white* gene disruptions resulting from insertions/deletions are indicated in red.

Table 1. Summary of the injection and mutagenesis mediated by independent single guide RNAs (sgRNAs) in *Culex quinquefasciatus*

		Survival			Somatic mosaicism (G_0)			Germline mosaicism (G_1)
sgRNA	#injected	♀	♂	Total (%)	♀ (%)	♂ (%)	Total (%)	G_1 mutants (%)
*w*sgRNA-1	50	15	20	35 (70)	7 (47)	13 (65)	20 (57)	128 (69)
*w*sgRNA-2	50	9	32	41 (82)	3 (33)	12 (38)	15 (37)	51 (61)
*w*sgRNA-3	50	17	15	32 (64)	7 (41)	8 (53)	15 (47)	157 (72)
*w*sgRNA-1/*w*sgRNA-2	50	7	16	23 (46)	5 (71)	12 (75)	17 (74)	123 (79)
*w*sgRNA-1/*w*sgRNA-3	50	13	9	22 (44)	10 (77)	6 (67)	16 (73)	72 (81)
*w*sgRNA-2/*w*sgRNA-3	50	17	10	27 (54)	13 (76)	8 (80)	21 (78)	101 (85)
*w*sgRNA-1/*w*sgRNA-2/*w*sgRNA-3	50	11	10	21 (42)	9 (82)	9 (90)	18 (86)	149 (86)

w, *white*; generation zero (G_0).

up to 43 bp (Fig. 2B). Healthy homozygous stocks were established for some of these *w* mutant lines indicating that this gene is not essential. Taken together, these results demonstrate that this method can generate germline mutations that can be inherited at a high rate and the efficiency of this inheritance is improved by synchronous injection of more than one sgRNA targeting different regions in the same gene.

Discussion

The Itokawa *et al*. study is a great example of the important and high impact research that can be achieved with CRISPR-Cas9 mutagenesis in *C. quinquefasciatus*; however, we aimed to provide more detail to allow researchers to effectively use this technology in functional genetic studies in this vector, including additional details on gRNA design and alternative egg handling and microinjection procedures. These details are particularly important as the unique biology of *Culex* species eggs, which are laid in raft structures, requires modifications from the standard microinjection procedures used for mosquitoes that lay single eggs, such as *Aedes* or *Anopheles* species. Proper separation, handling and injection of these egg rafts is not easy and it took us many rounds of refinement to achieve the high survival and mutagenesis rates in this study. Earlier work on Hermes transposable element-based transgenesis in *C. quinquefasciatus* still had consistently lower survival rates (range 5–40% survival; Allen *et al*., 2001) compared to our procedures (range 42–82% survival). Postinjection survival rates were unreported in the 2016 study (Itokawa *et al*., 2016), so it is unclear whether they were more successful in their study.

The few key differences between our work and these earlier studies are the timing of microinjection and the methods for collecting and handling of eggs, which are probably the causes of our higher mutagenesis and embryo survival rates, respectively. Our embryos were typically injected within 45 min postoviposition, so the embryos are early stage and white or cream coloured, whereas other protocols wait up to 45 min postoviposition to start injections when the eggs are later stage and a grey colour (Allen *et al*., 2001). It is crucial to deliver Cas9 protein and gRNA at early stages in embryonic development. Cas9 and gRNA delivery into the nucleus is difficult in later embryogenesis as cellular and nuclear membranes have limited permeability at these stages. Injection during early embryogenesis also ensures a higher likelihood of full penetrance of the mutant phenotype and germline mutagenesis as this is prior to extensive mitotic division, so there are fewer cells to mutate, and prior to the migration and differentiation of pole cells, the precursors of germ cells. Notably, there were also technical considerations for the selection of these earlier injection time points. In this study, early stage embryos (< 1 h postoviposition) had limited leakage of the egg cytoplasm and injection mixture (Cas9 protein and sgRNA mixture) into the injection needle, whereas later stage embryos (> 1 h postoviposition) had more cytoplasm and injection mixture leakage and consequently more issues with needle clogging and embryo survival. The drawback to this approach, however, is that these early stage eggs are more fragile, as they have none of the protective melanization of later stage eggs, so substantial effort was needed to develop methods for embryo collection and handling to reduce embryo mortality, which are described in detail herein.

Additionally, our work also outlines methods to optimize gRNA targeting and multiplexing to improve mutagenesis rates. Consequently, our design methods resulted in an increase in the mutagenesis rate of up to 26% for sgRNAs and up to 40% for multiplexed gRNAs compared to the previous study (Itokawa *et al*., 2016; Table 1). These improvements in survival and mutagenesis rates will make CRISPR-based technologies less laborious and more affordable to researchers and may even be generalizable enough to apply to other *Culex* species. Moreover, this is the first characterization of the *w* gene marker in *C. quinquefasciatus*, which is a common phenotypic marker used in other mosquito species (Coates *et al*., 1997). This easily screenable phenotypic marker can be used to simplify the generation of new genetic tools and future functional genetic studies in this species. Overall, these procedures provide step-by-step instructions for the CRISPR-Cas9 directed mutagenesis of *C. quinquefasciatus* in any laboratory with the appropriate insectary facilities and therefore we think this work is an important step towards bridging the gaps in research for this important, but frequently overlooked, disease vector.

Experimental procedures

Mosquito strain and rearing

We used the *C. quinquefasciatus* wild-type S-strain (Li and Liu, 2014) for these studies. Mosquitoes were raised at 25.0 $\pm$ 1 °C, 30% humidity and a 12-h light/dark cycle at the University of California, San Diego (UCSD). A 20% sugar solution was provided *ad libitum*. Females were provided a commercially sourced sodium citrate treated bovine bloodmeal (Colorado Serum Company, Denver, CO, USA cat# 31025) using the Hemotek (Blackburn, UK, model# PS5) blood feeding system.

sgRNA design and generation

sgRNAs were designed to target both the sense and antisense strand of exon 3 of the *w* gene (CPIJ005542). Target and protospacer-adjacent motif regions were selected using CHOPCHOP v. 2 software (http://chopchop.cbu.uib.no/) and CRISPR DESIGN (http://crispr.mit.edu/) (Xie *et al*., 2014). Target site conservation was confirmed in the CpipJ2 assembly of the Johannesburg strain of *C. quinquefasciatus* (http://www.vectorbase.org). Off-target

effects were evaluated with CHOPCHOP v. 2 (Labun *et al*., 2016), CRISPRDIRECT (https://crispr.dbcls.jp/) and a local sgRNA Cas9 package (Xie *et al*., 2014). Linear, double-stranded DNA templates for sgRNAs were generated by performing template-free PCR with Q5 high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA) using the forward primer of each gRNA, and universal-sgRNA. PCR conditions included an initial denaturation step of 98 °C for 30 s, followed by 35 cycles of 98°°C for 10 s, 58°°C for 10 s, and 72°°C for 10 s, followed by a final extension at 72°°C for 2 min. PCR products were purified with magnetic beads using standard protocols. gRNAs were generated by *in vitro* transcription (AM1334; Life Technologies, Rockville, MD, USA) using 300 ng purified DNA as template in an overnight reaction incubated at 37 °C. MegaClear columns (AM1908; Life Technologies) were used to purify sgRNAs, which were then diluted to 1 μg/μl, aliquoted, and stored at 80 °C until use. All primer sequences are listed in Table S1. Recombinant Cas9 protein from *Streptococcus pyogenes* was purchased from PNA Bio (Thousand Oaks, CA, USA, cat#: CP01-200) and diluted to 1 μg/μl in nuclease-free water with 20% glycerol, and stored in aliquots at −80 °C.

Preparation of sgRNA/Cas9 mixtures for microinjection

Our previous transgenesis work in *Aedes* (Li *et al*., 2017a) and *Anopheles* (Li *et al*., 2018), and our work in the parasitoid wasp *Nasonia vitripennis* (Li *et al*., 2017a), demonstrated that sgRNA/Cas9 directed mutagenesis is dose dependent. Therefore, as *C. quinquefasciatus* eggs are larger than *Aedes* and *Anopheles* mosquitoes eggs, we used slightly higher concentrations of gRNA and Cas9 protein (200 ng/μl sgRNA and 200 ng/μl Cas9) for our injections compared to these other taxa. To generate these mixtures, the stock Cas9 protein solution was diluted with nuclease free water and mixed with the purified sgRNAs at various concentrations (20–320 ng/μl). These mixtures were separated into 5–10 μl aliquots and stored at −80 °C to limit freeze–thaw associated degradation. Mixtures were then thawed as needed and maintained on ice while performing injections.

Preparation of needles for embryo microinjection

For effective penetration and microinjection into *C. quinquefasciatus* eggs, we experimented with several types of capillary glass needles with filament including quartz, aluminosilicate and borosilicate types. The quality of needles is critical for avoiding breakage/clogging during injection, embryo survival and high transformation efficiency. For each of these glass types we developed effective protocols to pull these needles on different Sutter Instruments micropipette pullers (Novato, CA, USA, cat#: P1000, and P-2000) to enable the needles to have the desired hypodermic-like long tip that we found effective for *C. quinquefasciatus* embryo microinjection. The parameters (filament, velocity, delay, pull, pressure) for the different types of capillary glass needles are listed in Table S2. Although all three types of needles were effective for *C. quinquefasciatus* injections, we preferred the aluminosilicate capillary glass needles, because the quartz capillary glass needles were more expensive, and the borosilicate capillary glass needles were a bit too soft and clogged easily. All needles were bevelled with a Sutter BV-10 beveller. Bevelling parameters to achieve appropriate bore size and needle shape are described in our previous work (Li *et al*., 2017c).

Embryo preparation and microinjection

Mixed sex pupae were allowed to eclose into a single (length 24.5 cm × width 24.5 cm × height 24.5 cm) cage. 3 to 6 days post-emergence, mated females (first gonotrophic cycle) were offered a bovine bloodmeal, using the Hemotek (model#: PS5) blood feeding system. Then females were kept for 3 days to allow oogenesis, after which oviposition cups with organically infused water (Karat, Chino, CA, USA, 9 oz clear plastic pet cup, cat#. C-KC9, filled with ~4 oz of nutrient rich larval pan water, including organic matter such as larval and pupal casings and larval food) were introduced into cages. Females were allowed to oviposit in the dark for 20–35 min by placing the cage into a laboratory cabinet. Fresh egg rafts (white colour) were transferred from the cup to a 100% wet filter paper with paintbrush. Single eggs were obtained by pushing down the egg rafts gently and slowly with the forceps (Dumont, Montignez, Switzerland, cat#: RS-5045) under a dissecting microscope. A wet paintbrush was used to align the anterior end of each embryo in the same orientation to facilitate higher precision injections on a coverslip with double-sided sticky tape (3M, Maplewood, MN, USA, cat#: 602710) to secure the eggs (Fig. S1). The eggs were then covered with water-saturated halocarbon oil, which consisted of a mixture of 9 ml halocarbon oil 700 (Sigma-Aldrich, St. Louis, MO, USA, cat#: H8898), 1 ml halocarbon oil 27 (Sigma-Aldrich, cat#: H8773) and 20 ml UltraPure™ DNase/RNase-Free distilled water (Invitrogen, Carlsbad, CA, USA, cat#:10977-015). The mixture was gently mixed to avoid forming an emulsion and then left overnight at room temperature (25 °C) to allow the water saturation of the halocarbon oil.

An Eppendorf Femtojet (Eppendorf, Hamburg, Germany, cat#: 5253000025) was used for the microinjections (pressure 30–60 psi), which were performed under a compound microscope at 100× magnification. Needles were filled with 2 μl injection mix and each egg was injected with a quantity of mixture approximately 10% of the egg volume. The injection volume was estimated by comparing the size of the oil immersed injection bubble to the embryo size under the microscope (100× magnification). The injection volume was then adjusted so that the size of the injection bubble was approximately 10% of the egg size. Injection volume was re-evaluated before each injection round. We injected approximately 20 eggs per round and then with a paintbrush, we carefully removed the halocarbon oil within 20 min postinjection. Eggs were then removed from the coverslip with a clean paintbrush and then floated on the surface of double-distilled H_20 in a small container. Eggs were monitored daily for hatching for up to 7 days postinjection.

Mutation screens

Screening was performed by standard methods (Lobo *et al*., 2006; Pondeville *et al*., 2014; Li et al., 2017b, 2018). Briefly, the phenotype of G_0 and G_1 mosquitoes was assessed and photographed under a Leica Biosystems (Nussloch, Germany) M165 FC stereomicroscope. To molecularly characterize CRISPR-Cas9-induced mutations, genomic DNA was extracted from a single mosquito with a DNeasy blood & tissue kit (Qiagen, Valencia, CA, USA) and target loci were amplified by PCR. For the T7 endonuclease

I (T7EI) assays in Fig. S2, 1 μl T7EI (NEB) was added to 19 μl of PCR product, digested for 15 min at 37 °C, and visualized on a 2% agarose electrophoresis gel stained with ethidium bromide. To characterize mutations introduced during NHEJ or microhomology-mediated end-joining (MMEJ), PCR products containing the sgRNA target site were amplified, cloned into TOPO TA vectors (Life Technologies), purified, and Sanger sequenced at Source Bioscience (https://www.sourcebioscience.com/).

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Data availability

Plasmids used to generate the mosquito strains produced here will be made available upon request.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Morphology of egg raft and single egg alignment of *Culex quinquefasciatus*. (A) *Culex quinquefasciatus* egg raft. (B) Egg alignment of *Culex quinquefasciatus*. Freshly laid eggs are lined up on a glass slide on a piece of sticky tape and injected one-by-one into the posterior end. Red arrows indicate injection site.

Figure S2. The T7 endonuclease assay can be used for rapid detection of clustered regularly interspaced short palindrome repeats-generated mutant alleles in *Culex quinquefasciatus*. PCR products from nonmosaic (C) and mosaic (1–3) mosquitoes digested with T7 endonuclease. Partial digestion of the PCR product is evident in all mosaic mosquitoes, whereas there is no digestion in nonmosaic mosquitoes.

Table S1. Primer sequences used in this study

Table S2. Needle puller settings for different capillary needles