Evaluation of pigmentation gene knock-outs by CRISPR as potential targets for the genetic engineering of *Culex quinquefasciatus*.

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Abstract

Culex mosquitoes are a globally widespread vector of several human and animal pathogens. Their biology and behavior allow them to thrive in proximity to urban areas, rendering them a constant public health threat. Their mixed bird/mammal feeding behavior further offers a vehicle for zoonotic pathogens transmission to people, and separately, poses a conservation threat to insular bird species. The advent of CRISPR has led to the development of novel technologies for the genetic engineering of wild mosquito populations, yet research in *Culex* has been lagging compared to other disease vectors, with only a few reports testing the functionality of CRISPR in these species. Here we use this tool to disrupt a set of five pigmentation genes in *Culex quinquefasciatus* that when altered, lead to visible, homozygous-viable phenotypes. We further validate our approach on two distinct strains of *Culex quinquefasciatus* that are relevant to potential future public health and bird conservation applications. Lastly, we generate a double-mutant line, demonstrating the possibility of combining multiple such mutations in a single individual. Our work provides a platform of five validated loci that could be used for targeted mutagenesis for research in *Culex quinquefasciatus* aimed at the development of genetic suppression strategies for this species. Furthermore, the mutant lines generated here could have widespread utility to the research community using this model organism, as they could be used as targets for transgene delivery, where a copy of the disrupted gene could be included as an easily-scored transgenesis marker.

Introduction

The *Culex pipiens* species complex includes several species and their hybrid forms, including *Cx. pipiens pipiens*, *Cx. pipiens pallens*, *Cx. pipiens molestus*, *Cx. quinquefasciatus*, *Cx. australicus*, and *Cx. globocoxitus*. While some of these species have limited distribution, *Cx. pipiens pipiens*, *Cx. quinquefasciatus*, and their hybrids are widespread globally and represent primary urban and suburban disease vectors. The ability of these species to produce fertile hybrids has been a source of an ongoing discussion around the taxonomy of these species [1] and has been proposed as a mechanism explaining the surprising adaptability of these mosquitoes, such as the "molestus" form of *Cx. pipiens* adapted to survive in the underground network of London [2]. Hybridization and high genetic diversity may further account for the rapid evolution of insecticide resistance across the *Culex pipiens* complex [3,4].

These mosquitoes are vectors for several medically-relevant RNA viruses such as West Nile (WNV), Japanese Encephalitis, Western Equine Encephalitis (WEEV), Eastern Equine Encephalitis (EEEV), Sindbis virus, and St. Louis Encephalitis virus, as well as nematodes that cause lymphatic filariasis [5]. Although fatalities in the United States due to these diseases are usually low in number, *Culex* mosquitoes are an established disease vector that represents a fertile ground for the establishment of new pathogens. It has been estimated that since its introduction in the United States in 1999, West Nile Virus hospitalizations alone amounted on average to ~$56M per year [6]. Furthermore, the mixed feeding behavior of these mosquitoes on birds and mammals creates seasonal cycles of pathogen amplification in wild animals [7], which combined with their ability to vertically transmit some of these pathogens to their offspring [8], could lead to a periodic increase in the risk of pathogen transmission to people and the potential of unpredictable outbreaks. The zoonotic nature of these and other viruses allows for unchecked reservoirs of viruses, mostly in wild birds, that could act as evolutionary melting pots for the development of new viral forms: for example, the Western Equine Encephalitis is considered to be the outcome of recombination between EEEV and a Sindbis-like virus [9]. Within this species complex, *Culex quinquefasciatus* is considered the disease vector with the greatest human health impact worldwide, mostly due to its widespread distribution in urban and suburban areas, due to its capability of tolerating polluted water reservoirs for its larval development often associated with human and livestock populations [10,11]. Furthermore, its genetic adaptability and capability to interbreed with other species have made it a uniquely challenging vector to keep at bay.

In addition to affecting human health, invasions of *Cx. quinquefasciatus* have been implicated in declines and extinctions of island avifauna and represent a serious threat to avian conservation. These effects are most apparent in the Hawai'i archipelago, where accidental introductions of *Cx. quinquefasciatus* in the mid-1800s contributed to extinctions of several Honeycreeper species (Drepanididae) and ongoing risks for those that remain [12–14]. In Hawai'i and elsewhere, *Cx. quinquefasciatus* is one of the main vectors of *Plasmodium relictum*, which causes avian malaria, as well as being a competent vector for the virus causing avian pox [15]. Honeycreepers evolved in isolation from these diseases, making them particularly susceptible. Most extant species survive only as fragmented populations in high elevation refugia where cooler temperatures negatively impact mosquito distributions and, in the case of avian malaria, pathogen development [16,17]. However, global warming is allowing mosquito-vectored diseases to invade these formerly protected areas, leading to rapidly collapsing endemic avian communities [18,19] and increasing concern by conservationists.

Genetic control tools have already been developed for other mosquito species, mainly belonging to the genera *Anopheles* and *Aedes,* and in the case of the latter have been implemented to control human diseases [20,21]. Recently the advent of CRISPR has fast-tracked the development of strategies for using genetically engineered vectors to modify or suppress wild populations of pests and invasive species [22,23], including technologies such as engineered gene drives that were proposed more than a decade ago [24]. Strategies have been proposed for the engineering of natural populations, such as the use of the endosymbiont *Wolbachia* [25,26] or engineered gene drive systems which have been successfully applied to a variety of

organisms including yeast [27], fruit flies [28,29], and *Anopheles* [30–32] and *Aedes* [33] mosquitoes, as well as the mouse [34].

Compared to *Anopheles* and *Aedes* mosquitoes, however, *Culex* has been somewhat neglected regarding the development of genetic suppression strategies, and only a few studies have evaluated the activity of CRISPR in this species [35–37]. Although no extensive CRISPR technology development has yet been achieved, previous work has shown the feasibility of using CRISPR by disrupting the *white* [36] or *kynurenine-hydroxylase* [35] genes causing a white-eye phenotype, as well as the altering a CYP450 gene involved in pesticide metabolism [37]. These studies highlight the potential for the development of genetic control strategies for *Culex* mosquitoes, as well as a need to identify and characterize additional CRISPR targets and protocols.

The scope of this study is to expand the available set of target genes that could be used in future work aimed at the development of genetic technologies for population control of *Culex* mosquitoes. As *Cx. quinquefasciatus* is both a human disease vector as well as a potential target for conservation efforts, we decided to focus on this species. We chose to target a set of pigmentation genes that have been previously targeted for disruption in diverse species [30,33,38,39], resulting in homozygous-viable mutations that could be used to track transgenes targeted without the use of fluorescent markers. We further evaluate our approach in two separate *Cx. quinquefasciatus* lines, demonstrating its flexibility of adaptation to different laboratory strains as well as the broad applications targeted to diverse genetic populations. For our study, we include two previously published genes (*white* and *kynurenine-hydroxylase*) for which we also provide new target gRNAs, and we target and evaluate an additional three genes (*cardinal*, *yellow*, and *ebony*).

Results

Targeting of *Culex quinquefasciatus* pigmentation genes for disruption.

In order to validate multiple gene targets that could be used in future *Cx. quinquefasciatus* research, we identified homologs of the fruit fly genes *white (w)*, *cinnabar* (*kynurenine hydroxylase, or kh*), *cardinal (cd)*, *yellow (y),* and *ebony (e).* For each gene, we first designed primers to PCR-amplify genomic sequences covering exons from our *Cx. quinquefasciatus* laboratory line sourced from California (CA line). Using the sequences obtained from such PCRs and the available genome for this species [40], we designed multiple gRNAs targeting each genes' coding sequences (**Fig. 1**). Remarkably for the *white* gene, we identified two different alleles with a quite extensive divergence, characterized by single nucleotide polymorphisms (SNP) every 10-20 bp (**Supplementary information**) and decided to design one of the gRNAs (*w4*) on a position covering one of these SNPs, to evaluate whether this could affect targeting.

We performed a preliminary test by injecting eggs with Cas9/gRNA mixes for each of the gRNAs, letting the embryos develop to adulthood to observe the adult mosquitoes for any mosaic display of the expected phenotypes: lighter eyes for *white* (**Fig. S1**) and *kh*, red-eye for *cardinal*, lighter body for *yellow* and darker body for *ebony*. For each gene we observed at least one gRNA yielding the expected phenotypes, and based on this preliminary analysis we were able to select a gRNA for each gene which was carried forward for further analysis of each mutant phenotype. Interestingly, the gRNA *w4* gave comparable results to the previously validated *w3* [41].

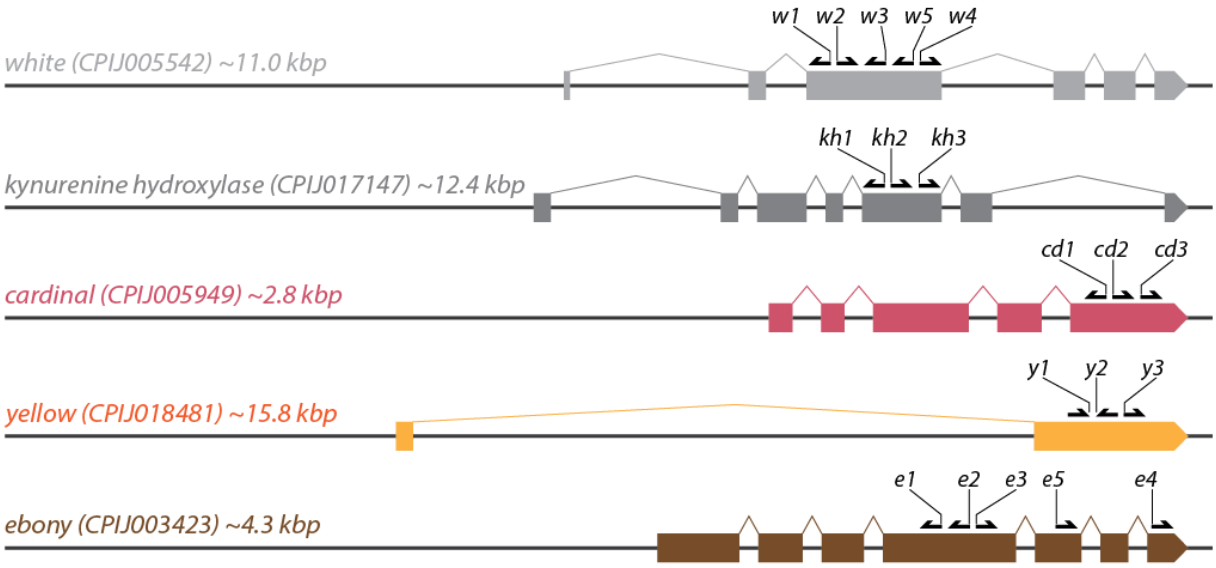

Figure 1 - *Culex quinquefasciatus* pigmentation loci targeted. Schematic outlining the coding sequence structure of the targeted genes, and the locations of the gRNA target sites used in the manuscript. The approximate locus size is indicated for each gene from the start to the stop codon. The gRNAs *w1*, *w2*, and *w3* were previously described as active [41] and were used here as controls. Drawings are not to scale.

Isolation of *Culex quinquefasciatus* pigmentation mutants.

In order to recover and characterize homozygous mutant lines, we then performed an additional round of injection for the selected gRNAs, as well as for the previously validated *w3*-gRNA targeting *white* as a control [41]. For each gene, we injected ~100-400 eggs using the same protocol used in our preliminary testing and scored the mosaic phenotype in the G0 injected animal at the pupal stage (**Table 1**). For the genes *w*, *kh*, *cd* we were able to clearly identify animals displaying a mosaic phenotype (**Fig. S1**), while for the *yellow* and *ebony* the mosaicism was not as obvious. For *ebony,* we were able to identify some animals displaying a darker body phenotype (**Fig. S1**), yet we decided to score them as wild-type in **Table 1** as it was difficult to make a clear distinction. This fact is probably due to the fact that, similarly to the fruit fly, *ebony* is likely to be a non-cell-autonomous phenotype in *Cx. quinquefasciatus*, affecting neighboring cells with a wild-type genotype, and resulting in varying gradients of the phenotype depending on the level of mosaicism.

To recover homozygous mutants of the targeted genes, we collected the G0 injected animals for each of *white* (*w4*), *cardinal* (*cd1*), *yellow* (*y1*), and *ebony* (*e4*), pool-crossed them to each other (one pool per gene), and scored their progeny for the expected phenotypic display. For all genes, except *ebony,* we were able to recover homozygous mutants that displayed the expected phenotypes: white eyes for *white*, red eyes for *cardinal*, and lighter body for *yellow* (**Fig. 2**). Remarkably for *white* 100% of the G1 progeny was mutant, suggesting very high activity of the *w4* gRNA, while for *cardinal* and *yellow* we instead recovered 33% and 45% mutant G0, respectively (**Table 1**). Since for the *kh* injection we recovered the highest number of G0 survival, and the phenotype was easily screened, we decided to generate two separate G0 crosses by pooling together either the animal displaying a mosaic phenotype or the ones having wild-type eye color. For the *kh*-mosaic pool, we recovered 100% G1 progeny displaying the expected white-eye phenotype (**Fig. 2**), while for the *kh*-wild-type pool, we did not observe any homozygous mutant animals in the G1 progeny (**Table 1**). While our data for the *kh*-mosaic pool comes from a single raft, and thus the high rate of mutant recovery could be due to an exceptional situation in which the two parents had high germline mutagenesis, our results from the *kh*-wild-type pool suggest that animals not displaying a phenotype had poor germline targeting. This underscores the

usefulness of visible markers during the development of genetic tools for population engineering, as they could help the recovery of transgenic animals.

To evaluate whether our analysis would be generalizable to different *Cx. quinquefasciatus* strains, which would need to be modified in the laboratory to pursue diverse applications, we also targeted a separate line sourced from Hawai'i (O'ahu and Hawai'i Islands). Here, we used the validated *w3* gRNA, which was chosen for its high transinfection rate and obvious phenotype, as noted in [41] and this study, and we performed a similar analysis to what was described above. For this line, ~34% of G0 injected animals displayed a white-/white+ mosaic phenotype, and an intercross of such G0 animals led to a recovery of mutants in the G1 progeny with ~92% rate (**Table 1**), comparable to what was previously described above using *w4*, as well as with the same gRNA in previous work [41]. We used the recovered G1 animals to establish a homozygous *white* mutant line (HI-*white*) to be used in further steps of this project.

Generation of a *white-/ebony-* double mutant line of *Culex quinquefasciatus*.

In our preliminary gRNA-efficiency analysis, we also attempted to recover mutants by intercrossing G0 injected animals, where we were able to recover a few *ebony-* larvae which unfortunately died at the pupal stage (**Fig. 2i**). Given that in our *e4* injection we did not recover any *ebony-* G1 mosquitoes, we attempted to increase mutagenesis efficiency by performing an additional injection for *ebony* employing a mix of three gRNAs (*e1*, *e2*, *e4*). We noticed that the survival rate of this injection was much lower than the one performed before with ~7% G0 surviving vs ~30% for e4, suggesting that there might be some lethality associated with this gene or with one of the gRNAs used. Nonetheless, we performed a pooled cross of the 15 surviving mosquitoes and obtained G1 animals that again did not display the *ebony-* phenotype (**Table 1**), underscoring the potential of a viability phenotype associated with the disruption of this gene.

To further attempt to obtain *ebony-* homozygous animals, we next made three changes to our approach: 1) we employed a different gRNA (*e5*) to avoid any toxicity that might be associated with the previously used ones; 2) we used a different *Cx. quinquefasciatus* background (HI), and 3) we started from the HI-*white* line. Our purpose in choosing the HI-*white* line was to additionally evaluate whether our single-mutant colonies could be further utilized for downstream studies. For example, in the model organism *Drosophila melanogaster*, single mutant lines are routinely used for subsequent transgenesis with synthetic constructs carrying a copy of the disrupted gene, which functions as a selectable marker for tracking integration efficiency [42]. Beginning with the HI-*white* line, we subjected it to an additional round of mutagenesis with the injections of the *e5* gRNA. To avoid a potential compounded weakness of the G0 animals (usually weaker due to the injection process), and putative viability issues that could be associated with *ebony* we modified our crossing strategy by backcross the *e5*-injected G0 mosaic individuals to wild-type (in contrast to how we previously pooled G0 males and females together). We then crossed these G1 animals to screen the G2 progeny for the *white*, *ebony*, and *ebony-white* phenotypes (**Fig. S2**). Indeed, we were able to recover *ebony-* homozygous mosquitoes using this strategy, as well as obtaining homozygous double-mutant animals that could be used as lines for future transgenesis.

injection			G0				G1	G2
Target Gene	**Line**	**gRNAs**	**Injected eggs**	**Adult survival**	**Wild-type phenotype**	**Mosaic phenotype**	**Mutants/Total (%)**	**Mutants/Total**
white	CA	*w4*	337	34 (10.1%)	3	31	174/174 (100%)	-
kh	CA	*kh3*	400	46 (11.5%)	13	33	0/389 (0%) *	-
							25/25 (100%) **	-
cardinal	CA	*cd1*	290	32 (11.0%)	0	32	28/84 (33.3%)	-
yellow	CA	*y1*	220	25 (11.4%)	25 ***	n/a ***	71/159 (44.7%)	-
ebony	CA	*e4*	288	90 (31.3%)	90 ***	n/a ***	0/375 (0%)	-
		e1 + e2 + e4	210	15 (7.1%)	15 ***	n/a ***	0/88 (0%)	-
white	HI	*w3*	258	41 (15.9%)	27	14	738/805 (91.7%)	-
ebony	HI-*white*	*e5*	533	34 (6.4%) §	15	19	n/a ‡	*white*: 82/558 *ebony*: 76/558 *ebony-white*: 52/558

Table 1. - Summary of injection information and phenotypic screening. The table summarizes the injection information and mutant screening data collected for both the G0 and the G1 animals. * G1 offspring of a G0 pool displaying wild-type phenotype. ** G1 offspring of a G0 pool displaying mosaic *kh-/kh+* phenotype. *** For these two injections we did not score the mosaic phenotype as it was hard to discern; therefore some of the individuals scored as wild-type, might have been mosaic. § the lower survival observed here could be due to a modified protocol for this injection (see Methods). ‡ The G0 animals generating this G1 offspring were outcrossed to wild type and therefore these animals were not scored for the *ebony-* phenotype as it would have been heterozygous.

Phenotypical and molecular characterization of the mutants generated.

As mentioned previously we were able to recover homozygous mutant mosquitoes for each of the targeted genes and establish lines. The observed phenotypes illustrated in **Fig. 2** paralleled previous work performed in the fruit fly, *Anopheles [30,38]*, and *Aedes [33]* species. We observed a distinct white-eye phenotype for *white* (**Fig. 2b**) and *kh* (**Fig. 2c**) mutants, and a red-ish eye for *cardinal* mutants (**Fig. 2d**) visible in all stages of development, but more obvious in the 4th larval instar, the pupa, and the adult. Interestingly, *cardinal-* mutants would darken after eclosing from the pupa and, over time (2-3 days), reach a phenotype indistinguishable from wild type (**Fig. 2f**) similarly to what was previously observed in *Anopheles* [38]. For mutants of the gene *yellow,* we could not see a major difference at the larval/pupal stages, but at the adult stages, the lighter body color phenotype becomes more obvious (**Fig. 2e-e'** vs. **2e''**). Contrary to the *cardinal* mutants, *yellow* mosquitoes, lighten over time, rendering the phenotype more obvious in older animals (**Fig. 2g**). Lastly, eggs laid by *yellow-* mosquitoes also appear lighter in color (**Fig. 2h**), probably due to the melanin pathway [43] being involved in the tanning of the eggs.

To confirm the editing at the correct location, we isolated genomic DNA from a few mosquitoes from each of the mutant lines generated and performed PCR amplification of the targeted region. Sanger sequencing revealed the makeup of the mutations generated during the editing process. Since we generated mutant lines by pooling G1 (or G2) mutant animals, we observed multiple alleles during this analysis, including

heterozygous mosquitoes having two different alleles. By using the ICE analysis tool (see methods) we were able to identify the different alleles present in the mixed Sanger traces for each of the *white* (**Fig. S3**), *kh* (**Fig. S4**), *cardinal* (**Fig. S5**) lines, while for *yellow* we observed only one allele (**Fig. S6**), most likely due to the establishment of the yellow line by a single raft carrying only one type of mutation. Indeed our molecular characterization revealed that each of the genes was successfully targeted for disruption, as all indels except the one for yellow disrupt the open reading frame of the genes. Interestingly the indel isolated for *yellow* lacks 3 bp, and therefore maintains the frame, so it is possible that this mutant is a hypomorph, maintaining a partial function of *yellow*.

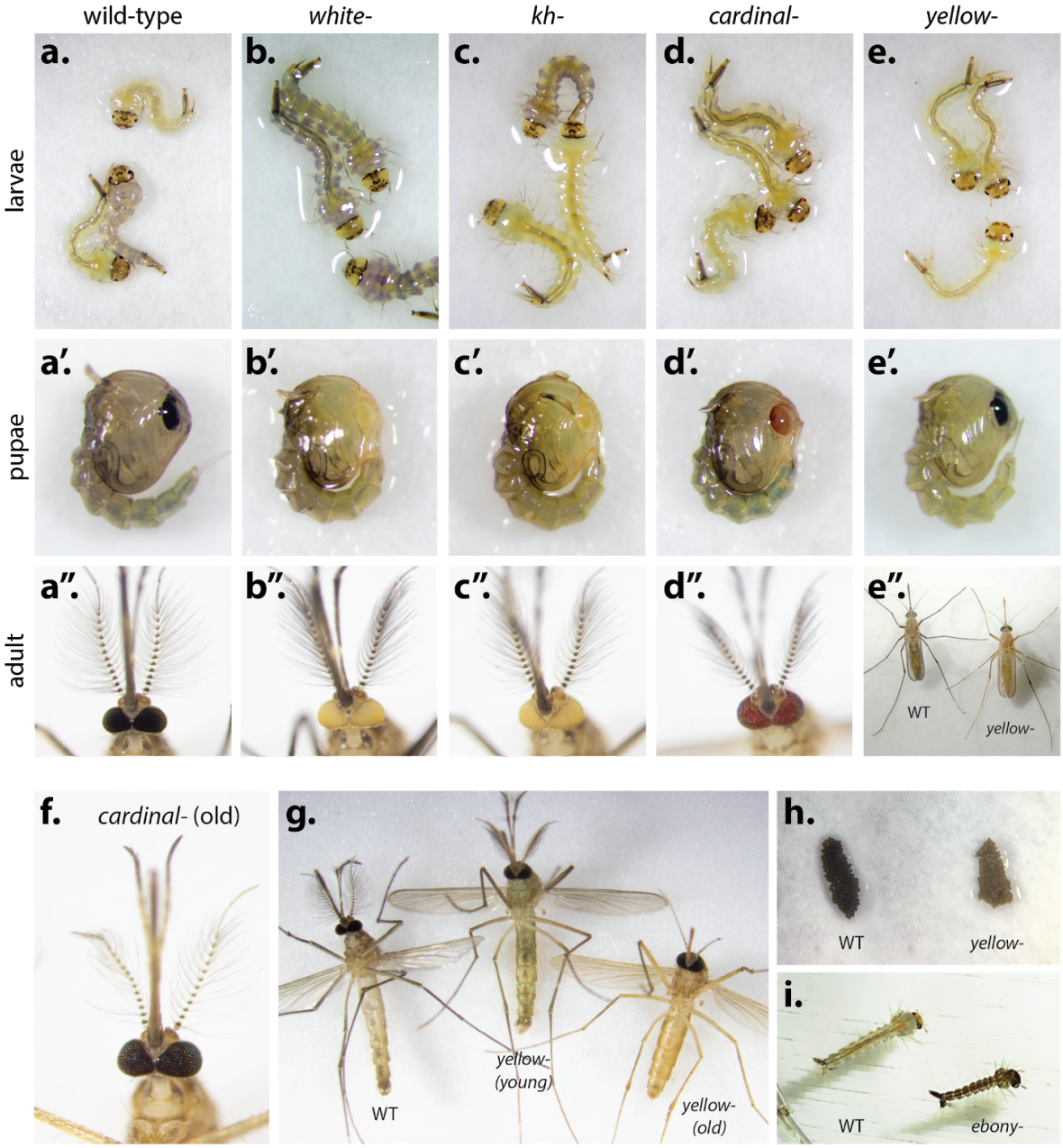

Figure 2 - Phenotypes of the generated mutant animals generated. (**a-e**) Larvae, (**a'-e'**) pupae and (**a''-e''**) adults of (**a,a',a''**) wild-type mosquitoes and (**b,b',b''**) *white-*, (**c,c',c''**) *kynurenine-hydroxylase- (kh-)*, (**d,d',d''**) *cardinal-*, and (**e,e',e''**) *yellow-* mutants. (**f**) cardinal mutants darken over time, acquires a phenotype similar to wild-type (**a''**). (**g**) *yellow-* mutants eclose from the pupae (center) with a phenotype similar to wild-type (left) and lighten over time to assume a more recognizable lighter color (right). (**h**) *yellow-* mutant females lay eggs that are lighter in color (left) compared to wild-type ones (right). (**i**) wild-type (left) and *ebony-* (right) larvae.

Discussion

Here we build on previous work demonstrating the feasibility of a CRISPR approach for targeted mutagenesis in *Culex quinquefasciatus* [35–37], by providing an in-depth mutagenesis analysis of several pigmentation loci in this species. The analysis described herein provides information for a set of five viable loci, *white*, *kynurenine-hydroxylase*, *cardinal*, *yellow,* and *ebony*, that can be efficiently targeted in future genetic strategy for the population suppression of these mosquitoes. As Anderson et al., we also target the *kynurenine-hydroxylase* gene for disruption [35]. However, we observe a much higher mutant G1 rate (100%) than in the previous study (2.6%) [35], a difference that could be due to our screening strategy, in which for *kh,* the G1 is obtained from a pool of G0s displaying mosaic phenotype. Alternatively, the discrepancy could be explained by a difference in the efficiency of the chosen gRNA. We also note that our data for such a pool was derived from a single raft that might have displayed an exceptional mutagenesis rate. Our mutagenesis of the *white* gene also yielded slightly higher mutagenesis rates (92%-100%) compared to what was shown by Li et al. (61%-86%), and contrary to the *kh* analysis where we picked different gRNA targets, for white we employed the same gRNA (*w3*) and obtained 92% instead of 72% G1 mutant rate observed in the previous study [41].

Furthermore, our analysis looks at validating the findings in two separate lines isolated from different locations. One line was sourced from the continental United States (California), while the second was obtained from invasive populations in Hawai'i. The validation of multiple targets for future work in diverse strains adds further confidence that laboratory analysis could be widely applied to locations where the genetic background might differ from the laboratory strain. While both the lines tested in this work could be targets of population engineering aimed at public health applications, our employment of a line recently sourced from Hawai'i underscores the technical feasibility of population engineering strategies aimed at bird conservation.

We purposely designed one of our gRNA (*w4*) on a location where our CA line was displaying heterogeneity in the population, therefore the gRNA would perfectly match some alleles but have mismatches on others. Nonetheless, we show efficient targeting of both alleles, suggesting that mutagenesis efforts in diverse lines of *Culex quinquefasciatus* and perhaps other organisms could tolerate allelic differences in a genetically heterogeneous population. In addition, this finding has implications for gene drive strategies using CRISPR, as genetic diversity in wild populations has been considered a potential hindrance to gene drive spread. Our data suggest that this issue might be more limited than previously thought [44] and could allow to widen further the pool of genetic targets available [45].

Our analysis furthermore shows that it is possible to target multiple genes, an aspect that could be valuable in downstream approaches requiring multiple marked transgenes. We show that it is possible to obtain and maintain a *white-*, *ebony-* double mutant line, suggesting that other combinations of the mutants described here could be generated. We observed that some of the mutant lines identified here seem to be slightly weaker than their wild-type counterparts, although there were no major hurdles in rearing them. In this study, we did not perform further analysis of the isolated strains, such as longevity, fecundity, or fertility, as this lays beyond the scope of this manuscript, which focused on validating novel CRISPR targets that could be valuable for future genetic engineering efforts in this species. Indeed, our work provides a set of validated genes that could be used in future *Culex quinquefasciatus* research to deliver targeted transgenesis. These findings help pave the way toward the development of population engineering technology for the control of this disease vector.

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Authors contributions

All authors contributed to the project planning and the design of the experiments. X.F., L.K., and J.H.K.N. performed the experiments and contributed to the collection and analysis of data. X.F, J.T.S., and V.M.G. wrote the manuscript. All authors edited the manuscript.

Competing interests

V.M.G. is a founder of and has equity interests in Synbal, Inc. and Agragene, Inc., companies that may potentially benefit from the research results. V.M.G. also serves on both the company's Scientific Advisory Board and the Board of Directors of Synbal, Inc. The terms of this arrangement have been reviewed and approved by the University of California, San Diego in accordance with its conflict of interest policies. X.F. J.A.D., L.K., F.A.R., J.T.S., J.H.K.N. declare no competing interests.

Methods

University of California San Diego Protocols

UCSD Mosquito strain

The *Culex quinquefasciatus* CA strain was kindly provided by Anton Cornell (UC Davis), the line was originally collected near the city of Merced, California in the 1950s. It was reared at 27 ± 1℃, 30% humidity, and a 12h light/dark cycle in the insectary room at the University of California, San Diego. The adults were fed with 10% sugar water. After mating, females were fed with defibrinated chicken blood (Colorado Serum Company, USA, Cat. # 31142) using the Hemotek blood-feeding system. Egg rafts were collected 4 days after blood feeding for microinjection. Larvae were fed with fish food pellets.

UCSD sgRNA design and synthesis

The sgRNA was designed using CHOPCHOP [46] and synthesized according to the protocol by Krisler et al. [47]. Briefly, the double-stranded DNA template for each sgRNA was synthesized by performing PCR with a specific forward primer for each sgRNA and a common reverse primer. The PCR product was later purified with QIAquick Gel Extraction Kit (Qiagen, Cat. # 28704). sgRNAs were then synthesized with the MEGAscriptTM T7 transcription kit (Thermo Fisher Scientific, Cat. # AM1334) by performing in vitro transcription reaction with purified DNA template incubated at 37°C overnight (12-16 hours). The MEGAClear Kit (Thermo Fisher Scientific, Cat. # AM1908) was later used to purify the in vitro transcription products. The synthesized sgRNAs were diluted to 1000ng/uL, aliquoted, and stored at -80°C.

UCSD Culex quinquefasciatus egg microinjection

- *Cas9/sgRNA injection mix*. The recombinant Cas9 protein (DNA Bio Inc., Cat # CP01) was dissolved in nuclease-free water, aliquoted, and stored at -80°C until use. The synthesized sgRNA was mixed with Cas9 protein (with a final concentration is 200ng/uL for each) and incubated at room temperature for 15 minutes before injection.
- *Eggs preparation for microinjection*. Four days after blood feeding, the mosquito cage was put in a dark place with an oviposition cup inside. After 30 minutes, the freshly laid egg rafts were collected and put into a Petri dish covered with a wet filter paper. Single eggs were separated from rafts and aligned with a paintbrush under a dissecting microscope. The aligned eggs were then fixed on a coverslip with double-sided sticky tape. A mixture of 700 and 27 halocarbon oil (1:1) was used to cover the aligned eggs to prevent dehydration. The cover slides with aligned eggs were fixed on glass slides and put under an inverted microscope (Olympus, BX43) for microinjection.
- *Needles preparation*. Injections were performed with Aluminosilicate glass (Sutter Instrument, Cat. # AF100-64-10) pulled by a micropipette puller (Sutter Instrument, P-1000) with program: Heat=516; Pull=65; Vel.=65; Delay=47; Pressure=500; Ramp=514. Before injection, the tips of needles were slightly opened by a Beveler (Sutter Instrument, BV-10) and then loaded with injection reagents by micro-loader (Eppendorf)
- *Microinjection Station*. All injections were operated in a microinjection station equipped with a FemtoJet 4 microinjector (Eppendorf).
- *Embryos and larvae rearing after injection*. After injection, the aligned eggs were rinsed with DI water and gently removed from the double-sided tapes with a paintbrush. The eggs were then transferred to a cup with clean DI water and incubated at 27C for 36 hours until they begin to hatch. Transfer all the hatched larvae to a larvae-rearing tray and fed with fish food pallets.

UCSD Phenotype screening and crossing

The phenotypes of G0 and G1 were screened under the dissecting microscope at the pupae stage. For *cardinal*, *white*, and *kh* phenotype, the G0s with mosaic phenotype were pooled, while for *yellow* and *ebony*,

with their mosaic phenotypes are hard to distinguish from G0, we pooled all the injected G0 together and screened the homozygous phenotype from G1. All the homozygous individuals from G1 were pooled to establish the homozygous colony. For yellow and ebony mutants, the heterozygous siblings were sibling crossed and the homozygous individuals were later screened from G2 to build the homozygous mutant lines.

UCSD Genotyping

To molecular characterize the knockout phenotypes, the genomic DNAs were extracted from ~10 individuals of each homozygous mutant line using a single-fly DNA extractions protocol described by Gloor et al. [48]. We added 50 uL of ddH2O to diluted extracted DNA samples to a final volume of 100 uL and used 1 uL of each sample as a template to run a 25 uL PCR reaction. The primers used for PCR amplification are designed based on the genomic sequences of genes identified in VectorBase.org. Oligos information is listed in the table below.

UCSD Primers used for sgRNA synthesis and PCR amplification

Gene	**Accession No.**	**sgRNA**	**sgRNA-F (5'-3')**	**PCR-F (5'-3')**	**PCR-R (5'-3')**	**Primers for sequence**
White	CPIJ005542	w4	GAAATTAATACGACTCACTATA**GGATCAAGGGTCTGTCGGGT**GTTTTAGAGCTAGAAATAGC	ACCCTCATCCAGGTGTGGAAACCG	CACCCGACCCTCCGCCATCAGCAG	CTGTCCTTCAGATCACCGCCG
Yellow	CPIJ018481	y1	GAAATTAATACGACTCACTATA**GGCTGGGCTGAACTTCCAGT**GTTTTAGAGCTAGAAATAGC	CTACGGGTTGATTGCGTACTCG	GTGCTGAGCGGGGCGCTGAAG	CTACGGGTTGATTGCGTACTCG
Kh	CPIJ017147	kh3	GAAATTAATACGACTCACTATA**GGAGTTTAGCGAAACTCGGT**GTTTTAGAGCTAGAAATAGC	CAGGCCGATCTGATCGTGGGCTGCG	CACGATTATGCTGCCTGATACTTCC	CACGATTATGCTGCCTGATACTTCC
Cardinal	CPIJ005949	cd1	GAAATTAATACGACTCACTATA**GGCTGATGGCATCCGAGTTG**GTTTTAGAGCTAGAAATAGC	GACCCTGATGAAACAAACACGC	CTTCAAGTGGCTGGCACCG	GACCCTGATGAAACAAACACGC
Ebony	CPIJ003423	e1	GAAATTAATACGACTCACTATA**GGGTTTGACCGGCTGGAGGT**GTTTTAGAGCTAGAAATAGC	GAAACGATCCTGAACCGTCTGCAG	ATGCTGATGGCGTGACCCTTTTGAC	GAAACGATCCTGAACCGTCTGCAG
		e2	GAAATTAATACGACTCACTATA**GGTCACGGAGGCAAAGTCTC**GTTTTAGAGCTAGAAATAGC			
		e4	GAAATTAATACGACTCACTATA**GGACATCCGAAAGCAACATTG**GTTTTAGAGCTAGAAATAGC			
sgRNA-R	AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC					

University of Hawai'i at Hilo (UHH) Protocols

UHH Mosquito Strain

The Hawai'i *Culex quinquefasciatus* strain was initially collected from Hilo and Honolulu in 2016 and reared at 26 ± 1°C, 70% humidity, and a 12h light/dark cycle that includes dawn and dusk in the insectary in University of Hawai'i at Hilo, Hilo. Adults were maintained on 3% sugar water. After mating, adults were provided commercially available bovine blood treated with sodium citrate (Lampire Biological Laboratories). Egg rafts for microinjection were collected after 1-3 opportunities for blood-feeding. Larvae were fed with fish food pellets.

UHH sgRNA design

A crRNA targeting *white* (w5 - GGACAGCGCGUUCAACAGGG) was based on *w*sgRNA-3 from [34] (target site on exon 3). A crRNA sequence targeting *ebony* exon 5 (e5 - CAGAGCAACUUCUACGAGCU) was selected manually and verified with CHOPCHOP and NCBI Blast. crRNA, tracrRNA, and Alt-R S. p. Cas9 Nuclease V3 were purchased from IDT.

Full *w5* crRNA as ordered from IDT: GGACAGCGCGUUCAACAGGGGUUUUAGAGCUAUGCU
Full *e5* crRNA as ordered from IDT: CAGAGCAACUUCUACGAGCUGUUUUAGAGCUAUGCU

UHH Culex quinquefasciatus egg microinjection

- *Cas9/sgRNA injection mix*. *Cas9/sgRNA injection mix*. crRNA and tracrRNA were eluted in a duplex buffer (100 mM Potassium Acetate, 30 mM HEPES, pH 7.5) to 10 pm/ul. 14.5 ul of each stock was combined, heated to 94C for 2 minutes in a thermocycler, and allowed to cool to RT. 10 ug of Cas9 was added to each 29 ul RNA aliquot tube for a final Cas9 concentration of 330 ng/ul. The mixture was kept at RT for 10 minutes prior to loading into Femtotip II microinjection capillaries.
- *Eggs preparation for microinjection*. Oviposition cups were placed in mosquito cages at least four days after blood feeding. After a minimum of 60 minutes, newly laid egg rafts were collected. Single eggs were separated from rafts and aligned against the edge of moistened filter paper on glass slides. No halocarbon oil was employed and all eggs were injected prior to full melanization.
- *Microinjection Station*. Microinjection took place under a non-inverted light microscope with a Femtojet 4i microinjector and Femtotip II microinjection capillaries (Eppendorf).
- *Embryos and larvae rearing after injection*. Slides with injected eggs were gently placed into larvae trays in such a way that the slides rested against the side of the tray at ~60-degree angles and the water's surface just reached the place where the eggs were aligned, with the filter paper wicking water up toward the eggs and allowing them to detach from the slide surface into the water. After hatching, larvae were maintained on fish food pellets in the same insectary as the adult cages.

Phenotype screening and crossing

Phenotypes were screened under a dissecting microscope in fourth instar larvae and/or pupae. For *white,* G0s with mosaic phenotype were pooled, and resulting homozygous G1s were pooled to establish the homozygous colony. G0 *ebony* males were separated from *ebony* G0 females and backcrossed to wildtype of the opposite sex (note that *ebony* G0s can be difficult to distinguish from wildtype). All G1s were presumed heterozygous and had fully wild-type phenotypes. G1s were pooled together to generate G2s with four different phenotypes: wildtype, *ebony*, *white*, and *ebony-white*.

Genotyping

Molecular characterization of UHH mutants was done by PCR detection of alterations in the typical Cas9 target region. Four *e5*-injected G0 individuals showed little or no amplification using a primer pair with one 3' end 4bp upstream of the PAM site (GAGCAACTTCTACGAGC-F, TCAATGTTGCTTTCGGATGTC-R) while showing strong amplification with a control reaction, indicating homozygous editing near the target PAM site.

ICE analysis

The analysis of mixed sequencing traces was performed by using the ICE analysis software by Synthego (Synthego Performance Analysis, ICE Analysis. 2019. v2.0. Synthego; 15 Oct. 2020), using a Sanger sequencing read performed on wild-type as a control.

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