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TITLE:

Embryo Microinjection Techniques for Efficient Site-Specific Mutagenesis in *Culex quinquefasciatus*

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KEYWORDS:

Culex quinquefasciatus, CRISPR/Cas9 genome editing, embryo alignment, embryo microinjection, genome modification, germline mutagenesis

SUMMARY:

This protocol describes microinjection procedures for *Culex quinquefasciatus* embryos that are optimized to work with CRISPR/Cas9 gene editing tools. This technique can efficiently generate site-specific, heritable, germline mutations that can be used for building genetic technologies in this understudied disease vector.

ABSTRACT:

Culex quinquefasciatus is a vector of a diverse range of vector-borne diseases such as avian malaria, West Nile virus (WNV), Japanese encephalitis, Eastern equine encephalitis, lymphatic filariasis, and Saint Louis encephalitis. Notably, avian malaria has played a major role in the extinction of numerous endemic island bird species, while WNV has become an important vector-borne disease in the United States. To gain further insight into *C. quinquefasciatus* biology and expand their genetic control toolbox, we need to develop more efficient and affordable methods for genome engineering in this species. However, some biological traits unique to *Culex* mosquitoes, particularly their egg rafts, have made it difficult to perform microinjection procedures required for genome engineering. To address these challenges, we have developed an optimized embryo microinjection protocol that focuses on mitigating the technical obstacles associated with the unique characteristics of *Culex* mosquitoes. These procedures demonstrate

optimized methods for egg collection, egg raft separation and other handling procedures essential for successful microinjection in *C. quinquefasciatus*. When coupled with the CRISPR/Cas9 genome editing technology, these procedures allow us to achieve site-specific, efficient and heritable germline mutations, which are required to perform advanced genome engineering and develop genetic control technologies in this important, but currently understudied, disease vector.

INTRODUCTION:

C. quinquefasciatus, commonly known as the southern house mosquito, is a competent vector of numerous pathogens including West Nile virus (WNV), Japanese encephalitis, Saint Louis encephalitis, and Eastern equine encephalitis. In particular, since it was first detected in New York in 1999, WNV has become a major vector-borne disease throughout the continental United States (US) with over 50,000 reported human cases resulting in around 2,300 deaths between 1999 and 2018¹ as well as over 4,500 reported equine cases between 2008-2019². In addition, at least 23 bird species found in North America have been impacted by WNV infections with at least 12 species classified as irrecoverable as a result of WNV³. The impact of WNV on human, equine, and avian populations is due to the opportunistic feeding behavior of its vectors. Typically, birds are the primary hosts for WNV, and humans and horses are incidental or dead-end hosts. Some pathogens vectored by *C. quinquefasciatus* only infect birds such as the avian malaria parasite, *Plasmodium relictum*. In Hawaii, *C. quinquefasciatus* is a principal vector of avian malaria and has caused the extinction of many native bird species^{4,5}.

To control diseases transmitted by *C. quinquefasciatus*, researchers and vector control agencies have used commonly established mosquito population control tools such as insecticide application⁶, however, these methods are costly, not species-specific, and have limited effectiveness as resistance to insecticides is high in many *C. quinquefasciatus* populations⁶⁻⁹. Other control techniques, such as *Wolbachia*-based population control strategies have been developed in recent years^{10,11}, but the fitness costs associated with *Wolbachia* infection limit the feasibility of this approach for this vector¹². There are also genetic-based control methods that have been developed in other mosquito species such as *Aedes aegypti*^{13,14}, *Anopheles gambiae*¹⁵ and *Anopheles stephensi*¹⁶, including the development of pathogen resistant mosquitoes¹⁷⁻¹⁹, that could also be developed for *C. quinquefasciatus* if the requisite genome engineering tools are developed for this species. However, *C. quinquefasciatus* biology differs greatly from other *Aedes* and *Anopheles* mosquito vectors which has made the development of similar genetic technologies difficult in this vector. With the advent of CRISPR-based genome engineering technologies, precise genome engineering has become increasingly trivial, affordable and adaptable and consequently has led to the development of novel genetic tools in a wide variety of species.

To generate mutations with CRISPR-based technologies, a mixture of Cas9 protein and synthetic guide RNA (sgRNA), complementary to the desired loci, is microinjected into pre-blastoderm stage embryos. Since *C. quinquefasciatus* females lay their eggs in groups attached in a floating raft structure (**Figure 1**), as opposed to ovipositing individual eggs, a trait of *Aedes* and *Anopheles* mosquitoes, embryo microinjections are increasingly complicated in this species. *Culex* larvae

also emerge from the anterior side of each egg, which is in contact with the water surface (**Figure 1**), so egg orientation post manipulation is important in this species. Here we describe a detailed protocol designed for the microinjection of Cas9 protein and sgRNA into *C. quinquefasciatus* embryos. This protocol has been designed to accommodate traits unique to *Culex* biology in order to improve embryo survival and genome mutation rates through certain steps that are key for timely egg collection and egg survival.

PROTOCOL:

1. *C. quinquefasciatus* colony rearing

1.1. Set up multiple colonies of *C. quinquefasciatus* adults in bug dorm cages.

NOTE: The colonies were provided by Dr. Laura Harrington at Cornell University²⁰. Detailed protocols for rearing *Culex* mosquitoes can be found in other literature²¹.

1.1.1. Maintain the mosquitoes at 25 ± 1 °C at 30% humidity with a 12:12 h day:night cycle.

1.1.2. Provide 20% sugar solution ad libitum by introducing a sugar solution container with a wick into the cage or by saturating cotton balls with the sugar solution and placing it within the cage.

1.2. Allow mosquitoes to mate for at least 3 days prior to blood feeding (**Figure 2A**).

2. Collection of *C. quinquefasciatus* pre-blastoderm stage embryos

2.1. After 3-6 days post eclosion, provide females with 1-2 mL of a citrated bovine blood-meal through a synthetic membrane and heated to approximately 40 °C in a blood feeding system (**Figure 2B**). There are also alternative blood feeding protocols that can be used for *Culex* mosquitoes (e.g., see ref²¹).

NOTE: *C. quinquefasciatus* is known for a preference for avian blood. Some labs use anesthetized live birds or avian blood for their blood meal source²¹⁻²³. However, colonies can be trained/selected to feed on bovine blood or small rodents if this feature is selected for over multiple generations.

2.1.1. Allow females to rest for a minimum of 3 days after blood feeding to undergo oogenesis and egg maturation before inducing oviposition for microinjection experiments.

2.2. On the day of embryonic microinjections, create an oviposition cup with organically infused oviposition water. Oviposition water should be made by fermenting rabbit feces (50 g/L), decomposing grass (4.5 g/L), or fish food (25 g/L) in water over the course of 5 or more days^{24,25}.

2.3. Place the oviposition cup into the cage and place the entire cage into a dark location (**Figure 2C**).

2.4. After every 30 min, check the cup for egg rafts.

2.4.1. If egg rafts are present, collect the rafts by scooping them with a paintbrush and place them on a wet filter paper (**Figure 2D,E**).

3. Alignment of *C. quinquefasciatus* pre-blastoderm stage embryos

3.1. Separate the eggs from the rafts by pressing down on the raft and teasing apart the eggs individually using a fine tip paintbrush and forceps (**Figure 2E**).

3.2. Align individual eggs on a thin strip of double-sided sticky tape placed across the top of a glass slide (**Figure 2F**).

3.3. While aligning, try to point the anterior side of each egg in the same direction for easier accessibility.

NOTE: An alternative egg alignment method without double sided sticky tape can be found in a previously published *Nasonia vitripennis* embryo microinjection protocol²⁶.

3.4. Prepare halocarbon mix by gently mixing the two halocarbon reagents and water (9:1:20, halocarbon 700 : halocarbon 27 : Ultrapure water) and then incubating the mixture overnight at 25 °C to facilitate water saturation of the halocarbon oil. Cover eggs with the halocarbon oil mix.

4. Needle preparation for microinjections

4.1. Generate the aluminosilicate capillary glass needles using a glass micropipette puller.

4.1.1. Place an aluminosilicate capillary glass into a needle puller, as per instructions from the needle puller's user manual.

NOTE: Quartz and borosilicate capillary needles can also be used, but for this experiment, aluminosilicate is preferred because of its relative affordability and durability.

4.1.2. Set Heat to 516, Velocity to 100, Delay to 70, Pull to 97, and Pressure to 500 on the needle puller.

4.1.3. Activate the needle puller, as per the puller's instructions and repeat as needed for additional needles.

NOTE: During the injection process, needles often become clogged or accidentally broken, therefore, pulling additional needles for a single experiment is highly recommended.

4.2. Bevel the needle tip by gently touching the tip of the pulled needle on the rotating diamond abrasive plate for around 10 s at a 25° angle.

NOTE: Beveling the needle opens it to allow fluid to flow through while also creating a sharper tip for easier penetration into the embryo. An example of a proper needle can be seen in a previous article²⁶.

4.3. Store pulled and beveled needles by embedding them into lines of modeler's clay in a Petri dish.

NOTE: To ensure the best quality for needle tips, needles should be freshly pulled and beveled as close to the time of injection as possible.

5. Loading the injection mixture

5.1. Prepare the injection mixture consisting of genome modification reagents (e.g., 200 ng/μL sgRNA and 200 ng/μL Cas9 mixture), or preferred injection solutions and keep it on ice.

NOTE: This mix can be prepared while waiting for eggs to be laid. More details on Cas9 and sgRNA production and preparation for microinjection can be found in previous publications^{27–30}.

5.2. Load 2 μL of injection mixture into the injection needle using a microloader tip.

6. Microinjection set up

6.1. Place the filled injection needle into a micromanipulator set up linked to an electronic microinjector.

6.2. Place the glass slide containing the aligned eggs on the stage of a compound microscope.

6.3. Using the micromanipulator and compound microscope, align the needle to aim at the posterior end of the embryo at a 25–35° angle.

7. Embryo microinjection (Figure 2G)

7.1. Carefully insert the needle into the embryo and inject the mixture at a quantity of about 10% of the volume of the embryo (700–800 pL depending on the size of the eggs).

NOTE: When injecting, the egg should slightly swell, however, if too much fluid is injected, eggs may burst, or cytoplasmic fluid may leak out.

7.2. Inject around 20 eggs at a time then stop and perform embryo recovery procedures.

NOTE: While injecting, there is a high chance of needles to either clog or break. If a clog occurs, which can be determined by a lack of injection fluid flowing through the needle, try either cleaning out the needle with the clean function on the electronic microinjector or try re-beveling the needle. If neither step works, quickly change to a new needle while ensuring the prepared eggs remain moistened.

8. Embryo recovery and hatching

8.1. Leave the eggs undisturbed for at least 5 min. Within 20 min post-injection, carefully remove the halocarbon oil by brushing lightly with a clean paintbrush (**Figure 2H**).

8.2. Lift the eggs gently using a paintbrush and place them into a cup of double-distilled water (**Figure 2I**). Take care to keep the eggs on the surface of the water.

8.3. Check the eggs daily for 7 days for hatching (**Figure 2J**).

8.4. Follow normal larval rearing procedures²¹.

9. Screening for genome modification

9.1. Screen the injected mosquitoes for the mutant phenotypes using a stereoscope (**Figure 2K**).

9.1.1. Verify mutations that do not have an easily recognizable phenotype, by PCR amplification, T7 Endonuclease I assay, and sequencing by subcloning of the target region³¹.

9.2. Set up additional crosses between injected individuals to detect heritable mutations within the germline.

REPRESENTATIVE RESULTS:

In previously published experiments this method was used to successfully generate somatic and germline mutations of a gene critical for the development of dark eye pigmentation, *white* (CPIJ005542)³⁰ (**Table 1**). CRISPR/Cas9 generated somatic mutations were scored by screening for loss of pigmentation in pupal stage eyes of injected individuals (G_0). Somatic mutations were generally present as mosaic phenotypes where some but not all the cells have the mutant phenotype. For example, somatic mutations of the *white* gene resulted in a mixture of ommatidia with phenotypes that lack pigmentation and those with a wildtype pigmented phenotype³⁰. When there was a germline mutation of the *white* gene, however, the next generation (G_1) inherited the full white eyed phenotype. Germline mutation rates were determined by intercrossing mosaic G_0 individuals and scoring for completely white eyed offspring (G_1). These experiments resulted in a 64% to 82% embryo survival rate, as well as a 37% to 57% somatic mutagenesis rate and a >61% germline mutagenesis rate (**Table 1**). By multiplexing sgRNAs to target multiple loci in the same gene, somatic and germline mutagenesis rates increased upwards to as much as 86% (**Table 1**). In addition, for many generations, viable homozygous stocks of the white mutants have been successfully kept in the lab.

FIGURE LEGENDS

Figure 1: *C. quinquefasciatus* egg raft and single egg morphology. Dorsal (A), lateral (B), and ventral (C) views of *C. quinquefasciatus* egg rafts. *C. quinquefasciatus* females lay their eggs with the more bulbous anterior side touching the surface of the water while the more pointed posterior away from the water's surface (D). A- anterior; P- posterior

Figure 2: Timeline for creating *C. quinquefasciatus* mutants by microinjection. Timeline of *C. quinquefasciatus* colony preparation and embryo collection for microinjection (A-D). Collected eggs are then separated (E) aligned in the same orientation and covered with halocarbon oil (F). The eggs are then injected (G) and allowed to rest for a minimum of 5 min before removing the halocarbon oil (H). Eggs are then transferred to a clean water source (I) for hatching (J) and screened for mutant phenotypes (K).

Table 1: Survival and mutation rates of embryos injected with single and multiplexed gRNAs targeting white. The table is reprinted with permission from³⁰

DISCUSSION:

With recent efforts to generate genetically engineered tools for mosquito vector control, there is a need to develop and optimize embryo microinjection protocols for common mosquito disease vectors. Although methods have been developed for *Aedes* and *Anopheles* mosquitoes, a protocol designed specifically for *Culex* has been minimally explored. In general mosquito embryo injection protocols can be divided into 3 general steps: 1) embryo collection and preparation, 2) embryo injection, and 3) post-injection recovery. In order to successfully generate mutants, all three steps must be optimized for the target species. This modified protocol for embryo microinjection is specific for successful genome engineering of *Culex* mosquitoes.

Maximizing embryo collection is a common bottleneck in embryo microinjection protocols. In order to increase the number of eggs collected in a short period of time, mosquitoes were restricted from an oviposition substrate for 2-3 days post blood meal. It is important to note that *C. quinquefasciatus* tend to prefer avian blood sources as opposed to mammalian sources or membrane feeding with mammalian blood. However, many researchers have difficulty acquiring a reliable source of live avians or avian blood for blood feeding, so laboratory stocks can be adapted to feed on more accessible blood sources. It is also crucial to use nutrient-rich water for the oviposition substrate as it provides oviposition cues for *C. quinquefasciatus* and most other *Culex* vectors. There are many methods to generate nutrient-rich water, which may need to be optimized between labs to ensure an appropriate number of eggs are available for microinjection. For example, while this method was the most successful with rabbit feces fermented in deionized water for 5 or more days, other researchers have more success with grass or fish food as the nutrient source and some even with distilled water²¹.

Since *Culex* mosquitoes lay groups of eggs in rafts, collecting eggs is fairly simple. Eggs can simply be collected by scooping the entire raft with a paintbrush. Egg separation is more complicated,

but essential for successful injection. Eggs can be carefully separated from the raft by applying gentle downward pressure between eggs with paintbrush or forceps. With some practice, multiple users were reliably able to separate single eggs from egg rafts. After separating each egg, the eggs are aligned in the same orientation on double-sided sticky tape, which stabilizes the eggs during microinjection. Eggs are injected into the tapered posterior end and the addition of halocarbon oil keeps the eggs moist during manipulation.

To limit embryo damage during microinjection, injection needles need to be of appropriate strength and beveled at an appropriate angle. Aluminosilicate needles beveled for 10 seconds at a 25° angle had the best results, but borosilicate and quartz needles may also work, even though they may be less durable and more expensive. In addition, proper needle beveling facilitates better flow of the Cas9 and sgRNA mixtures and creates a sharper point for easier penetration into the embryo. In many cases, beveling is also an efficient way to quickly fix clogged needles instead of spending the time and effort to replace clogged needles.

After injection, eggs should remain undisturbed for at least 5 minutes with the halocarbon oil being removed immediately afterward by gently brushing it away with a clean paintbrush. Following removal of the halocarbon oil, the injected eggs can be placed in water to hatch, which typically occurs within 3 days post-injection. With practice and sufficient numbers of eggs, this protocol can achieve consistent somatic and germline mutations in *C. quinquefasciatus* and is versatile enough that it should be easily adaptable to other *Culex* mosquitoes.

ACKNOWLEDGMENTS:

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DISCLOSURES:

None

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Figure 1

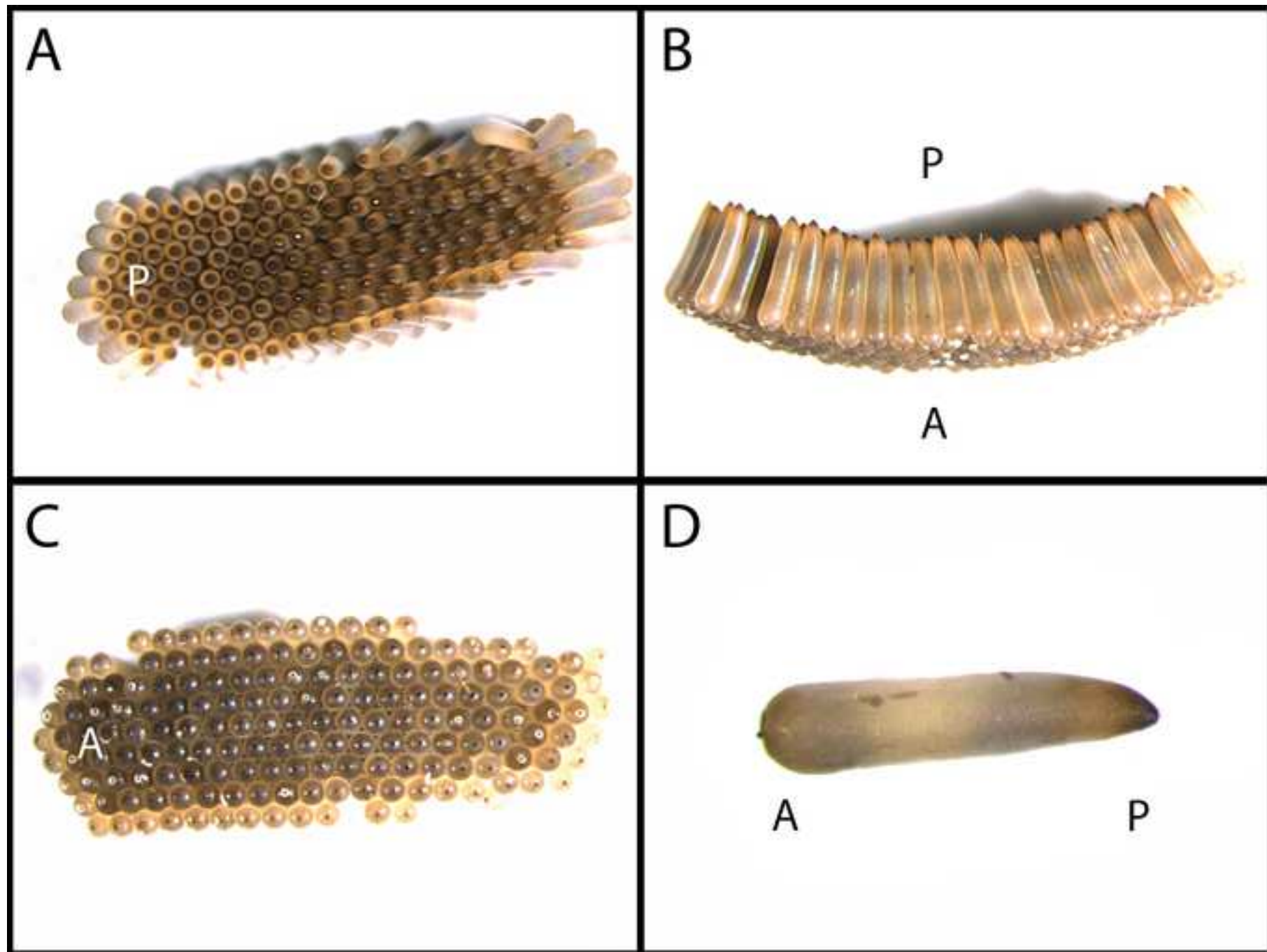


Figure 2



sgRNA	#injected	Survival	Somatic Mos
		♀	♂
w sgRNA-1	50	15	20
w sgRNA-2	50	9	32
w sgRNA-3	50	17	15
w sgRNA-1/w sgRNA-2	50	7	16
w sgRNA-1/w sgRNA-3	50	13	9
w sgRNA-2/w sgRNA-3	50	17	10
w sgRNA-1/w sgRNA-2/w sgRNA-3	50	11	10

data from: Li, M., Li, T., Liu, N., Raban, R.R., Wang, X., Akbari, O.S. Methods for the generat

Germline Mosaicism (G_i)

Total (%)	♀ (%)	♂ (%)	Total (%)	G _i mutants (%)
35 (70)	7 (47)	13 (65)	20 (57)	128 (69)
41 (82)	3 (33)	12 (38)	15 (37)	51 (61)
32 (64)	7 (41)	8 (53)	15 (47)	157 (72)
23 (46)	5 (71)	12 (75)	17 (74)	123 (79)
22 (44)	10 (77)	6 (67)	16 (73)	72 (81)
27 (54)	13 (76)	8 (80)	21 (78)	101 (85)
21 (42)	9 (82)	9 (90)	18 (86)	149 (86)

tion of heritable germline mutations in the disease vector *Culex quinquefasciatus* using clustered r

regularly interspaced short palindrome repeats-associated protein 9. *Insect Molecular Biology* . doi:

10.1111/imb.12626 (2019).

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
9 oz clear plastic pet cup	Karat	C-KC9
Aluminosilicate glass capillary tubing 1mm(outside diameter) X 0.58mm (inner diameter)	Sutter Instruments	BF100-58-10
Blood	Colorado Serum Company	31025
Bugdorm	Bugdorm	4F2222
Cas9 Protein with NLS	PNABio	CP01
Compound Microscope	Leica DM-750	
Diamond abrasive plate (0.7u to 2.0u tip sizes)	Sutter Instruments	104E
DNase/RNase-Free distilled Water	Invitrogen	10977-015
Double-sided Tape	Scotch	B084NVQGXD
Femtojet 4x or 4i programmable microinjector	Eppendorf	
Femtotips Microloader tips	Fisher Scientific	E5242956003
Filter Paper	Whatman	1001-090
Fine-tip paintbrush	ZEM	2595
Halocarbon oil 27	Sigma-Aldrich	H8773
Halocarbon oil 700	Sigma-Aldrich	H8898
Hemotek	Hemotek	PS5
Microelectrode Beveler	Sutter Instruments	BV10
Micropipette Puller	Sutter Instruments	P-1000 or P-2000
Microscope Slides	Fisherbrand	12-550-A3
Non-Drying Modeling Clay	Jovi	B0025Z71IM
Stereo Microscope	Olympus	SZ51
Sugar	Domino	
T7 Endonuclease I	NEB	M0302
TOPO TA Cloning Kit	ThermoFisher Scientific	K451020

Ultra-fine tip forceps	Fisher Scientific	16-100-121
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Comments/Description
Insect Rearing and Egg Collection
Microinjection, borosilicate and quartz needles could also be used but we preferred aluminosilicate
The colony took multiple generations to adapt to this blood source. Other blood sources are likely just as appropriate. See protocol notes on blood source selection.
Insect Rearing Cage
Microinjection
Microinjection, for embryo injection
Microinjection, to be used with beveler
Microinjection
Microinjection, embryo alignment
Microinjection
Microinjection
Microinjection, embryo collection
Microinjection, embryo alignment
Microinjection, embryo alignment
Microinjection, embryo alignment
Line Maintenance
Microinjection, needle beveling
Microinjection, needle pulling
Microinjection, embryo alignment
Microinjection, needle storage
Microinjection, for embryo alignment
20% sugar solution for adult sugar source.
Preparation of microinjection materials
Preparation of microinjection materials


Microinjection, embryo alignment

April 2nd, 2020

Dear JOVE Editorial Team,

Thank you very much for providing us with the opportunity to revise our manuscript, entitled, “**Embryo microinjection techniques for efficient site-specific mutagenesis in *Culex quinquefasciatus***”, for consideration as an article in JOVE. We greatly appreciate your edits and the comments, as we believe these have significantly improved our manuscript. We have addressed each of the reviewer points in the response below - reviewer comments are in gray text, our responses are in blue text, and any significant textual changes are pasted below responses for clarity. Thank you for your consideration, and please let us know if we can provide any other information.

With best wishes,



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Please see the editor’s comments in gray below and the author’s responses are in blue. We would like to thank the editors for their helpful suggestions and edits to improve the manuscript.

Comment 1:

The editor has formatted the manuscript to match the journal’s style. Please retain

Please proofread the manuscript well before submission

[We have retained the formatting and made additional grammatical and technical corrections to the document.](#)

Comment 2:

Please number all the references in the order of it being cited in the text. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

[This has been corrected.](#)

Comment 3:

I have highlighted part of the protocol section to be used in the video. Please check and edit.

Please ensure that the highlighted part forms a cohesive story and is in line with the title and do not exceed 2.75 pages including headings and spacings.

Please include all actions associated with your protocol as if you are explaining someone how to do the technique. Please use imperative tense throughout.

Please do not use personal pronouns in the manuscript.

The highlighted protocol looks like the appropriate content and length for the video.

We have added additional actions and action details and made edits to ensure instructions are now in imperative tense.

We removed personal pronouns from the manuscript.

Comment 4:

How do you provide this?

We have noted that we place sugar solution saturated cotton balls within the cage or by using a container of sugar with a wick to provide sugar for the mosquito colony.

Comment 5:

Please label the panels as A, B, C etc. for all the figures and change accordingly in the text.

We have changed the figure panel labeling as well as the corresponding references in the manuscript

Comment 6:

Volume? How is this done- please include in brief. How do you visually identify mated females?

It depends on the membrane feeding system, but we described the method we used. Females are assumed to be mated if housed with males for at least 3 days post eclosion. We removed the word "mated" as this is not necessarily verified and as the author suggests causes confusion.

Comment 7:

Please move this to the table of materials. We cannot have commercial terms in the manuscript.

We have made this correction.

Comment 8:

So basically they just rear the mosquito colonies on the bird directly. Please include citations.

We have added multiple citations that use live birds or bird blood.

Comment 9:

Please reword for clarity.

We have reworded for clarity.

Comment 10:

How is this done. Please include volumes. Please use imperative tense throughout..

We have added more details on how we generate oviposition water. We have edited this to use imperative tense.

Comment 11:

Steps are now written in order. Please check. What is the volume used? Do you place the glass slide in a Petri dish?

The steps are in an appropriate order. We added the volume used and do not use a petri dish.

Comment 12:

How?

We have changed the wording and provided the how throughout this section.

Comment 13:

Where in the needle puller.

We have now indicated that this is specific to the needle puller and the manufacturer's instructions should be used.

Comment 14:

If we cant state what needle puller we use should we tell them these values with are puller specific?

We have now indicated what is specific to the needle puller and suggest the manufacturer's instructions should be followed where this applies.

Comment 15:

How?

We have now indicated that this is specific to the needle puller and the manufacturer's instructions should be used.

Comment 16:

This can be moved to the discussion instead.

We have moved this to the discussion.

Comment 17:

What volume?.

We have added this information.

Comment 18:

How do you visualize identify this?

We have included details on how this is determined.

Comment 19:

Citations?

Citations have been added for this technique.

Comment 20:

Please remove the figure legends from the uploaded figures.

We have done this.

Comment 21:

Please mark the panels as A, B, C etc. to maintain consistency.

[We have done this.](#)

Comment 22:

Please number the references in the order.

[We have done this.](#)



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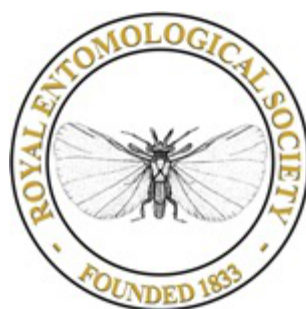
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Gene duplication and subsequent functional diversification of sucrose hydrolase in *Papilio xuthus*

Sucrose is the main product of photosynthesis in plants, providing a rich carbon and energy source for the physiological growth of insects. In *Papilio xuthus*, sucrose hydrolase (SuH) is a key enzyme in the sucrose metabolism pathway. The SuH gene family has expanded in *P. xuthus* through gene duplication and subsequent functional diversification. This study provides insights into the evolution and functional diversification of the SuH gene family in *P. xuthus*.
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