INTEGRATION OF A "SELF-DOCKING SITE" GENETIC CONSTRUCT IN THE SOUTHERN HOUSE MOSQUITO (*CULEX QUINQUEFASCIATUS*) AS A STEP TOWARD GENETIC CONTROL STRATEGIES

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Abstract

Background: Since its initial introduction to Hawai‘i, *Culex quinquefasciatus*, continues to threaten native birds by vectoring *Plasmodium relictum*; the parasite responsible for avian malaria. Avian malaria will become a larger threat with global warming expanding the range in which both the vector and disease can develop. Conventional methods to mitigate mosquito threats are not feasible on a large, landscape scale. Genetic modification of mosquito populations may be more efficient and longer lasting than smaller scale or more traditional mosquito control methods.

Study focus: We propose to create an underdominance-based gene drive system in *Cx. quinquefasciatus*. This gene drive system could transform wild mosquito populations in Hawai‘i to carry a *Plasmodium* refractory gene, which will offer a new tool that could potentially mitigate the decline of native Hawaiian birds. In this study, we aimed to develop the first step in creating an underdominance-based gene drive system, which was to integrate and express a phiC31(ΦC31) integrase construct that includes a “self-docking site”. We used a Restriction Enzyme Mediated Integration (REMI) strategy to integrate a ΦC31integrase construct with a *DsRed2* phenotype marker (a red florescent protein) into wildtype *Cx. quinquefasciatus.* Integration was confirmed via PCR and sequencing, establishing that REMI, which has never been used to genetically modify mosquitoes before, is a viable strategy for germline genetic modification in mosquitoes. However, due to problems with accurate screening of the *DsRed2*

phenotype, we also microinjected wildtype *Cx. quinquefasciatus* with an edited version of the ΦC31plasmid, called the "pBattP-EGFP" construct, to attempt to improve screening efficiency and to better assess efficacy of the use of REMI. This edited construct contained an alternative phenotype marker, a gene for a green florescent protein (*EFGP*), under control of a promoter (*3xP3*) that was expected to drive expression in the eye regions, for easier diagnostic screening. However, there was no success using the pBattP-EGFP construct to transform *Cx. quinquefasciatus*. Future research should focus on increasing transformation and screening efficiency by exploring alternative construct components and alternative genome editing methods.

Broader impacts: The ΦC31 plasmid is an important component because it contains a self-docking site, which will allow site-specific integration of additional genes of interest. Subsequent microinjections could include possible effector genes, such as an avian malaria refractory gene. The self-docking site can be used to attach any other type of refractory gene, such as a human West Nile Fever refractory gene, for which *Cx. quinquefasciatus* is a primary vector. Successful integration and expression of the ΦC31construct in *Cx. quinquefasciatus* will open up many different possibilities to control wild mosquito populations through the use of the self-docking site, whether it is to control for a human or animal disease.

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Introduction

Background

Over 75% of native Hawaiian forest birds have been driven to extinction or are currently listed as endangered, and a major cause of these losses and declines are presumed to be due to introduced disease, especially avian malaria (caused by *Plasmodium relictum*) and avian pox (caused by *Poxvirus avium*; Warner 1968; van Riper *et al*. 1986; Atkinson *et al*. 1995; Liao *et al*. 2017). Birds affected by avian malaria show significantly less food consumption, less body mass, and upwards of 90% mortality rate after a single transmission from a mosquito (Atkinson *et al*. 1995; Woodworth *et al*. 2005). In the Hawaiian Islands, introduced birds do not appear to be as susceptible to avian malaria as native species. The native *ʻiʻiwi* (*Vestiaria coccinea*), for example, is extremely vulnerable to malaria, and continues to be affected worse than other species, such as the nonnative Japanese white-eye (*Zosterops japonicus*; van Riper *et al*. 1986; Woodworth *et al*. 2005; Atkinson & LaPointe 2009). The vector for the introduced avian malaria in Hawaiʻi, the southern house mosquito (*Culex quinquefasciatus*), is widespread throughout the Hawaiian Islands, but malaria transmission is limited at higher elevations, due to colder temperatures inhibiting the vector and pathogen development (Benning *et al*. 2002; Atkinson & LaPointe 2009; Fortini *et al*. 2015; Liao *et al*. 2017). These higher elevation regions have become the last refuges for many of Hawaiʻi's native forest birds.

Global climate change is predicted to increase temperatures by about 2℃ sometime within the next 100 years, which will allow *P. relictum* and *Cx. quinquefasciatus* to survive in higher elevations and therefore increase malaria transmission in habitats where native birds were once relatively safe (Benning *et al*. 2002; Atkinson & LaPointe 2009; Garamszegi 2011; Liao *et al*. 2017). One dire scenario of possible environmental range expansion is in the Alakaʻi Plateau on the island of Kauaʻi, which is already within the typical elevational range in which the vector and disease can survive (Paxton *et al*. 2016). Currently, temperatures in Alakaʻi Plateau forests are uniquely cooler compared to similar elevations on other islands (LaPointe *et al.* 2010), which may be why there are still native forest bird populations that have not shown high prevalence of avian malaria (LaPointe *et al*. 2010; Paxton *et al*. 2016). However, inevitable seasonal warming and climate change are anticipated to allow this summit to become completely invaded by avian malaria (Benning *et al*. 2002; Paxton *et al*. 2016). Two endangered Hawaiian honeycreepers, the *ʻakikiki* (*Oreomystis bairdi*) and the *ʻakekeʻe* (*Loxops caeruleirostris*), have experienced sudden recent declines in their core ranges and population numbers within the Alakaʻi Plateau and are predicted to go extinct within the next 10 years due to habitat degradation, invasive species competition and habitat alternations, and mostly range expansion of mosquito-vectored disease (Paxton *et al*. 2016). With the impending consequences of climate change rapidly approaching, it is urgent to develop and act upon plans to control mosquito populations.

Typical mosquito control methods, such as removing still water sources in urban areas that serve as larvae habitat or widespread use of insecticides in natural forests, are not feasible for large-scale control of populations living in and near native bird habitat (Pratt 2009; Bellini *et al*. 2014). There are also concerning side-effects of pesticide application including: harming non-

target organisms, harming native ecosystems, harming humans through chemical contamination of upland farms and streams, and the potential for evolution of resistance to the used pesticides (Pratt 2009). Reduction of larval habitats has been shown to be successful on government land and test plots where regulations can be more easily enforced compared to residential and public land (Pratt 2009). However, on a landscape scale, it is not feasible with the limited resources available to conservation and community cooperation (Pratt 2009). Another control method would be to suppress and control pig populations in an attempt to reduce larval habitats in forested areas. In Hawai‘i, pigs create cavities in native flora which serve as larval habitats, but eradication of pigs and construction and maintenance of fence lines throughout the islands would not be cost-efficient (Hone & Stone 1989). Passing a plan to eradicate feral pigs in the hopes of reducing larval habitat that feral pigs create would be difficult (Tummons 1997), because pigs are also a popular game animal for hunters within the Hawaiian Islands (Pratt 2009). Mosquito management in the Hawaiian Islands needs to have a solution that can be long-lasting and efficiently control populations on a landscape scale.

Gene Drive Systems as Control Strategies

Development of a synthetic gene drive system is one option for landscape level mosquito management. A gene drive system ensures the spread of a gene throughout a population and throughout generations (Champer *et al*. 2016). A release of a population that is genetically modified to carry a synthetic gene of interest associated with a gene drive mechanism could theoretically be used to change a wild population by fixing every wild individual to be homozygous for the transgene. For example, a possible gene of interest can be a refractory gene

for a disease, which is a gene that can inhibit or prevent transmission of the disease (Marshall & Taylor 2009). There are different types of synthetic gene drive systems that utilize different mechanisms to ensure that a gene of interest will spread throughout a population. For example, there are synthetic gene drive systems that utilize Transposable Elements, Homing Endonuclease Genes, RNA Interference (RNAi) genes, and more (reviewed in Sinkins & Gould 2006). Certain properties of different mechanisms may make them more ideal than others for particular situations, such as the ability to remove the synthetic gene from the population, ability to be stable in the target organism and population over time, and the ability to be geographically stable.

The type of gene drive system that we are interested in is a synthetic underdominance-based gene drive mechanism, called RPM-Drive (Reed *et al*. 2018). The RPM-Drive can be used to effectively transform a wild population to carry a gene of choice, such as a refractory gene for disease resistance (Reed *et al*. 2013; Reeves *et al*. 2014; Reed *et al.* 2018). Underdominance is when heterozygous individuals have lower fitness than homozygous individuals, resulting in an unstable allele frequency equilibrium (Curtis 1968; Reed *et al*. 2013; Figure 1). An underdominance-based gene drive is an ideal mechanism due to its ability to be reversed, ability to be stable over time, and its ability to be geographically stable (Reed *et al*. 2013). A transformed wild population can be reversed back to its original state through the same mechanism that transformed it in the first place: release of homozygous wildtype populations at a higher frequency than the unstable equilibrium will drive the wildtype allele to fixation and drive the transgene out of the wild population (Sinkins & Gould 2006; Reeves *et al*. 2014). Stability of underdominance as a gene drive mechanism has been shown in Reed *et al*. (2018), where a

colony of transformed *Drosophila melanogaster* with an underdominance construct was stable for over 200 generations. Underdominance is also geographically stable, because migration from a transformed population to a geographically independent wildtype population would result in heterozygous offspring which, because of the underdominance construct, would have lowered fitness, causing the transgene allele to be removed (Altrock *et al*. 2010). As long as the migration between populations is at a lower frequency than the unstable equilibrium frequency (Figure 1), then neither population will be changed (Reed *et al*. 2013). Keyghobadi *et al*. (2006) has found genetic differentiation among *Cx. quinquefasciatus* populations on the island of Hawai‘i, some populations less than 5 kilometers apart showing significant genetic differentiation, thus implying minimal rates of migration among the spatially isolated populations. These smaller target populations that do not show substantial migration are ideal for transformation using an underdominance-based gene drive.

This type of underdominance-based gene drive is not meant to immediately eradicate a target population, but instead, the purpose is to make all individuals within the population carry and spread a synthetic gene throughout a population so that future generations will also carry the transgene. Release of homozygous transgenic populations at a frequency larger than the unstable equilibrium frequency will drive the transgene allele to fixation, thus transforming the wild population (Altrock *et al*. 2010; Reeves *et al*. 2014; Figure 1). This frequency is determined by the fitness of each of the genotypes and the projection of the allele frequencies over time (Reed *et al*. 2013; Figure 1). The gene drive system can also allow managers and decision makers to have the choice of what gene of interest or effector gene they want to be spread throughout the wild populations. An effector gene that can inhibit the ability to transmit disease can be spread

among the mosquitoes (Ito *et al*. 2002; Marshall & Taylor 2009) or an effector gene that can suppress and collapse the population (Kyrou *et al*. 2018) of mosquitoes can be used. An underdominance-based gene drive could efficiently control disease-spread because the system can ensure the gene drive construct and the accompanying effector gene are driven to fixation within the population.

Risks

Along with the proposed benefits of genetic control strategies, there are risks. Common concerns regarding release of genetically modified arthropods include environmental risks, stability of the construct within the transgenic organism, human health risks, and genetically modified organism (GMO) concerns involved with social, cultural, and economic areas (FAO/IAEA 2006). There are three main factors of an underdominance-based gene drive system that address some of these concerns, which may make it more likely to be favorable among the public and policy makers compared to some other types of gene drive systems. Underdominance will contain a transgene within target populations, decreasing all risk of unwanted spread, due to its geographic stability. Underdominance also allows a mechanism to reverse wild populations back to their natural state if opinions ever change or if results are not showing high success. The RPM-Drive has also already been shown to be a stable construct within a transgenic organism (Reed *et al.* 2018). Other synthetic gene drive systems may be less realistic for wild releases if they lack safety features and efficiency that underdominance possesses. For example, gene drive methods relying on transposable elements or natural meiotic drive systems typically lack mechanisms to remove the synthetic gene from a wild population and lack a mechanism to keep

the transgene from spreading outside the target population (reviewed in Sinkins & Gould 2006). Although underdominance does have ideal properties, one limitation is that the initial release of mosquitoes has to be relatively large (Figure 1) compared to other mechanisms (e.g., Medea, Meiotic drive systems, Transposable elements; reviewed in Alphey 2014). However, the population transformation efficiency of an underdominance-based gene drive system is much greater when compared to Sterile Insect Technique (SIT) strategies such as one that takes advantage of *Wolbachia* induced cytoplasmic incompatibility. SIT requires a much larger release (up to 10 times the size of the wildtype population) and repeated releases to attempt population suppression (Harris *et al.* 2012; Dyck *et al.* 2006). Underdominance-based gene drives combine all the properties of 1) requires a single release to effectively drive a transgene through a population, 2) has a built in safety mechanism (i.e., frequency threshold > 50%) to prevent global spread of a transgene, and 3) allows a simple mechanism to eliminate a transgene and restore wild populations if desired.

Objectives

In this study, we worked on developing the first step of generating an underdominance-based gene drive system in *Cx. quinquefasciatus* populations, which is the successful integration of a plasmid containing a self-docking site into *Cx. quinquefasciatus* and utilizing the process of Restriction Enzyme Mediated Integration (REMI) to undergo genetic modification. In order to create a gene drive system, the target organism must first be successfully genetically modified. To do this, REMI can be used to integrate synthetic plasmid DNA. REMI is the use of restriction enzymes to first cut a plasmid DNA containing a gene of interest, and then injecting an

individual with the linearized DNA fragments and active restriction enzyme mix (Kuspa 2006). The active restriction enzymes will then cleave the genomic DNA and the linearized fragments will be integrated into the individual's genome (Kuspa 2006). REMI has been used to create genetically modified organisms such as amphibians (Sparrow *et al*. 2000) and fungi (Schiestl & Petes 1991), but to the best of our knowledge, this method has not been used to genetically modify mosquitoes before. REMI has also been shown to increase transformation efficiency in some cases (Kuspa 2006; Thon *et al*. 2000). This process can simply and efficiently integrate a gene of interest into an individual's genome. The plasmid used in this study was the phiC31 (ΦC31) integrase construct (developed by Meredith *et al*. 2013; kindly provided by Paul Eggleston, Keele University). This synthetic gene construct includes *DsRed2*, a *nanos*-integrase promoter from *Anopheles gambiae*, and a self-docking site (Meredith *et al*. 2013). The self-docking site is a phage attachment site (*attP*) that recognizes gene constructs that have a bacterial attachment site (*attB*; Meredith *et al*. 2013). This means that a subsequent injection of a plasmid with a gene of interest including a bacterial attachment site, such as the underdominance construct or a refractory gene, can be site-specifically integrated and attached to the original construct. Attaching a subsequent gene to an exact chromosomal site of the original transgene is important because if the original transgene is integrated into the genome at a site that results in no deleterious side-effects as a homozygote, then it can be reasonably assumed that fitness levels will not be affected when adding an additional gene at the same site. The self-docking site feature of the construct is ideal, because of its flexibility to allow future additions from a wide range of genes of interest, instead of an alternative gene of interest. *DsRed2* is a phenotype marker that serves the purpose to physically confirm successful integration of the gene of interest through red fluorescence expression. The *nanos* promoter was chosen to be a part of the overall

ΦC31 design in the Meredith *et al*. (2013) study because *nanos* has been shown to increase transformation efficiency and success in *Drosophila melanogaster* (Bischof *et al*. 2007). Choosing an ideal promoter is crucial, as promoters are part of the initial process in gene transcription. This study shows the efficacy of injecting this gene construct using REMI and the potential to rear transgenic colonies of *Cx. quinquefasciatus* that are homozygous for the synthetic gene to facilitate the next steps of integrating the rest of the gene drive construct.

Among the practical and important reasons previously mentioned surrounding the specific self-docking site gene, we chose to genetically modify *Cx. quinquefasciatus* using this ΦC31 integrase construct because: 1) *Cx. quinquefasciatus* has been successfully genetically modified before (Allen *et al*. 2001; Allen & Christensen 2004), 2) the ΦC31 integrase construct was successfully expressed in *An. gambiae*, which is a relative of *Cx. quinquefasciatus* (Reidenbach *et al.* 2009; Meredith *et al*. 2013; Marinotti *et al*. 2013), and 3) the *nanos* gene, which comes from *An. gambiae*, is already incorporated in the ΦC31 integrase construct (Meredith *et al*. 2013; Marinotti *et al*. 2013). While successful genetic modification of *Cx. quinquefasciatus* has been done twice before (Allen *et al*. 2001; Allen & Christensen 2004), genetic modification of the ΦC31 integrase construct in *Cx. quinquefasciatus* is novel research, and the use of REMI to genetically modify a mosquito species is also novel research.

Development of this first step of engineered underdominance in *Cx. quinquefasciatus* will allow not only site-specific integration of the underdominance-based gene drive construct and the possibility of adding a refractory avian malaria gene, but the self-docking site will also allow the possibility of addition of any other gene such as a refractory gene for human diseases like

Zika Virus or West Nile Fever. The successful integration of the ΦC31 integrase construct in *Cx. quinquefasciatus* shows that this construct may be used outside of the *Anopheles* and *Culex* genera and possibly be used in other species of mosquitoes that are vectors for other human diseases. The successful use of REMI to genetically modify a mosquito species shows the potential to use REMI as a simple method to genetically modify other mosquito species for other areas of research. For example, these methods and synthetic constructs potentially can apply to *Aedes aegypti*, a major vector of Dengue Fever, which has about 50 million cases of infection per year (World Health Organization 2009). This study has direct implications to prevent the extinction of native Hawaiian birds through transformation of Hawai‘i mosquito populations as well as human health implications, as an underdominance-based gene drive system could be applied to other mosquitoes that are vectors for human diseases.

Methods

Mosquito Maintenance

Wildtype *Cx. quinquefasciatus* egg rafts were collected from aged water sources during October 2016 in Hilo, Hawai‘i. Wild egg rafts were collected every 4-5 generations and added to lab colonies to minimize colony inbreeding or lab condition effects. Egg rafts were transferred to larval rearing trays inside the laboratory. Emerged larvae were visually confirmed to be *Cx. quinquefasciatus*. Larvae were reared in temperatures of 25℃ – 27℃ and 70% – 75% humidity. A light cycle consisting of 1-hour dawn, 12-hours daylight, 1-hour dusk, and 10-hours nighttime was implemented using timed night-lights to imitate day and night light conditions inside the

laboratory. Pupae were transferred into water-filled containers and put into 1-foot x 1-foot mesh cages to contain emerging adults. Adults were reared in identical temperature, humidity, and light conditions as the larvae. Adults were fed fresh 3% sugar water solutions three times per week. Protein meals in the form of bovine whole blood in sodium heparin (Lampire Biologicals) were fed to the adults to allow females to develop eggs. Approximately seven days after bloodmeals were provided, oviposition water created from aged tap water, synthetic hay infusion (Synthetic Hay-Infusion *Culex* Lure, BioQuip), and fish food tablets were placed in adult cages before dusk as a substrate for females to lay eggs. After all eggs were collected, the adults were killed by freezing the cages for 24 hours.

ΦC31 Plasmid Preparation

The ΦC31 plasmid (Figure 2) was transformed into *Escherichia coli* (strain MM294; Carolina Biological) via heat shock transformation (Appendix A) and cloned on LB-agar-ampicillin plates. Positive *E. coli* colonies were selected for plasmid purification (see Appendix B for purification protocol; Figure 3), after which the purified plasmid was stored at -20°C prior to linearization for REMI.

The restriction enzyme used for linearization was AclI (New England Biolabs), which recognizes the sequence "AACGTT", splitting the ΦC31 plasmid into one 373 bp fragment and a larger, 12,248 bp fragment (Figure 2; Figure 4) containing the genes of interest. The restriction enzyme digest solution of 6.5 µL molecular-grade water, 2 µL purified ΦC31 plasmid, 0.5 µL AclI restriction enzyme, and 1 µL of 10X CutSmart buffer (New England Biolabs) was mixed

and then incubated at 37°C for 1 hour. The resulting digest solution was then stored at -20°C for later use or immediately used for microinjections. All steps for the restriction enzyme digest were done on ice.

ΦC31 Microinjections

Oviposition water was provided about 30 minutes prior to the dusk light cycle and was checked approximately every hour after the start of the night cycle for the presence of freshly laid egg rafts. Microinjections were conducted using egg rafts that were collected within the hour they were laid. Egg rafts that were collected were split in half, one half to maintain the laboratory colonies and one half to use for microinjections. A moist filter paper was laid on top a microscope slide, covering about ½ of the slide. The eggs were set up on the bare part of the microscope slide, braced up against the edge of the filter paper. Eggs were lined up vertically in this fashion from the top of the slide to the bottom (Figure 5). The microinjection needle (Femtotip II Microinjection Capillaries, Eppendorf) was loaded with the restriction enzyme and linearized ΦC31 integrase plasmid solution that was mixed with colored dye to help with visual confirmation of an injection. The ΦC31 plasmid solution had a concentration of ~280 ng/µL. Using the microinjection apparatus, the needle pierced the posterior end of each egg and injected the contents using varying injection time and pressure parameters on the Eppendorf FemtoJet 4i (Eppendorf). This process was then repeated to inject all eggs on the slide. Injected eggs were then carefully placed into larval rearing trays and followed the previously mentioned rearing procedures. Injected eggs were labeled as generation 0 (G0). Larvae that survived to eclose were pooled together into a single cage to propagate the following generation (G1).

ΦC31 Screening

G1 larvae were screened for the *DsRed2* phenotype using a stereomicroscope fluorescence adapter (NightSea Model SFA). Larvae that fluoresced red were selected to start transgenic colonies. Larvae that did not express the *DsRed2* phenotype were considered negative and were killed by freezing. To assess integration of the self-docking site, mosquito larvae and adults that were positively screened for *DsRed2* expression were sampled and stored in 95% ethanol prior to DNA extraction (DNeasy Blood and Tissue Kit, QIAGEN). DNA quality and quantity were assessed through gel electrophoresis (1.5% agarose in 1X TAE, visualized using GelRed® dye [Biotium] under UV light) with a 10kb ladder (Thermo Fisher Scientific GeneRuler™ DNA Ladder Mix #SM0333). A Polymerase Chain Reaction (PCR) was run in 20 µL final volumes consisting of 0.4 µM of both forward and reverse primers, 1X Taq buffer (New England Biolabs Taq 2X Master Mix M0270S), and ~15ng/µL of DNA. The primers used in the PCR identified unoccupied *attP* self-docking sites (attP-int-fwd and attP-int-rev; Meredith *et al*. 2013). The thermal parameters for the self-docking site PCR began with a pre-heat step of 94°C for 60 seconds followed by an initial denature at 94°C for 30 seconds. This was followed by 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds, and 68°C for 45 seconds. Lastly, a final extension step consisted of 68°C for 5 minutes. Gel electrophoresis (1.5% agarose in 1X TAE, visualized using GelRed® dye [Biotium] under UV light) was then used to assess if the ΦC31 integrase construct was integrated into the mosquito genome (Figure 6). Verification of success was visualized by gel electrophoresis that indicated bands of the expected size (approximately 437 bp) as well as two more larger bands (around 900 and 800 bp) in *DsRed2* positive larvae and

a single band (approximately 437 bp) for the plasmid positive control (Figure 6). The 437 bp bands were excised and purified using GeneJET Gel Extraction Kit #K0691 (ThermoScientific; Figure 8). Gel electrophoresis was run on the mosquito samples, the plasmid positive control, and a wildtype sample for further comparison and confirmation (Figure 7). The two mosquito samples and the ΦC31 plasmid sample were then Sanger sequenced (Advanced Studies in Genomics, Proteomics and Bioinformatics (ASGPB) facility at the University of Hawai‘i at Mānoa). Using Geneious version 11.1.2 (Kearse *et al*. 2012), sequences were then analyzed and compared against the purified plasmid sample and sequences from Meredith *et al*. 2013 as positive controls (Figure 9).

Larvae were continually screened for positive *DsRed2* expression (Figure 10) and transgenic colonies as they were reared until the sixth generation of the transgenic line (Table 2; Table 3). However, at around the sixth generation, the larvae that were screened and selected as positive *DsRed2* expression showed up as negative results in the PCR using primers targeting the self-docking site (Figure 11). To confirm the previous results, a new PCR was done in identical volumes and concentrations as mentioned previously for the self-docking site PCR, but the primers used in this new PCR identified the *DsRed2* gene (dsRed_F and dsRed_R; Table S1).The dsRed_F and dsRed_R primers were based off the original ΦC31 plasmid and were designed for this study. The thermal parameters for this *DsRed2* PCR began with an initial denature at 95°C for 30 seconds. This was then followed by 32 cycles of 95°C for 15 seconds, 54°C for 15 seconds, and 68°C for 40 seconds. Lastly, a final extension step consisted of 68°C for 2 minutes. Gel electrophoresis (1.5% agarose in 1X TAE, visualized using GelRed® dye [Biotium] under UV light) was then used to assess if the *DsRed2* gene was present or absent in

the mosquito genome. The PCR and following gel electrophoresis indicated that the G6 samples were negative for *DsRed2* (Figure 12). This potential false-positive, background florescence that was similar to the *DsRed2* fluorescence expression made it difficult to continue screening for the ΦC31 construct (Figure 13). Thus, the ΦC31 construct was modified by the addition of a green fluorescent marker (EGFP) under the control of a 3xP3 promoter to improve the efficiency of the marker gene screening process. The modified plasmid is hereafter referred to as pBattP-EGFP.

pBattP-EGFP Plasmid Preparation

The 3xP3-EGFP cassette was amplified from a pMi(3xP3-EGFP) plasmid (Pavlopoulos *et al*. 2004) using 3xP3F and EFGPR primers (Table S1) that were designed during this study. The ΦC31 plasmid was digested with XhoI restriction enzyme (New England Biolabs) and AvrII restriction enzyme (New England Biolabs) and then the 3xP3-EGFP cassette was ligated into the plasmid. The new pBattP-EGFP plasmid was transformed into *E. coli* and cloned in the same process as mentioned for the ΦC31 plasmid preparation. The restriction enzyme digest for REMI also followed the same process and used the same enzyme as previously mentioned.

pBattP-EGFP Microinjections

Microinjections using the new pBattP-EGFP plasmid was conducted as described above for the initial ΦC31 microinjections. The restriction enzyme and pBattP-EGFP linearized solution had a concentration of ~400 ng/µL. Injected eggs were labeled as generation 0 (G0). Larvae that survived to eclose were separated by sex and immediately backcrossed with the

opposite sex of wildtype adults in attempt to establish isofemale lines. Two separate cages were made for the two combinations of backcrossing (Table 5) and then the resulting offspring from these cages were G1 individuals. Each G1 egg raft produced from the backcrosses were reared in separate larval rearing trays and larvae were screened independently at around the 4th instar stage.

pBattP-EGFP Screening

The pBattP-EGFP construct was screened for integration similarly to the ΦC31 confirmation methods. G1 larvae were screened for the *EGFP* phenotype using a stereomicroscope fluorescence adapter (NightSea Model SFA). Larvae that did not express the *EGFP* phenotype were considered negative and were killed by freezing.

Several mosquito larvae that had possible *EGFP* expression were chosen to be stored in 95% ethanol. These individuals were then used for DNA extraction using DNeasy Blood and Tissue Kit (QIAGEN). DNA quality and quantity were assessed through gel electrophoresis (1.5% agarose in 1X TAE, visualized using GelRed® dye [Biotium] under UV light) with a 10kb ladder (Thermo Fisher Scientific GeneRuler™ DNA Ladder Mix #SM0333). To assess integration of the new 3xP3-EGFP cassette, a Polymerase Chain Reaction (PCR) was run in identical concentrations and parameters as mentioned previously for the self-docking site PCR. The primers used were 3xP3F and EGFPR (Table S1). Gel electrophoresis (1.5% agarose in 1X TAE, visualized using GelRed® dye [Biotium] under UV light) was then used to assess if the pBattP-EGFP construct was integrated into the mosquito genome (Figure 14).

Results

ΦC31

For ΦC31 injections, 2,228 eggs were injected of which 342 survived to at least 4th instar, resulting in a 15.4% survivorship (Table 1). These individuals represented the G0 population and they were placed into a single cage to mate and create the G1 offspring. The G1 eggs were reared until they developed into about 4th instar larvae. Once the G1 larvae were at this stage, they were screened for *DsRed2* expression (Figure 10). From 76 G1 egg rafts, there were 6,167 larvae screened and 139 positive results (Table 2). A transformation success rate of 7.9% was estimated using the number of rafts that produced positive larvae and the total number of G1 rafts, assuming that each raft with positive larvae came from a single transformed adult. DNA was extracted from 19 G2 larvae that expressed the *DsRed2* fluorescent phenotype (Figure 10). A PCR amplifying the self-docking site (*attP*) was done and two G2 larvae samples and the ΦC31 plasmid positive control produced an approximately 437 bp band (Figure 6). These positive samples also produced two fainter bands at approximately 900 and 800 bp (Figure 6). The 437 bp band, which is the expected fragment size for the self-docking site, was then excised from the agarose gel, purified, and then visualized through gel electrophoresis (Figure 8). Sequences from these two mosquito samples aligned and matched with a positive control containing a ΦC31 plasmid sample and a reference sequence (Figure 9) obtained from a study from Meredith *et al*. (2012), confirming the successful, germline integration of the self-docking site into the *Cx. quinquefasciatus* genome.

Following confirmation of the integration of the ΦC31 construct, transgenic colonies were built with all larvae that positively expressed the *DsRed2* fluorescent phenotype. Screening and selection for positive individuals continued to attempt to create a homozygous colony. Proportion of screened individuals that expressed *DsRed2* gradually increased until the 3rd generation of larvae where only a single egg raft was laid from the 2nd generation adults (Table 2). This single egg raft was screened to contain 100% of all larvae expressing the *DsRed2* gene (Table 2). The proportion of positive individuals gradually decreased for every subsequent generation until the 6th generation where about 49% of all screened larvae were considered to be positive (Table 2). This unexpected trend led us to run a PCR amplifying the self-docking site using larvae samples from the 6th generation that were screened as positive and it was found that all samples were negative for the self-docking site (Figure 11). A PCR amplifying the *DsRed2* gene was done using the same larvae samples as the previously mentioned PCR and all samples were also found to be negative for the *DsRed2* gene (Figure 12). The larvae that were being screened and considered positive may have been expressing a type of background fluorescence (Figure 13), resulting in the selection for false-positive colonies for potentially a few generations. The presence of background fluorescence that was similar to the *DsRed2* fluorescence led us to modify the ΦC31 plasmid to contain a new promoter (*3xP3*) and marker gene (*EGFP*).

pBattP-EGFP

New wildtype eggs were used for pBattP-EGFP microinjections. The resulting pBattP-EGFP injected eggs are considered to be pBattP-EGFP Generation 0 (G0). Individuals that developed to at least the 4th instar stage were considered to survive to recruitment. For pBattP-

EGFP injections, there were 2,055 eggs injected and 59 survivors, resulting in a 2.9% survivorship (Table 4).

The backcrossed pBattP-EGFP injected females (30 individuals) and wildtype males (133 individuals) produced 24 rafts (Table 5). The backcrossed 3xP3-EGFP injected males (29 individuals) and wildtype females (109 individuals) produced 21 rafts (Table 5). The offspring of the backcrosses were considered to be pBattP-EGFP Generation 1 (G1). There were 1,972 pBattP-EGFP G1 larvae screened and 0 positive results, resulting in a 0% transformation success (Table 6). Several larvae from the G1 population were used for DNA extractions and a PCR was run (Figure 14). The PCR confirmed that any potential fluorescence from the *EGFP* screening was not actual *EGFP* expression. Integration of the new pBattP-EGFP construct was not achieved in this study.

Discussion

Overall Results

The objective of this study was to test whether an important component, the self-docking site (*attP*), of an underdominance-based gene drive system (e.g., RPM-drive; Reed *et al*. 2018) can be integrated into *Cx. Quinquefasciatus* via REMI. The PCR and sequence analysis confirmed there was successful germline transformation of *Cx. quinquefasciatus* using the ΦC31 construct and through the use of REMI as a genetic modification method (Figure 7; Figure 9). While the expression of the self-docking site was not assessed in this study, the self-docking site

gene was confirmed to be integrated into the mosquito genome (Figure 9). A specific location within the *Cx. quinquefasciatus* genome to integrate the ΦC31 construct was not chosen for this study as the use of REMI will integrate the desired construct randomly at one of the restriction enzyme cut sites present in the genome. Our integration event, while random, was successful because it can be assumed to be at a non-lethal site as we were able to propagate subsequent genetically modified generations. This study shows the first successful use of REMI to genetically modify a mosquito species and the first successful transformation of *Cx. quinquefasciatus* using the ΦC31 construct.

Confounding Factors

The use of the ΦC31 *DsRed2* gene as a marker gene did cause issues with background fluorescence, making *DsRed2* expression difficult to detect in the screening process. In a study with *D. melanogaster*, screening for a *DsRed2* gene resulted in comparably more background fluorescence than cyan or green marker genes, making marker gene detection more difficult (Sarkar *et al*. 2006). It may have been possible that we inadvertently selected for off-target genetic factors (e.g., phenotypic variation) or non-heritable factors (e.g., microbiota within the mosquitoes or the rearing environment) that happen to fluoresce in a similar manner as *DsRed2*. The screening difficulties led us to modify the ΦC31 plasmid to include a new promoter and marker gene. The new *3xP3* promoter was believed to help increase fluorescence screening efficiency because of our background fluorescence issue. We previously did not observe any type of fluorescence in the headcap region of the larvae (Figure 10; Figure 13) so we expected that the *3xP3* eye-specific promoter, which has been used in *D. melanogaster* (Horn *et al*. 2000)

and in *Anopheles stephensi* (Ito *et al*. 2002), in the pBattP-EGFP construct would reduce uncertainty in screening. However, we were unable to confirm the practical use of this new pBattP-EGFP construct as we were unable confirm a positive transformation in any of the larvae (Table 6; Figure 14).

There was a large drop in survivorship for the injected eggs after moving to the new pBattP-EGFP plasmid (Table 1; Table 4), which, with a higher mortality rate, there could have been a resulting lowered chance of a successful germline transformation. There could have been issues with the new pBattP-EGFP plasmid causing lethality as it has been found that green fluorescent proteins (GFP) can be toxic and lethal in cells (reviewed in Ansari *et al*. 2016). Another possibility that comes from the REMI process is that the concentration of the restriction enzyme may have been too high, resulting in too many cut sites in the mosquitoes' genome and causing lethality (indicated in Barnes & Rine 1985). Control injections were done with different people operating the injections, new needles being switched out periodically throughout injections, use of an injection solution without any plasmid, and use of an injection solution without any restriction enzymes and accompanying buffer. The average survival for these control injections was approximately 3.2%. This survival rate was not much greater than what has been occurring with the regular pBattP-EGFP injections (Table 4). The wildtype colonies that were being used for egg collections may have been fairly inbred, suffering from colony effects, despite bringing in new eggs from the wild in an attempt to offset any colony effects. This was done about every 4-5 generations, but that may have been too long and resulted in inbreeding depression occurring, possibly reducing fitness and hatching success post-injections. These are issues to account for in future studies, but depending on the actual cause of the lowered

survivability, the practical use of the new pBattP-EGFP plasmid and REMI may not be disregarded.

Future Directions

Possible considerations for future work can look at redesigning the pBattP-EGFP construct, specifically the promoter and marker genes. With the initial ease of screening for *DsRed2* expression and confirmation of a successful transformation due to individuals picked out from the screening process, it may be worthwhile to retain one of the original components from the ΦC31 construct. Use of the IE1 promoter from the original ΦC31 construct (Meredith *et al.* 2013) paired with a new marker gene (e.g., *EGFP*) may prove more diagnostic since this study shows the IE1 promoter does work in *Cx. quinquefasciatus*, but possibly changing the marker gene will reduce potential background fluorescence that has been shown to interfere with *DsRed2* fluorescence (Sarkar *et al.* 2006). However, with *EGFP* being known to be more toxic than other marker genes (reviewed in Ansari *et al.* 2016), it may be ideal to revert back to using *DsRed2* or potentially another marker gene such as *ECFP* and pair it with a new promoter. Pairing the *3xP3* promoter with a marker gene besides *EGFP* may be beneficial as the specific targeting of the *3xP3* promoter would still be expected to increase screening efficiency even with the background fluorescence. However, the lack of transformation success with the pBattP-EGFP may have been due a lack of ability to identify *EGFP* fluorescence because of issues with the *3xP3* promoter driving the *EGFP* gene. The *3xP3* promoter has never been used before in *Cx. quinquefasciatus*, so possibly a promoter that has been paired with *EGFP* to stably germline transform *Cx. quinquefasciatus* in previous studies would solve our screening issues. With the

two germline transformations of *Cx. quinquefasciatus* that we are aware of, the studies used an *actin5C* promoter paired with *EGFP* (Allen *et al.* 2001) and an *act88F* promoter also paired with *EGFP* (Allen & Christensen 2004). With the cause of the extreme decrease in survivorship when using the pBattP-EGFP construct (Table 4) being unknown and the potential causes of the background fluorescence (Figure 13) being unknown, the design of the promoter and marker genes needs to be assessed in order to increase overall screening efficiency.

The ΦC31 construct has been reported to have differing levels of efficiency where an increase from initial integration to site-specific transformation using the *attP* self-docking site was reported in *Ae. aegypti* (Nimmo *et al*. 2006) and *An. gambiae* (Meredith *et al*. 2013) and a decrease from initial integration to site-specific transformation using the *attP* self-docking site was reported in *An. gambiae* (Meredith *et al*. 2011). The studies that have shown an increase in efficiency utilized a *piggyBac* transposable element for the initial integration (transformation efficiency averaging 11% in Nimmo *et al*. 2006) and then utilized site specific integration with the *attP* self-docking site and *attB* attachment site (transformation efficiency averaging 23% in Nimmo *et al*. 2006). REMI has been reported to have increased transformation efficiencies compared to circular or linearized plasmid injections without restriction enzymes included in the injection mix (Thon *et al*. 2000). In this study, we intended to test the efficacy of these two novel aspects, the ΦC31 construct and REMI, in *Cx. quinquefasciatus*. However, moving forward with future studies, other constructs and genetic modification methods may prove to be more efficient for germline transformation of *Cx. quinquefasciatus* as this study did not find comparable transformation and injection survival efficiencies as the studies previously mentioned. One possible alternative construct that is currently being worked on, the “RPM-drive”, is an

underdominance-based gene drive system that has been reported to be successful in a laboratory colony of *D. melanogaster* (Reed *et al*. 2018). There may be potential to modify this system to include the self-docking site component of the ΦC31 plasmid and port the system to *Cx. quinquefasciatus* as a gene drive system to control vector-borne disease. A possible alternative method of genetic modification that could be used in place of REMI to initially transform *Cx. quinquefasciatus* with the RPM-drive or other construct is the use of a CRISPR-based mechanism (e.g., Jinek *et al*. 2012). *Cx. quinquefasciatus* has been genetically modified using a *Hermes* transposable element-based transformation system (Allen *et al*. 2001; Allen & Christensen 2004) and using REMI in this current study, but it has never been transformed using CRISPR to the best of our knowledge. A CRISPR-Cas9 system has been applied to at least two other mosquito species, *An. stephensi* (Gantz. *et al* 2015) and *An. gambiae* (Hammond *et al*. 2012; Kyrou *et al*. 2018), with the objective of developing a gene drive system to control disease spread. REMI itself is not highly specific when it comes to integrating a gene of interest as there can be multiple cut-sites in the target organism's genome (Kuspa 2006), whereas a CRISPR-Cas9 system can be highly specific for a single target in the organism's genome (Jinek *et al*. 2012). For the purpose of this study, exact integration location was not highly prioritized as this study was for a proof-of-concept. However, future gene-drive system studies for *Cx. quinquefasciatus* may find it efficient to utilize a CRISPR-based system, even though a CRISPR-based system may have more preparation work prior to experimentation than REMI, to integrate an underdominance-based gene drive construct. A CRISPR-based system may increase efficiency since it would be important for underdominance to target a specific non-detrimental region in the genome to integrate the synthetic construct as to not affect the fitness of the transformed mosquitoes (Figure 1). Nevertheless, CRISPR-based systems have seen unintended

results (reviewed in Reed 2017) such as off-target cleaving, so optimization of CRISPR or REMI methods may ensure greater efficiency when it comes to genetic modifications. This study shows the potential for REMI to transform mosquito vector species; however, a study to compare the efficiency of the different genetic modification methods and possible optimizations may prove useful, as the threat of mosquito borne diseases is an urgent issue that should be mitigated or even eliminated as soon as possible.

Implications

Successful transformation of *Cx. quinquefasciatus* using the ΦC31 plasmid shows that REMI and the plasmid used are potential methods and components for the future of *Cx. quinquefasciatus* genetic modification, especially gene-drive related studies because of the important self-docking site that is a part of the ΦC31 plasmid. Testing the expression of the self-docking site in future studies is crucial to show that this ΦC31 integrase construct is a viable transgene to complete an underdominance-based gene drive construct in *Cx. quinquefasciatus*. Due to the self-docking site, addition of the next transgene – an effector gene that can control the spread of disease – will be assumed to not affect fitness levels as the underdominance-based gene drive construct would ideally be placed into a non-detrimental site in the genome. The addition of an effector gene, such as an avian malaria disease refractory gene will then allow the application of this underdominance-based gene drive system to be applied to the conservation of the native forest birds of Hawai‘i through mitigating the damage being done by *Cx. quinquefasciatus* as an avian malaria vector. Successful integration and expression of the self-docking site component can also lead to future research using the same constructs used in this

study to create gene drive systems in other prominent disease vector mosquitoes. Control of other mosquito species that are vectors for human diseases may be possible using this study's constructs since it was shown that they are viable transgenes in a species other than what it was originally tested on. The success of this study is one step towards the immediate goal of preserving the native Hawaiian forest birds, and also adds to the growing suite of possible methods to apply an underdominance-based gene drive system to possibly control mosquito populations carrying human diseases.

Tables

Table 1. Survivorship of ΦC31 microinjected larvae to 4th instar life stage. Wildtype *Cx. quinquefasciatus* injected eggs and resulting surviving injected individuals are Generation 0 (G0).

Total Injected	Total Survived	Proportion Survived
2228	342	0.1535

Table 2. ΦC31 transformation success in all generations of larvae. Screened individuals were around the 4th instar life stage. The Generation 1 (G1) population are the offspring from the G0 population and each subsequent generation followed up until generation 6.

Generation	Total egg rafts	Total individuals screened	Total positive	Proportion transformed
1	76	6167	139	0.0225393222
2	18	460	148	0 0.3217391304
3	1	42	42	1
4	21	842	631	0.7494061758
5	50	612	425	0.6944444444
6	60	528	259	0.490530303

Table 3. Adult density of each ΦC31 generation's cage.

Generation	Male	Female	Total adults
1	14	23	37
2	57	43	100
3	19	17	36
4	315	256	571
5	82	193	275
6	92	108	200

Table 4. Survivorship of pBattP-EGFP microinjected larvae to 4th instar life stage.

Wildtype *Cx. quinquefasciatus* injected eggs and resulting surviving injected individuals are Generation 0 (G0).

Total Injected	Total Survived	Proportion Survived
2055	59	0.0287

Table 5. Density of each pBattP-EGFP backcross cage. Initial backcross generations are labeled as G0. Two "types" of backcross cages were made: 3xP3 ♂ x wt ♀ which consists of injected males mated with wildtype females and 3xP3 ♀ x wt ♂ which consists of injected females mated with wildtype males.

Generation	Type	Male	Female	Total adults	Rafts produced
G0	3xP3 ♂ x wt ♀	21	109	130	21
G0	3xP3 ♀ x wt ♂	133	30	163	24

Table 6. pBattP-EGFP transformation success in all generations of larvae. Screened individuals were around the 4th instar life stage. The Generation 1 (G1) population are the offspring from the G0 backcross population.

Generation	Total egg rafts	Total individuals screened	Total positive	Proportion transformed
1	32	1972	0	0

Figures

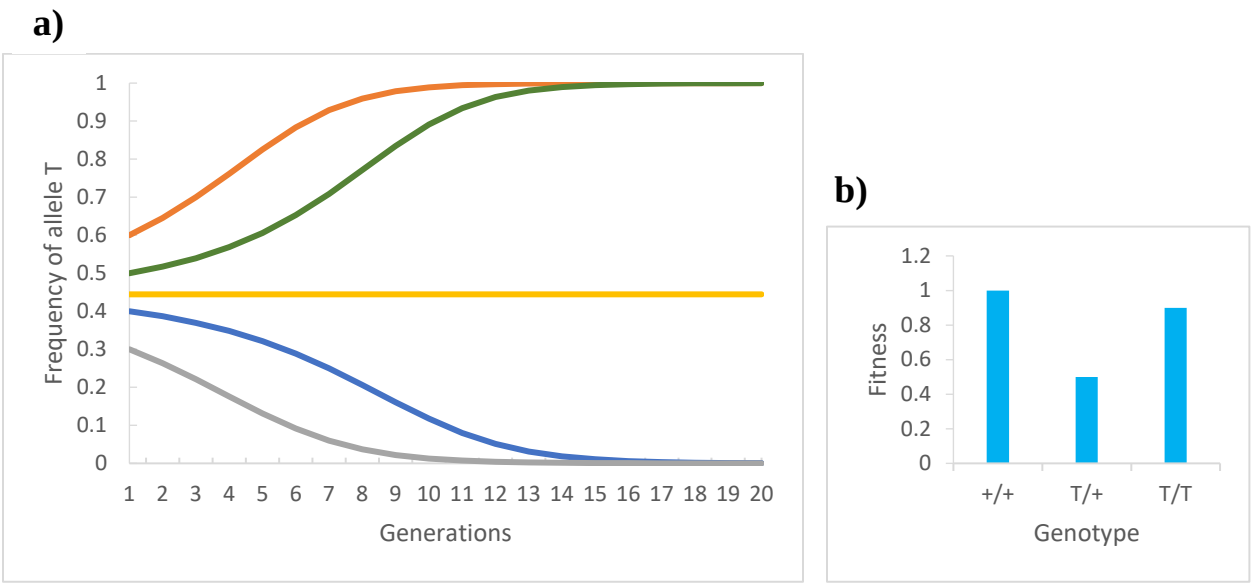

Figure 1. Theoretical evolution of allele T in an underdominance system. (A) 20 generations of allele T frequency trajectories at different starting allele frequencies. Starting allele frequencies are 0.3 (gray line), 0.4 (blue line), $0.4\acute{4}4$(yellow line), 0.5 (green line), and 0.6 (orange line). A frequency of $0.4\acute{4}4$ is the unstable equilibrium. The transgenic allele is lost within a population when starting frequencies are below the unstable equilibrium and the transgenic allele proceeds to fixation within a population when the starting allele frequency is above the unstable equilibrium. (B) Fitness levels of homozygous and heterozygous individuals in an underdominance system. In both plots, allele T represents the transgene and + represents the wild-type allele. Wild-type homozygotes would have a fitness of 1, transgenic homozygotes would have a fitness almost equal of the wild-types (~0.9), and heterozygotes would experience a relatively lower fitness (~0.5). [Adapted from Figure 2 in Reed *et al*. 2013]

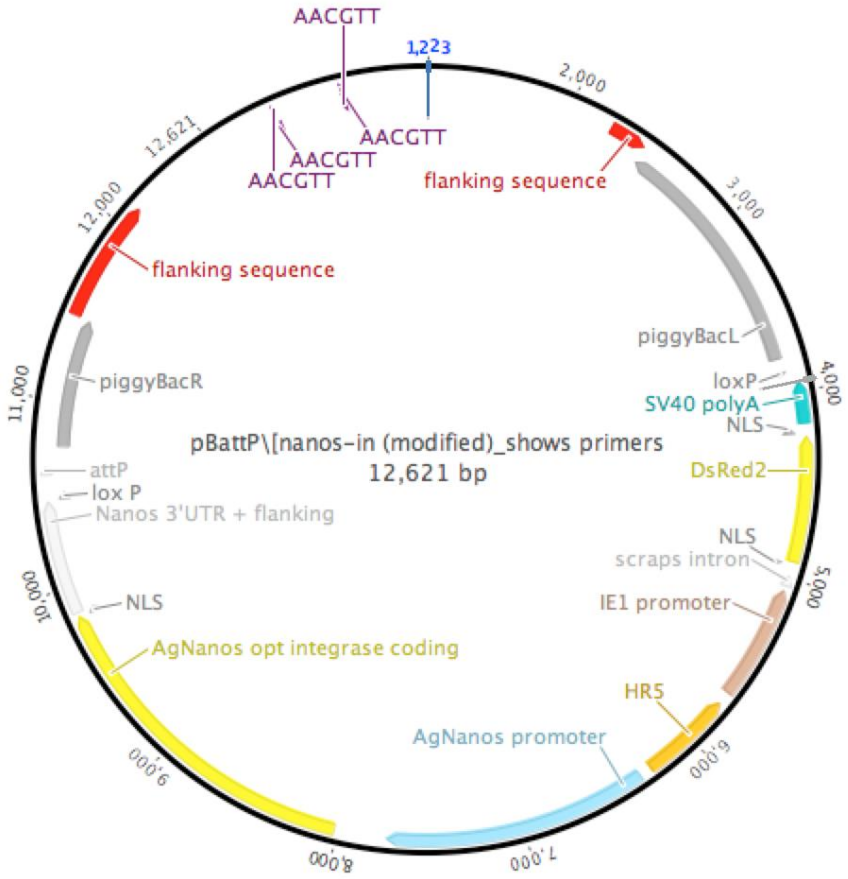

Figure 2. Annotated sequence of the ΦC31 plasmid. AclI restriction enzyme and the respective cut sites annotated by the recognition sequence (AACGTT) in purple font. Self-docking site annotated as the "attP" region. Sequence coding for the phenotype marker annotated as "DsRed2". Sequence of the *nanos*-integrase construct annotated with a blue and yellow color. Figure made using Geneious version 11.1.2 (Kearse *et al*. 2012).

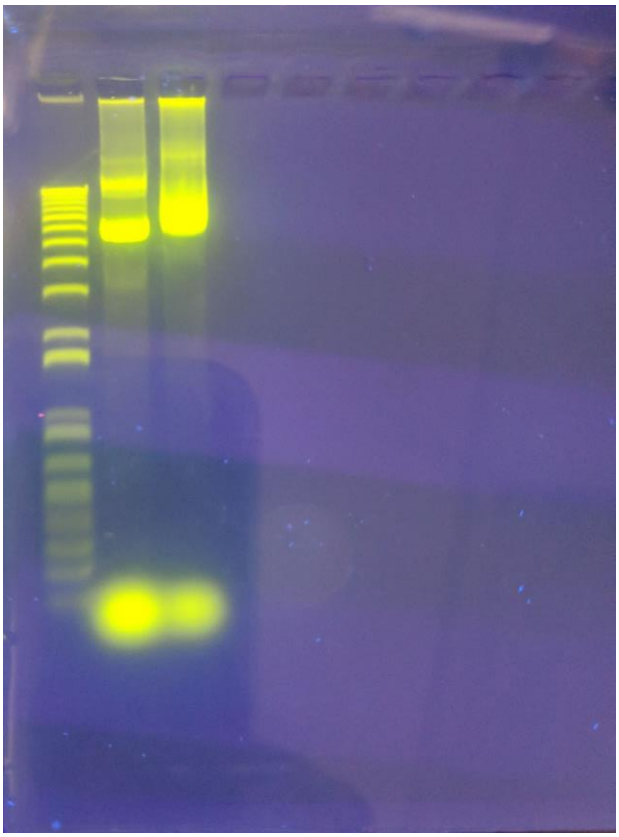

Figure 3. Purified ΦC31 plasmid product. The furthest left lane contains a 12,000 base pair ladder. The next two lanes contain two separate samples of the ΦC31 purified plasmid. The second lane more clearly shows the size of the nicked plasmids (~12,000 bp band) and the size of the supercoiled plasmids (~9,000 bp band). Gel was run using 1.5% agarose in 1X TAE buffer at 90V for 20 minutes, visualized using SYBR-Gold dye under UV light.

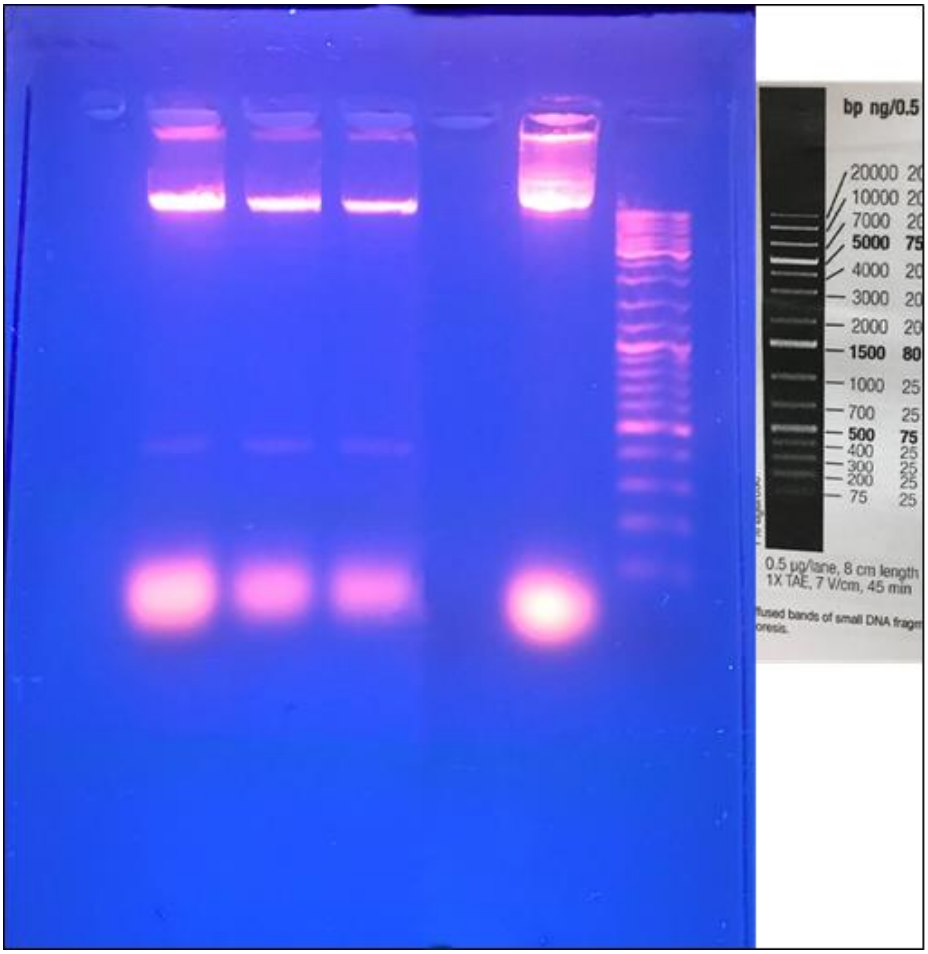

Figure 4. Gel visualization post-restriction enzyme digest. Lanes are 1-8 from the left. Lane 8 contains the ladder used and ladder details are provided next to the gel picture. Lanes 1, 2, and 3 were restriction enzyme digests using the AclI enzyme. Two distinct bands can be seen in these lanes, a large band over 12,000 bp and a small band at around the 400 bp mark, confirming a successful digest with expected fragment sizes. Lane 4 was a negative control. Lane 5 was a positive control containing an uncut plasmid with the ΦC31 construct. Gel was run using 1.5% agarose in 1X TAE buffer at 90V for 20 minutes, visualized using GelRed dye under UV light.

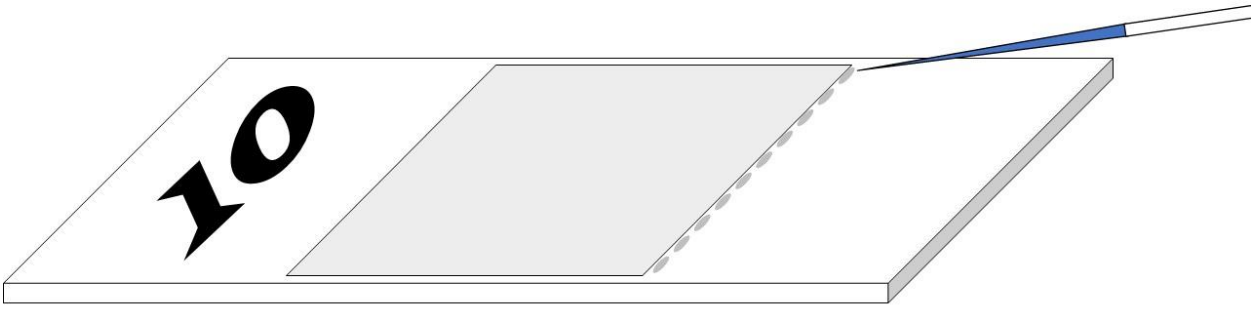

Figure 5. Microinjection slide setup. The white base is the microscope slide. The gray square on top the slide is a moistened filter paper. The gray ovals are the freshly laid mosquito eggs. They are braced against the moistened filter paper, arranged vertically. The number "10" denotes the number of eggs being injected on each slide. The white and blue tip is the microscopic needle that is used to inject the linearized plasmid and restriction enzyme solution. The solution is dyed blue in order to visually confirm a successful injection into the egg.

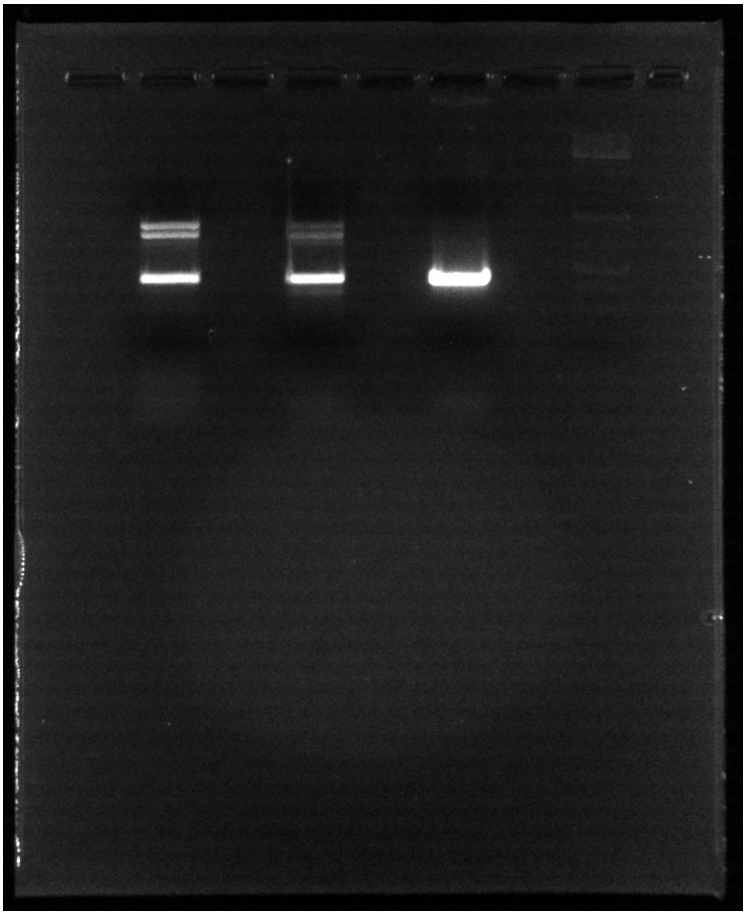

Figure 6. Gel visualization of PCR products used to confirm integration of the ΦC31 gene construct. Lanes are 1-8 from the left. Lane 8 contains the ladder used (Gene Ruler DNA Ladder Mix #SM0333). Lanes 2 and 4 were two different samples used that had successful extractions (PIP 5 & PIP 9). Three distinct bands can be seen in these lanes. Lane 5 was a negative control. Lane 6 was a positive control containing purified ΦC31 plasmid product. Gel was run using 1.5% agarose mixed in with 10,000X SYBR-safe dye diluted 1:10,000 in 1X TAE buffer at 90V for 30 minutes, visualized under blue light.

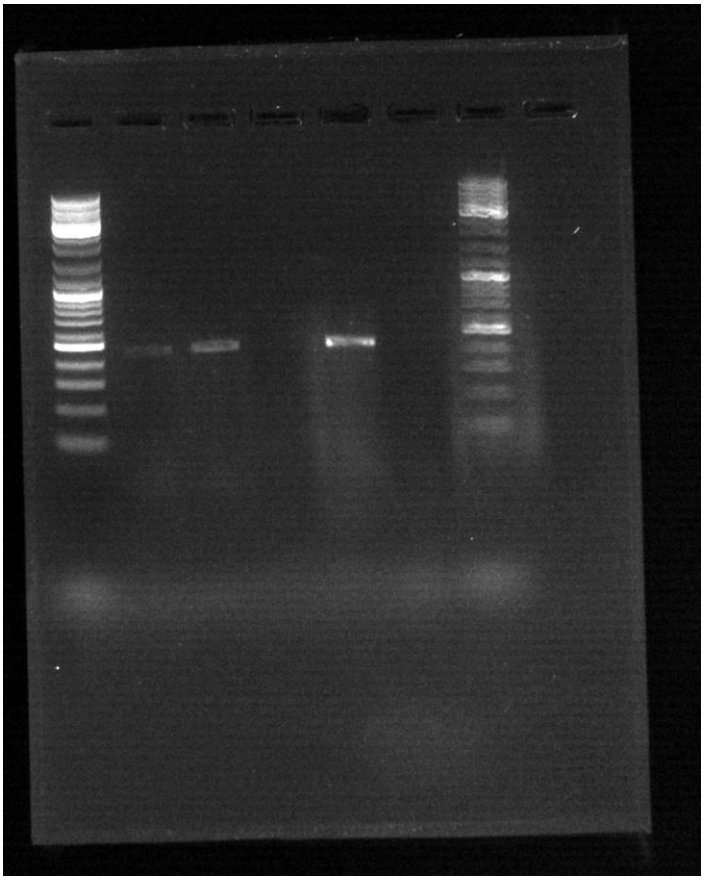

Figure 7. Gel visualization of post PCR products including controls used to confirm integration of the self-docking site (*attP*) gene. Lanes are 1-8 from the left. Lanes 1 and 7 contains the ladder used (Gene Ruler DNA Ladder Mix #SM0333). Lanes 2 and 3 were the two different mosquito samples (PIP 5 & PIP 9) used in the original PCR (Figure 6). Lane 4 was a wildtype *Cx. quinquefasciatus* control. Lane 5 was a positive control containing purified ΦC31 plasmid product. Lane 6 was the negative control. Gel was run using 1.5% agarose in 1X TAE buffer at 90V for 20 minutes, visualized using GelRed dye under UV light.

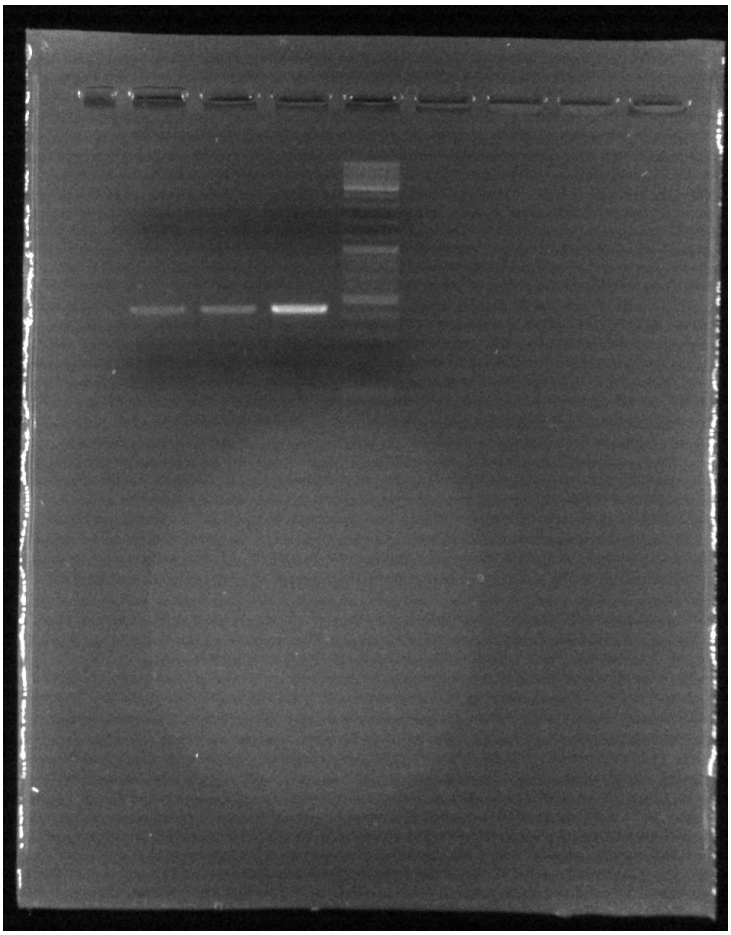

Figure 8. Gel visualization of post gel excision purification of PCR products used to confirm integration of the ΦC31 gene construct. Lanes are 1-8 from the left. Lane 4 contains the ladder used (Gene Ruler DNA Ladder Mix #SM0333). Lanes 1 and 2 were the two different samples (PIP 5 & PIP 9) used in the PCR (Figure 6). Lane 4 was a positive control containing purified ΦC31 plasmid product. The two samples and the positive control all line up at the same size (in-between the 400 and 500 bp bands on the ladder). Gel was run using 1.5% agarose in 1X TAE buffer at 90V for 20 minutes, visualized using GelRed dye under UV light.

Figure 9. Visualization of sample sequences lining up with a reference sequence. The first sequence (attP_reference) is the reference sequence of the *attP* gene acquired from Meredith *et al*. 2013. The following second-fifth sequences (PIP5_F_C02.ab1 & PIP5_R_D02.ab1; PIP9_F_E02.ab1 & PIP9_R_F02.ab1) are from DNA extraction samples of two separate larvae individuals that expressed the *DsRed2* phenotype. The last two sequences (PO_R_H02.ab1, PO_F_G02.ab1) are sequences of the purified plasmid product that was obtained from the plasmid-prep protocol (Appendix B). Sequences were uploaded and matched using Geneious version 11.1.2 (Kearse *et al*. 2012).

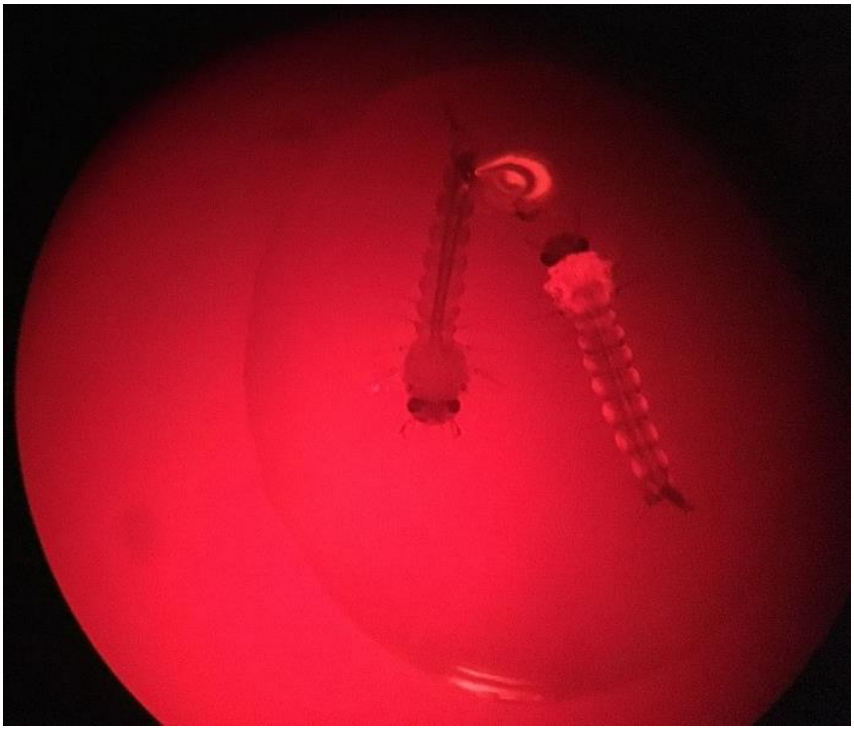

Figure 10. Positive expression of the ΦC31 plasmid *DsRed2* phenotype in a transgenic *Culex quinquefasciatus*. Larvae on the left is a wild-type *Cx. quinquefasciatus* for comparison, and the larvae on the right that is fluorescing is the ΦC31 modified *Cx. quinquefasciatus*.

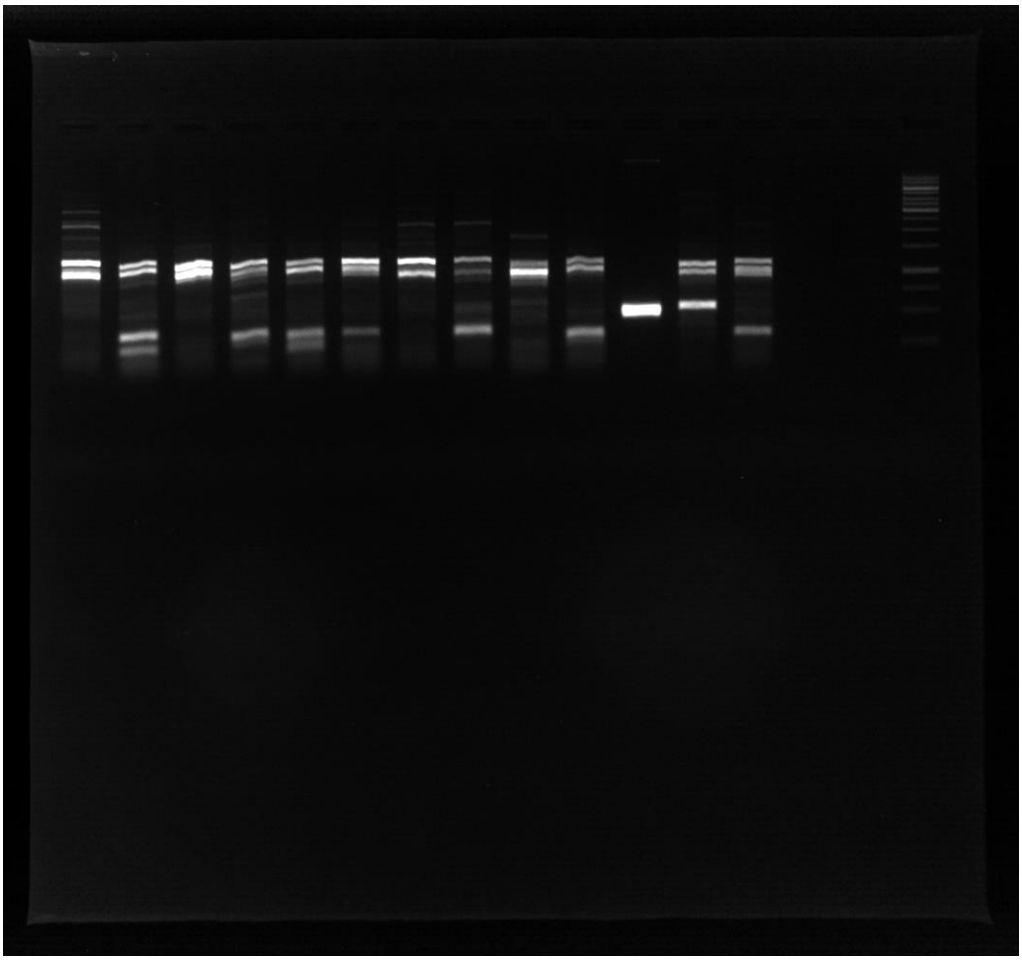

Figure 11. Gel visualization of PCR products used to assess presence of the self-docking site (*attP*) gene in G6 larvae. Lanes are 1-16 from the left. Lanes 1-10 contain G6 larvae that appeared to express *DsRed2* fluorescence. Lane 11 is a positive control consisting of a 1:10 dilution of the purified ΦC31 plasmid. Lane 12 is a positive control consisting of previously confirmed transgenic mosquito DNA (PIP 5). Lane 13 is a negative control consisting of pure wildtype mosquito DNA. Lane 14 is a PCR negative control. Lane 16 contains the ladder used (Gene Ruler DNA Ladder Mix #SM0333). Gel was run using 1.5% agarose in 1X TAE buffer at 90V for 20 minutes, visualized using GelRed dye under UV light.

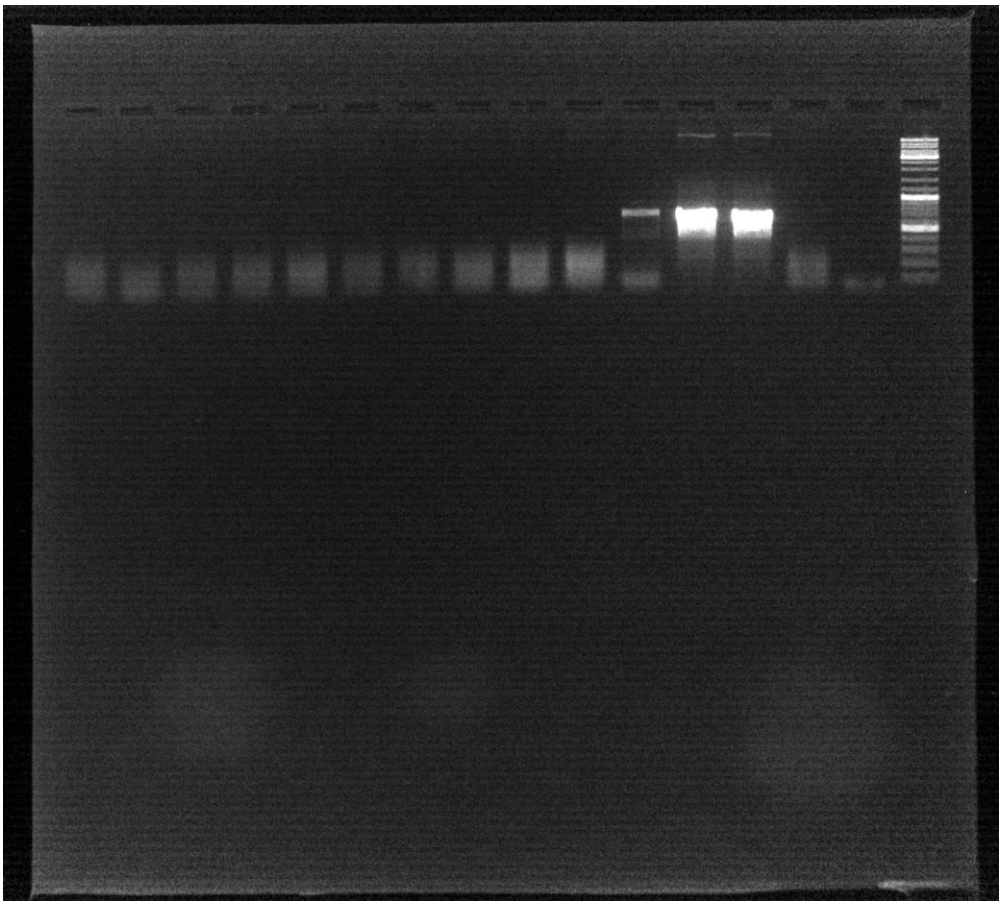

Figure 12. Gel visualization of PCR products used to assess presence of the *DsRed2* gene in G6 larvae. Lanes are 1-16 from the left. Lanes 1-10 contain G6 larvae that appeared to express *DsRed2* fluorescence. Lane 11 is a positive control consisting of previously confirmed transgenic mosquito DNA (PIP 5). Lanes 12 and 13 are both positive controls consisting of a 1:10 dilution of the purified ΦC31 plasmid. Lane 14 is a negative control consisting of pure wildtype mosquito DNA. Lane 15 is a PCR negative control. Lane 16 contains the ladder used (Gene Ruler DNA Ladder Mix #SM0333). Gel was run using 1.5% agarose in 1X TAE buffer at 90V for 20 minutes, visualized using GelRed dye under UV light.

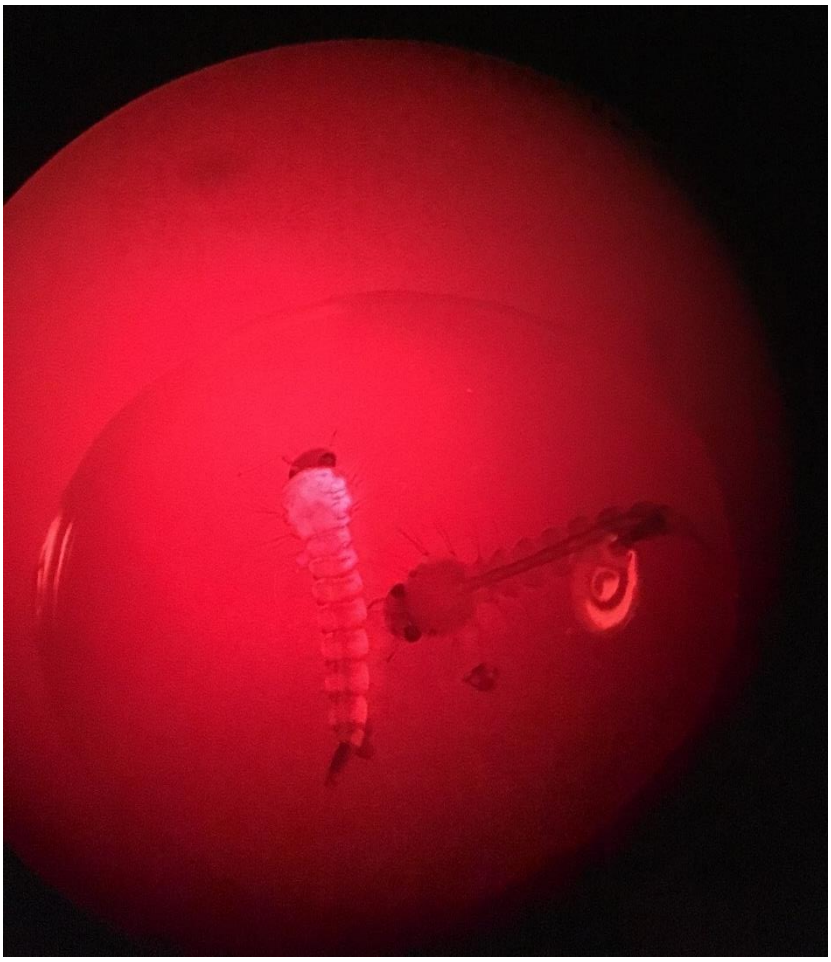

Figure 13. False-Positive *DsRed2* expression in a wildtype *Culex quinquefasciatus*. Larva on the left is a wild-type *Cx. quinquefasciatus* that isn't expressing any form of background fluorescence and the larva on the right is also a wildtype *Cx. quinquefasciatus*, but it is expressing a background fluorescence that is similar to the *DsRed2* fluorescence.

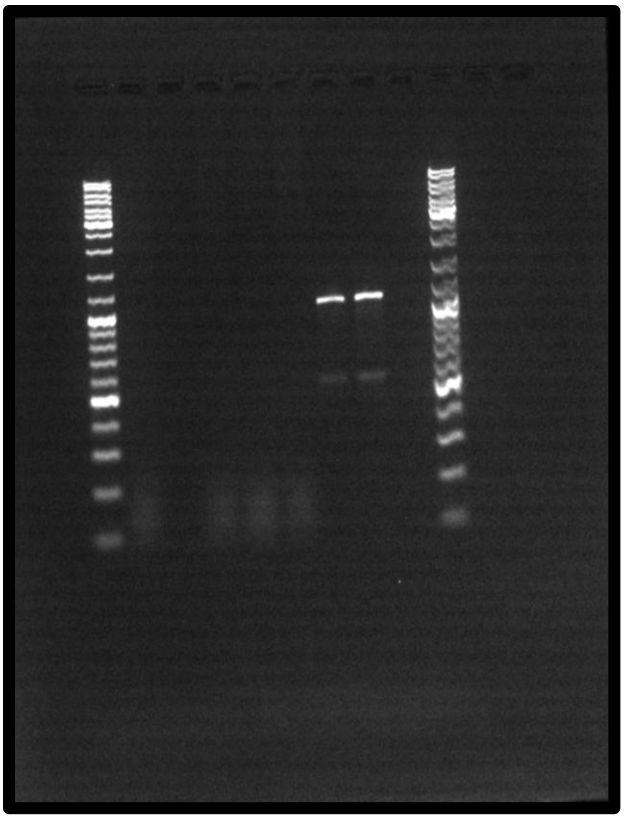

Figure 14. Gel visualization of PCR products used to assess presence of the *EGFP* gene in G1 larvae. Lanes are 1-10 from the left. Lanes 2-5 contain G1 larvae that appeared to express *EGFP* fluorescence. Lane 6 is a negative control consisting of pure wildtype mosquito DNA. Lanes 7 and 8 are both positive controls consisting of a 1:10 dilution of the purified pBattP-EGFP plasmid. Lane 9 is a PCR negative control. Lanes 1 & 10 contains the ladder used (Gene Ruler DNA Ladder Mix #SM0333). Gel was run using 1.5% agarose in 1X TAE buffer at 90V for 20 minutes, visualized using GelRed dye under UV light.

Appendices

Appendix A

Cell Heat Shock Transformation and Cloning: creates numerous copies of the plasmid containing a gene of interest

1. Transfer *E. coli* onto a petri dish containing Luria Broth and agar (LB/agar plate) using an inoculating loop.
2. Incubate the LB/agar plate at 37°C overnight.
3. The next day, scrape up an *E. coli* colony from the LB/agar plate using an inoculating loop.
4. Swish the colony in 500 µL of chilled Calcium Chloride (CaCl2) solution. Keep on ice.
5. Pipette 5 µL of purified ΦC31 integrase plasmid to the *E. coli*/CaCl2 mixture and incubate on ice for 15 minutes.
6. Quickly immerse the tube containing the mixture into 42°C water for 90 seconds.
7. Immediately transfer the tube back onto ice for 1 minute.
8. Add 100 µL of warm SOC (Super Optimal broth with Catabolic repression) medium to the tube and then mix.
9. Pipette 50 µL of the cell suspension onto a LB/agar plate containing Ampicillin.
10. Incubate the plate at 37°C overnight.

Appendix B

Plasmid-Prep: purifies the plasmid from *E. coli* bacteria

1. Scrape up an *E. coli* colony from the LB/agar plate with Ampicillin and transfer to a 50 mL tube containing 10 mL of a Luria Broth/Ampicillin solution.
2. Incubate the tube in a shaker at 30°C and 178 RPM for about 16 hours.
3. After incubation, centrifuge the tube at 2700 RPM for 7 minutes.
4. Pour out supernatant.
5. Add 200 µL of Solution 1 (50mM glucose, 25 mM Tris-HCl, 1 mM EDTA, 100 µg/mL RNase A, pH 8.0) and mix.
6. Add 270 µL of the solution into a 1.5 mL tube.
7. Add 400 µL of Solution 2 (1% SDS, 0.2 M NaOH) and mix.
8. Let the tube sit on ice for 5 minutes.
9. Add 300 µL of Solution 3 (3.0 M Potassium Acetate, pH 5.5) and mix.
10. Let the tube sit for 5 minutes on ice.
11. Centrifuge at maximum speed (~14,000 RPM) for 4 minutes.
12. Pipette 700 µL of supernatant into a new 1.5 mL tube.
13. Add 700 µL of 100% isopropanol and mix.
14. Centrifuge at maximum speed (~14,000 RPM) for 5 minutes.
15. Dispose of supernatant.
16. Add 600 µL of 70% ethanol and mix.

17. Dispose of supernatant.
18. Add 300 µL of 100% ethanol and mix.
19. Dispose of supernatant.
20. Dry the resulting pellet by placing the un-capped tube into a sterile fume hood for 30 minutes.
21. Resuspend the pellet by pipetting 80 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and mix until the pellet dissolves.
22. Store in -20°C.

Appendix C

Plasmid-Prep with Phenol-Chloroform Cleanup: purifies the plasmid from *E. coli* bacteria

1. Scrape up an *E. coli* colony from the LB/agar plate with Ampicillin and transfer to a 50 mL tube containing 10 mL of a Luria Broth/Ampicillin solution.
2. Incubate the tube in a shaker at 30°C and 178 RPM for about 16 hours.
3. After incubation, centrifuge the tube at 2700 RPM for 7 minutes.
4. Pour out supernatant.
5. Add 200 µL of Solution 1 (50mM glucose, 25 mM Tris-HCl, 1 mM EDTA, 100 µg/mL RNase A, pH 8.0) and mix.
6. Add 270 µL of the solution into a 1.5 mL tube.
7. Add 400 µL of Solution 2 (1% SDS, 0.2 M NaOH) and mix.
8. Let the tube sit on ice for 5 minutes.
9. Add 300 µL of Solution 3 (3.0 M Potassium Acetate, pH 5.5) and mix.
10. Let the tube sit for 5 minutes on ice.
11. Centrifuge at maximum speed (~14,000 RPM) for 4 minutes.
12. Pipette 700 µL of supernatant into a new 1.5 mL tube.
13. Add 700 µL of 100% isopropanol and mix.
14. Centrifuge at maximum speed (~14,000 RPM) for 5 minutes.
15. Dispose of supernatant.
16. Add 600 µL of 70% ethanol and mix.
17. Dispose of supernatant.
18. Add 300 µL of 100% ethanol and mix.
19. Dispose of supernatant.
20. Dry the resulting pellet by placing the un-capped tube into a sterile fume hood for 30 minutes.
21. Resuspend the pellet by pipetting 400 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and mix until the pellet dissolves.
22. Pipette 270 µL of phenol-chloroform into the same tube and mix.
23. Let solution sit for 5 minutes.
24. Centrifuge at maximum speed (~14,000 RPM) for 3 minutes.
25. A bilayer will form, pipette out 350 µL of the top layer and add it to a new tube.
26. To the new tube, add 40 µL of Sodium Acetate (3M, pH 6.0) and mix.
27. Add 390 µL of 100% isopropanol and mix
28. Centrifuge at maximum speed (~14,000 RPM) for 6 minutes.
29. Pipette out supernatant and dispose.

30. Add 500 µL of 70% ethanol and mix.
31. Centrifuge at maximum speed (~14,000 RPM) for 3 minutes.
32. Pipette out supernatant and dispose.
33. Add 200 µL of 100% ethanol and mix.
34. Centrifuge at maximum speed (~14,000 RPM) for 3 minutes.
35. Pipette out supernatant and dispose.
36. Dry the resulting pellet by placing the un-capped tube into a sterile fume hood for 30 minutes.
37. Resuspend the pellet by pipetting 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and mix until the pellet dissolves.
38. Store in -20°C.

Appendix D

Restriction Enzyme Digest: creates a solution containing restriction enzymes and plasmid fragments

1. On ice, pipette 2 µL of purified ΦC31 plasmid and 6.5 µL of molecular-grade water into a sterile PCR tube.
2. Pipette 1 µL of 10X CutSmart buffer into the tube.
3. Pipette 0.5 µL of the AclI restriction enzyme into the tube.
4. Tap/spin to mix.
5. Incubate at 37°C for 1 hour.
6. Store at -20°C.

Appendix E

PCR Parameters: reagent concentrations and cycle parameters used in PCR to confirm successful integration of the ΦC31 plasmid in *Cx. quinquefasciatus*.

Thermal program

1. 94°C for 60 seconds (pre-heat block)
2. 94°C for 30 seconds (initial denature)
3. 94°C for 30 seconds (denature)
4. 50°C for 30 seconds (anneal)
5. 68°C for 45 seconds (extend)
6. Repeat 35 times steps 3 to 5
7. 68°C for 5 minutes (final extension)

Reagent concentrations

1. Forward primer (attP-int-fwd)—0.4 µM
2. Reverse primer (attP-int-rev)—0.4 µM
3. New England Biolabs Taq 2X Master Mix M0270S—1X
4. DNA—15 ng/µL

Supporting Information

Table S1. Primers.

Oligonucleotide	Sequence
dsRed_R	5’ – GGTCGAGATCTCAGGAAC – 3’
dsRed_F	5’ – GAACGTCATCACCGAGTTC – 3’
3xP3F	5’ –ATTCGAGCTCGCCCG– 3’
EGFPR	5’ –TTTATCTAGATTACTTGTACAGCTCGTCC– 3’

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