ABSTRACT

Genomic Analysis of Stenogamy Mating Behavior in the *Culex pipiens* Complex

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The *Culex pipiens* complex mosquitoes are major vectors for several arboviruses including West Nile virus in the United States. Within *Cx. pipiens* complex there are three biotypes that differ mainly in eco-physiological and behavioral characteristics such as habitat selection, blood meal preference, mating behavior and overwintering strategy and yet are difficult to differentiate morphologically. The three biotypes are *Cx. pipiens* form molestus F., *Cx. pipiens* f. pipiens L. and *Cx. pipiens quinquefasciatus* (aka *Cx. quinquefasciatus*).

The low number of genetic markers has been the key obstacle in performing genetic studies in the *Cx. pipiens* complex. In post genomic era plethora of single nucleotide polymorphisms (SNPs) were discovered from carious genomic data and provide a valuable resource to develop informative molecular markers. In the species complex, the bioytpes' morphological similarity creates a need for accurate identification methods to differentiate between biotypes. Utilizing the novel SNPs found, here we show high resolution melting curve analysis as a method which distinguish biotypes efficiently at low cost.

Reproductive behavior of vector mosquitoes is important to understanding the processes of disease transmission. Hybridization of these biotypes is known to occur, even though form pipiens mate above ground in larges spaces (eurygamy) and form molestus preferentially in small spaces (stenogamy). Hybridization allow gene flow of biotype-specific traits that are crucial in the disease transmission cycle including host preferences, diapause and autogeny. In this study, we have also examined the mating behaviors, insemination rates, fecundity and fertility in parental and F1 hybrids between *Culex pipiens* form pipiens and *Cx. pipiens* f. molestus in stenogamy conditions to look at mate competitiveness and specificity for the sterile insect program.

In summary, manipulating genes that control either directly or indirectly to the mating behavior of *Culex pipiens* complex mosquitoes is crucial for the sterile insect technique (SIT). In this context, a functional genomics study can elucidate the potential genes to be utilized for a genetically driven SIT program. Here we have performed RNA-seq to quantify genetic variations by linking SNPs and transcripts of two *Culex pipiens* biotypes that exhibit stenogamy and eurygamy mating behavior.

Genomic Analysis of Stenogamy Mating Behavior in the *Culex pipiens* Complex

by

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ABBREVIATIONS

Cx. pipiens	*Culex pipiens*
WNV	West Nile virus
HRM	high-resolution melting
SIT	sterile insect technique
DV/D ratio	dorsal-ventral/dorsal ratio
SNP	single nucleotide polymorphism

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ATTRIBUTIONS

Chapter Three:

Sungshil Kim: Designed and carried out the experiments and wrote the manuscript.

Cheolho Sim: Designed and wrote the manuscript.

Rahmi Lee: Carried out the experiment and helped write the manuscript.

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Sungshil Kim: Designed and carried out the experiments and wrote the manuscript.

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CHAPTER ONE

Background

Culex pipiens Complex

The *Culex pipiens* complex mosquitoes are well known vectors of West Nile virus, Rift Valley fever, lymphatic filariases and other diseases (Monath 1988; Fonseca, Keyghobadi et al. 2004; Hamer, Kitron et al. 2008). The *Culex* complex consists of *Cx. quinquefasciatus* and *Cx. pipiens* among others. The latter has two known biotypes, namely *Cx. pipiens* form pipiens and *Cx. pipiens* form molestus that are almost identical morphologically, relying on distinct eco-physiological traits to separate the two (Clements 1992). Understandably, all three members of the complex are evolutionarily closely related which could be the reason for the difficulty in morphological identification and thus, give cause to one of the most complex taxonomy debate in mosquitoes (Clements 1992; Harbach et al. 1984).

Cx. pipiens originated from the area stretching from South Africa all the way to the northern European countries (Harbach, Dahl et al. 1985). Human travel allowed both biotypes of the *Cx pipiens* to makes its way to North America, starting from the temperate northeastern states to eventually making the entire continent their home (Vinogradova 2000). Their ability to adapt to most conditions contributed to the rapid species expansion.

The *Cx. pipiens* taxonomy is highly debated but recent advances in genetic tools and methods have been developed in hopes of better understanding. The WGS physical and genetic maps of *Cx. quinquefasciatus*, provides a reference genome that allows for future genetic studies of the unique traits of these biotypes. With the aid of a reference genome and advancement in genomic technology, the bioytpes' unique preferences for mating behavior, host selection, oviposition sites and other traits can be important targets for vector control. This background information will discuss the geographic dispersal, morphological identification, unique traits focusing especially on mating behavior to understand the factors that contribute to effective vector control programs such as the Sterile Insect Technique (SIT).

Geographic Distribution

While other members of the *Cx. pipiens* complex members are limited geographically, *Cx. pipiens* and *Cx. quinquefasciatus* have dispersed to almost every continent making them the primary vector for WNV (Vinogradova 2000). In North America, *Cx. pipiens* complex mosquitoes are found in both temperate and tropical climates, urban and suburban areas. Specifically, *Cx. pipiens*, also known as the northern house mosquito, are found in the colder northern states whereas *Cx. quinquefasciauts*, also known as the southern house mosquito, are found in the warmer southern states (Vinogradova 2000; Smith and Fonseca 2004). *Cx. pipiens* f. molestus on the other hand are usually found parallel to *Cx. pipiens* populations inhabiting underground areas such as sewers and subways (Harbach et al. 1984; Vinogradova 2000a). All three members are commonly found in urban centers allowing human hosts to be readily available targets.

Although at first glance, there seems to be a division of the northern and southern *Culex* mosquitoes, hybridization zones exist and are prevalent in all parts of North America (Fonseca et al. 2004). Hybridization is especially common between *Cx. pipiens* and *Cx. quinquefasciatus*, but introgression of all three members are found, as evidenced by genetic studies (Bahnck and Fonseca 2006; Fonseca et al. 2004). Indeed, hybridization of the *Culex* mosquitoes favored opportunistic host preference of both avian and human potentially serving as a bridge vector. Additionally, populations showing stronger introgression of *Cx. pipiens* form molestus were more likely to feed on humans posing a threat to higher rates of disease transmission (Kilpatrick, Kramer et al. 2006; Huang, Hamer et al. 2009).

Cx. pipiens Morphology

The proper identification of active vectors is critical for determining the level of risk of disease transmission. In the past, the *Cx. pipiens* complex was mainly distinguished by their morphology including wing shape, thorax, abdominal patterns and the male phallosome ratio (DV/D). However, this was difficult for accurate identification as the standard for these characteristics varied depending on location (Cornel et al. 2003). Also, studies in DV/D ratio of males proved to be unreliable and impossible for identification of females (Rueda, Patel et al. 1990; Dodson, Kramer et al. 2012). In the temperate northern region of the U.S., identification between the two *Cx. pipiens* biotypes are based on key eco-physiological traits such as autogeny: the ability to lay their first batch of eggs without a blood meal. Hybridization between the members of the *Cx. pipiens* complex adds to the frustration of identification due to the intermixing of behavioral and phenotype characteristics (Cornel, McAbee et al. 2003; Sanogo, Kim et al.

2008). Indeed, genetic studies have also shown the varying levels of introgression between parent populations resulting in intermediate hybrids and further complications for identification (Smith and Fonseca 2004).

Biotype-specific Traits

In contrast to their similar morphology, the *Cx. pipiens* complex mosquitoes diverge in their survival strategies in nature. The main trait that differentiates between the two biotypes in particular is the necessity of a blood meal for oviposition (anautogeny). *Cx. pipiens* f. molestus is known to lay their first batch of eggs without the need of a bloodmeal (autogeny). In contrast, *Cx. pipiens* f. pipiens and *Cx. quinquefasciatus* are anautogenous thus requiring a blood meal for the females to oviposit. The *Cx. pipiens* form pipiens mosquitoes are known "bird biters", *Cx. pipiens* f. molestus prefer mammalian hosts for their second batch of eggs and *Cx. quinquefasciatus* are opportunistic between avian and mammals. Aside from host preferences, diapause is another distinct trait that is most likely due to the habitats they live in. The *Cx. pipiens* f. pipiens, or "above ground" mosquitoes, undergo ovarian arrest (diapause) during the winter months while *Cx. pipiens* f. molestus, "underground" mosquitoes, does not overwinter (non-diapause) as they are protected from the harsh elements in sewers and subways for example (Harbach, Harrison et al. 1984). Mating behavior in the *Cx. pipiens* biotypes also attests to the adaptability of their surroundings where *Cx. pipiens* f. molestus mate without nuptial flight (stenogamy) while the other two types of mosquitoes need large open spaces to mate (eurygamy). The last trait is discussed more in depth below.

Mating Behavior

Mosquito biology is critical in developing effective methods for vector control. In particular, genetic control strategies thrive on the basic knowledge of mosquito biology. Mate choice, male competition and female monogamy are all important aspects that contribute to successful mosquito control methods (Koyama, Kakinohana et al. 2004).

Pre-copulatory Elements

The *Culex pipiens* complex mosquitoes have two distinct reproduction strategies namely eurygamy and stenogamy. Eurygamy, or also known as swarming, requires ample space for the mosquitoes to fly around to mate. In swarms, males typically create clusters made of tens to thousands of mosquitoes, around a landmark designated as "the marker" (Downes 1969; Gibson 1985). This phenomenon occurs around dusk or dawn when the lighting is dim. Once this swarm is initiated, the song of the male wing beats attracts females to fly into the aggregation of conspecific males (Warren, Gibson et al. 2009). Male and female contact initially occurs in the swarm where the pair will soon after leave the group of males once copulation occurs (Downes 1969). This behavioral characteristic is common in the field and factors regarding swarms have previously been studied across *Anopheles*, *Aedes* and *Culex* mosquitoes (Charlwood and Jones 1980; Reisen, Knop et al. 1985; Ponlawat and Harrington 2009)

Opposite of eurygamy, some *Culex* mosquitoes mate in small spaces which is called stenogamy. Stenogamous mosquitoes do not need nuptial flight as males and females mate in confined spaces. In contrast to the primary importance of flight tone in eurygamy, previous studies in other insects show olfactory and gustatory factors to play a crucial role in mating behavior where nuptial flight is a secondary behavioral

characteristic (Polerstock, Eigenbrode et al. 2002; Yamamoto and Koganezawa 2013; Diabate and Tripet 2015). The close proximity of interactions involved in stenogmay mating behavior could be supported by the involvement of olfactory and gustatory receptors. Additionally, male wing, clasper size and clasper movement are also thought to effect stenogamy mating ability in other mosquitoes (Wijit et al. 2016).

Post-copulatory Elements

Once mating occurs, other aspects such as insemination rates, fertility and fecundity all play a role in the overall reproductive scheme (South and Catterucia, 2016). Mating molecules created by both females and males collectively contributes to ultimate mating success (Polerstock et al, 2002). In *Anopheles* and *Aedes* mosquitoes, mating plugs from the male causes females to go through a refractory period (Giglioli and Mason 1966). Studies of the male accessory gland and the mating plug have identified steroid hormone 20E and matrone to *Anopheles* and *Aedes* respectively as the primary signal for the decrease in female mating behavior interest (Becker, Petric et al. 2010; Gabrieli, Kakani et al. 2014)). Although, both *Anopheles* and *Aedes* show transference of a mating plug, their content vary and thus is difficult to determine the composition of the mating plug for the *Cx. pipiens* complex. Like *Aedes*, *Culex* mosquitoes have three separate spermathecae where females store the sperm (Pascini et al, 2012). This is thought to exist for polyandrous insects where multiple sperm storage space means a complex post-copulatory selection compared to pre-copulatory selection (South and Catterucia, 2016). Overall, knowledge of both pre and post-copulatory elements will contribute to the success of vector control strategies.

Sterile Insect Technique

Considering that there are limited methods for the control of mosquitoes, innovative tools needs to be developed. Currently, vaccines and drugs that specifically target vector borne diseases are still underdeveloped (Kules, Horvatic et al. 2016). Insecticides are widely used for both adult and larvae mosquitoes, but resistant population to these conventional tools are difficult to keep up with (McGraw and O'Neill 2013). Aside from resistance, biological methods can cause environmental damage and health concerns in the long run creating other problems while trying to solve one. These reasons recently prompted interest in alternative methods that are environmentally friendly.

The sterile insect technique (SIT) method is an alternative environmentally safe method of insect control that has shown to be successful in various field studies (Knipling 1955; Krafsur 1998; Dyck et al., 2005a). Success in using this method on agricultural pest insects were shown in the early 1950's till the 1980's and have gained interest in mosquitoes due to advances in genetic technology that improves on the current SIT method (Knipling 1955; Handler 2002; Coleman and Alphey 2004). Indeed, release of sterile males in malaria vector *Anopheles* and the dengue vector *Aedes* have had some success in the field (Phuc, Andreasen et al. 2007; Galizi, Doyle et al. 2014). SIT mass produces sterile males that have undergone ionizing radiation to be released to mate with wild females. Mated field females will experience a decrease in their reproductive capabilities thus controlling the vector population (Alphey et al., 2010). Although in theory, the SIT program seems straightforward, challenges for an effective SIT program

exist including: accurate sexing ability and understanding of species specific mating behavior.

Development of genetic sexing strains is a key limiting factor of SIT (Gilles, Schetelig et al. 2014). Mass production of males require proper egg production and sex separation preferably at an early developmental stage reducing the cost and labor that goes into supporting the foundation of a SIT program. Another key underlying factor that is needed for this technique is the understanding of mating behavior phenotypes and their corresponding genotype. This is due to the likelihood of genetic mutation effects of the sterile males (Brown, Alphey et al. 2014). Since SIT is species specific, each species of mosquito will likely exhibit varying mate competitiveness that will contribute to the success of this method (Alphey, Benedict et al. 2010). Previous efforts of sterile mosquito release have shown a decrease in male mating fitness due to irradiation (Harris, McKemey et al. 2012). Other times, high numbers are not as effective as smaller releases over a long period of time (Harris, McKemey et al. 2012). Factors that go into the SIT program all goes back to basic mosquito species specific mating biology. Advances in modern technology and continued efforts of mating behavioral studies, steps to improving the efficacy and efficiency of the SIT seems possible for the *Culex pipiens* complex.

CHAPTER TWO

Isolation and Characterization of SNP Markers in the WNV Vector *Culex pipiens* Complex

Abstract

The *Culex pipiens* complex is one of the most important mosquito vectors for the spread of diseases including lymphatic filariasis and the West Nile virus. Vector competence, host preference, mating behavior and diapause are important traits to understand the disease transmission cycle between host and vector. Elucidation of a link between genes and these traits may provide a novel way for both suppressing these vectors and blocking pathogen transmission. The low number of genetic markers is one of the key obstacles in performing genetic studies in *Cx pipiens* complex. Single nucleotide polymorphisms (SNPs) are one of the most abundant variations available and have been widely used as molecular markers for genetic studies. We selected candidate regions among the largest 100 supercontigs in size ranging from 3.08Mb to 939Kb from *Culex quinquefasciatus* genome, a sister species of *Culex pipiens*. Primers were designed in exons flanking the introns of the candidate genes. Genomic DNA was then extracted from twelve females of *Cx. pipiens* form *pipiens*, originally from Columbus, Ohio and *Cx. pipiens* form *molestus*, from Chicago, Illinois and was amplified by PCR separately. SNPs were then characterized from the sequence traces of the PCR products, providing thirty SNP-based markers for future studies on *Culex pipiens* complex.

Keywords: *Culex pipiens* f. molestus, *Culex pipiens* f. pipiens, *Culex quinquefasciatus*, single nucleotide polymorphisms, genetic markers, vector control, *Culex* biotypes

Introduction

The *Culex pipiens* complex mosquitoes are among the most widespread and important vectors of West Nile Virus, St. Louis encephalitis virus and other diseases (Monath 1988; Fonseca, Keyghobadi et al. 2004; Hamer, Kitron et al. 2008). Within *Cx. pipiens* complex there are two biotypes that differ mainly in behavior and are not reliably distinguishable through morphological differences (Mattingly 1967; Spielman 1967). These two biotypes include *Culex pipiens* f. molestus and *Culex pipiens* f. pipiens. To name a few key differences concerning pathogen transmission, *Culex pipiens* f. molestus does not undergo diapause, lays eggs without a blood meal (autogeneous), and mates in confined spaces (stenogamous) (Harbach, Harrison et al. 1984). On the other hand, *Culex pipiens* f. pipiens goes through diapause to overwinter, lays eggs only after a blood meal (anautogeneous) and needs extensive space for mating (eurygamous) (Harbach, Dahl et al. 1985). These biotypes are considered sympatric although *Cx. pipiens* f. molestus mostly inhabits underground areas in cities while *Cx. pipiens* f. pipiens resides above ground creating distinct ecological niches (Byrne and Nichols 1999; Huang, Molaei et al. 2008). Additionally, a host-feeding study of *Cx. pipiens* complex showing preference for various species including mammalian human blood, emphasizes the need for accurate methods to not only distinguish the two forms, but to further study the distinctive traits of the two biotypes (Savage and Kothera 2012). These traits are important factors that may determine the outcome of disease transmission to the human population (Savage, Aggarwal et al. 2007).

So far there has been relatively little work done on the genetic basis of these traits; the development of informative genetic and molecular markers is an important first

step. Many genetic marker development studies such as isolating microsatellite and RFLP markers in different organisms have been studied, (Hinomoto, Higaki et al. 2006; Nakahara, Kobashigawa et al. 2008) but dense and informative SNP marker development is still lacking even though it serves to be valuable due to its high throughput capabilities. Microsatellite markers of *Cx. pipiens* complex including form pipiens and form molestus are also reported (Mori, Severson et al. 1999; Bahnck and Fonseca 2006; Hickner, Mori et al. 2013) which includes comparative microsatellite studies with other species of mosquitoes (Mori, Severson et al. 1999; Hickner, Debruyn et al. 2010). Studies using single nucleotide polymorphisms (SNP) markers have also been initiated to identify *Cx. pipiens* complex species using methods such as high-resolution melting analysis (Kang and Sim 2013) for ecotype comparison (Lee, Seifert et al. 2012; Arthofer, Bertini et al. 2015). Many mosquito strains, including *Cx. pipiens*, have been sequenced in pursuit of SNP based identification methods to help clarify evolutionary mechanisms (Engdahl, Larsson et al. 2013). These studies have helped establish a genetic basis behind phenotypic differences thus far by quantitative trail loci (QTL) mapping in various organisms and will provide useful genetic markers for future studies (Saavedra-Rodriguez, Strode et al. 2008; Veyrieras, Kudaravalli et al. 2008; Edwards and Mackay 2009).

In this study, we characterized a set of SNP markers from 30 loci obtained from both biotypes in *Cx. pipiens* complex mosquitoes as an addition to the marker development for this mosquito complex. These biotype-specific SNPs may provide informative resources for high-resolution genetic mapping and QTL analysis to link between genes and biotype-specific traits from *Culex pipiens* complex.

Materials and Methods

Mosquito Colony

The insectary chamber for *Cx. pipiens* f. pipiens was set at 26°C with 75% relative humidity. The daily light cycle for the chamber was set for a 15 h light: 9 h dark (L:D) rotation. Larvae were given Tetramin fish food (Tetra holding Inc., Blacksburg, VA, USA) and were kept in de-chlorinated water through their pupa stage. Adults were fed diluted honey absorbed by cotton balls and were kept in large screened cages with dimensions 22×26×24 inches. The adult females were fed chicken blood using a Hemotek feeding apparatus for 1 h (Discovery Workshops, Accrington, Lancashire, UK). The colony was first established in 2000, from Columbus, Ohio and was transferred over from Dr. David L. Denlinger's lab at the Ohio State University.

Much like *Cx. pipiens* f. pipiens, the insectary chamber conditions for *Cx. pipiens* f. molestus were as follows: 25°C and 75% relative humidity under a 15 h light: 9 h dark (L:D) daily light cycle. Adults were also fed diluted honey partially absorbed by cotton balls, but the larvae were fed Tetramin fish food mixed in with liver powder (MP Biomedicals, LLC, Solon, OH, USA) in a 4:1 ratio. The colony was kept in smaller screened cages with dimensions 10×14×12 inches to mimic its original underground population from the Calumet Water Reclamation Plant in Chicago. Although it originated from Chicago, the main colony of *Cx. pipiens* form molestus that we have here at Baylor University was provided by Dr. Linda Kothera's lab at the Centers for Disease Control and Prevention Division of Vector-Borne Infectious Diseases in Fort Collins, Colorado.

SNP Discovery and Validation

Based on the genome of *Cx. quinquefasciatus*, a sister species, candidate genes for SNP selection were picked from among the largest 100 supercontigs. The supercontigs for *Cx. quinquefasciatus* were selected by size that ranged from 3.08Mb to 939Kb. These candidate genes were obtained from Vectorbase (http://cquinquefasciatus.vectorbase.org/) and Primer3 (http://frodo.wi.mit.edu/primer3/) was used to design primers for each candidate gene.

Genomic DNA was extracted from 10 females of each biotype, *Cx. pipiens* f. pipiens, and *Cx. pipiens* f. molestus, using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the protocol in the kit. Once the purity of the genomic DNA was measured against the elution buffer on a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) at 260/280 nm, samples were amplified by polymerase chain reactions (PCR) using the Taq PCR Kit (New England BioLabs, Ipswich, MA). 50 µl PCR amplifications were assayed utilizing 200 µM dNTPs, 10 x PCR buffer, 2 mM MgSO4, 10 mM primers and 10 ng of gDNA. Amplifications were then performed on a T100 thermal cycler (Bio-Rad, Hercules, CA) with an initial denaturation at 94°C for 2 minutes, followed by 39 cycles at 94°C for 15 seconds, 50°C for 15 seconds, 72° for 45 seconds, then one cycle of 72°C for 5 minutes. PCR products and a negative control were then visualized on 2% agarose with a 100 bp molecular weight ladder (Invitrogen, Carlsbad, CA). Amplified PCR products were purified using two different cleaning protocols, including ExoSAP-IT (GE Healthcare, Little Chalfont, UK) or the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) removing unincorporated primers, nucleotides and salts before being sent to MacrogenUSA Inc. (Maryland) for direct DNA

sequencing. The SNPs found were further validated by resequencing using reverse primers. Each candidate gene was sequenced at least twice.

Bioinformatics Analysis

SNPs were identified by analyzing sequences of the two biotypes first aligned with *Cx. quinquefasciatus* by using the assemble sequences function of CLC Main Workbench 6 (CLC bio, Aarhus, Denmark). Once assembled, the sequences of the two biotypes aligned against each other were then manually checked for SNPs to validate each SNP location. Candidate SNP positions were examined between members of *Culex pipiens* complex. These validated SNPs were also positioned on the relative locations of each chromosome, which is estimated from the genetic map of *Culex pipiens* constructed in previous studies (Naumenko, Timoshevskiy et al. 2015).

SNPs were then categorized into transitions or transversions in coding and non-coding regions. The coding region SNPs were subdivided further as synonymous or non-synonymous and their codon position was determined. The nucleotide diversity analyses were done by using the DnaSP 5.0 program (Librado and Rozas 2009). Some other calculations for further analysis of SNPs include: the average number of nucleotide substitutions per site between two sequences from two biotypes (π), the average number of synonymous substitutions per synonymous site (Ks) and nonsynonymous substitutions per nonsynonymous site (Ka) (Librado and Rozas 2009).

Results

Gene Amplification

Thirty loci out of 100 primer pairs that were tested by PCR showed amplification and were used for SNP identification. The PCR amplicon location information from the reference genome of *Cx. quinquefasciastus* is shown in Table 2.1. The sizes of PCR products from the thirty-selected primer pairs in the study were 220-498 bp. There was a total of 5,646 bp of coding region and 2,132 bp of non-coding region to make up of a total of 7,778 bp sequenced. Out of the 7,778 bp sequenced, 253 SNPs were identified ranging from 1-30 SNPs per amplicon. The data given here concerning the SNPs can be accessed in NCBI and their ss (submitted SNP) numbers are ss947848635 - ss947848878.

Table 2.1. PCR amplicon location information from the *Culex* genome

Gene ID	Accession no	Function	Forward primer	Reverse primer	Expected fragment size	SNPs
CPIJ000123	947848635-947848637	pyrroline-5-carboxylate reductase	GGCCAGTGATGGAAAGTTGT	AAGTTTACCCGAACCAAGCA	456	3
CPIJ000290	947848638-947848648	hypothetical protein	TGTCAACATCGATTGCCTGT	AGACTCCACATTCGCTGCTT	447	11
CPIJ000408	947848649	hypothetical protein	CAAGGAGGTAGTCGGAGCAG	TACGAGGGACTTGAGCGATT	374	1
CPIJ000494	947848650-947848652	hypothetical protein	CATCCTCCTGCAATCCATTT	TCGCTGAAGTTCTCCGTACA	483	3
CPIJ000595	947848653-947848656	hypothetical protein	CCTTACCTGCCCTCTCTTCC	GAGTTCGAGCGCTGAGATTT	432	4
CPIJ000874	947848657-947848674	carbohydrate sulfotransferase	ACCCGTACAAAACCACCAAA	ACGTCATCAGCGGGTTAAAG	340	18
CPIJ000778	947848675-947848683	phospholipase c	AGGACATGGAACCAATCGAG	GTGTGTCCTTTGCTGTCGTG	441	9
CPIJ001132	947848684-947848692	elongation factor 2	CTACGGTGTGCTCAATCGTC	TACGTCATCGTGCAGGACAT	487	11
CPIJ001573	947848693-947848695	hypothetical protein	GCTGCTCTACCCAAAGCAAT	ACAGGTTGAGTTGTACCCGC	498	3
CPIJ001336	947848696-947848708	rRNA large subunit methyltransferase J	CTAAACGATCCCTTCGTGGA	TTTGTTATTCGGTGTTGCGA	480	13
CPIJ001674	947848709-947848715	exocyst complex component 2	TGTGCTGTCTCTCGAATTGC	AAAAGTTCAACGTGAACGGC	476	7
CPIJ002017	947848716-947848738	cell adhesion molecule	CGATTTCAGTGCGATTCTGA	GTAATGTTCATCCGGGCACT	410	23
CPIJ801721	947848739-947848740	-	TCTACCTTCCGGTGTTCCAC	GGACGTCCTCAGTGTTGGAT	475	2
CPIJ801769	947848741-947848742	-	AATTGGTCCAGGATCAGACG	AAGACCCAATCCGAAGACCT	413	2
CPIJ801866	947848743-947848759	-	ATCTCAGAACGAACCGATGG	GAGCGTGGGATGGAAATCTA	402	17
CPIJ002361	947848760	sodium/solute symporter, putative	CAACCGGGATGATTGTCTCT	TAAATGGCCGGATGTAGGAG	456	1
CPIJ003251	947848761-947848764	hypothetical protein	GAACCGGTCAAGATTCGAGA	CACCCTGACCGTAATTTGCT	457	4
CPIJ003297	947848765-947848765	bhlhzip transcription factor max/bigmax	CGTGACCACATCAAGGACAG	CTGCTGGTGGGAGTTGTTCT	220	1
CPIJ003806	947848766-947848768	hypothetical protein	AGCTACTCGATTCCGCACAC	GCTGCTGAAGGTAGGTCGTC	496	3
CPIJ003693	947848769-947848772	glucosyl/glucuronosyl transferases	GTGGCAAATCCCATCACTTT	GTTGAAGTGCTGCGCCAAC	418	4
CPIJ004052	947848773-947848780	prospero	AGAAGTACGCCCGCCAGT	CCAGGAAGTTGGGAGACTTG	404	8
CPIJ004085	947848781-947848794	adenylate cyclase, putative	TCGACATCGTCAACGAGAAG	GCTGAACTCGACCTCACTCC	480	14
CPIJ004120	947848795-947848824	huntingtin	ATCAAGGACGGTGACTTTCG	AAAGCTGTTCCGGTGTGTTC	461	30
CPIJ802176	947848825-947848829	-	CAGTGGCTCGTTCCAGTGTA	GAGTGGCGGAGTTCAAATGT	429	5
CPIJ802210	947848830-947848841	-	GACAACGTCGAGTCGATCTG	GTTCTGCCTGCTCAACCTTC	465	12
CPIJ004636	947848842-947848845	para-nitrobenzyl esterase	ATTGGAGGACATCGCTTCAC	GGCTGTTGTACTGGGCTGAT	464	4
CPIJ802260	947848846-947848866	-	TGGACTTCACCATACTAATTCTA ACC	TACAGAATCGCGTCTGTGCT	407	22
CPIJ005102	947848867-947848869	voltage-dependent p/q type calcium channel	AAAAATGGGCCCCAAAAAG	CACCACGAAAAAGTCCATCA	441	3

CPIJ005487	947848870-947848877	tubulin gamma-1 chain	CGAAATCATCACCCTCCAG	TTAGCATACGGCGAGGTCAT	305	8
CPIJ005773	947848878	actin binding	ACCAAGCTGGACAAGCTGAC	TATTTGGTGGATGCGACGTA	413	1

Table 2.2. Synonymous and non-synonymous polymorphisms in coding and non-coding region regarding distribution of transitions (Ts) and transversions (Tv).

Coding		
Synonymous	#	%
Ts	50	46.7
Tv	22	20.6
Non Synonymous	#	%
Ts	15	14.0
Tv	20	18.7
Non-Coding		
Ts	61	47.3
Tv	68	52.7

Polymorphism Classification and Characteristics

Categorization of synonymous and non-synonymous polymorphisms in the coding and non-coding region regarding the distribution of transitions (Ts) and transversions (Tv) is shown in Table 2.2. The coding region had an overall larger number of Ts compared to the number of Tv. Out of a total of 106 SNPs found in the coding region, 65 were transitions (61.3%) and 41 were transversions (38.6%). For the non-coding regions, there were 61 (41.4%) transitions, 68 (46.2%) transversions and 18 indels (12.24%) out of a total of 147 polymorphisms. The frequency of transitions between coding and non-coding region were significantly different (60.7% vs. 47.3%, $\chi^2 = 4.26$, P=0.02). The results in Table 2.3 showed no significant difference between the frequency of transversions at fourfold degenerate codon positions and at non-coding regions (67.9% vs. 52.7%, $\chi^2 = 2.13$, P = 0.05).

Table 2.3. Transition (Ts) and transversion (Tv) polymorphisms for different classes of DNA

	Polymorphism			Probability	
	Ts	Tv	%Tv	Coding Region	Fourfold
Non-coding regions	61	68	52.70%	P = 0.02	P = 0.05
Coding regions	65	42	39.30%		
Wobble Position	51	19	27.10%		
Fourfold degenerate sites	9	19	67.90%		

In the non-coding region, transversions slightly outnumber transitions. Transitions C/T (35 %) and A/G (30%) in the coding region were most abundant compared to the transversion possibilities of A/C, A/T, C/G, and G/T (Figure 2.1). The frequency of transitions in the coding was 61.32% and non-coding region was 41.49%. Overall, SNPs were found more abundantly as transitions vs. transversions and transition SNPs were

more commonly found in coding regions rather than non-coding (Table 2.2). There was a higher probability that the SNPs are located at the third codon position (72.5%), which results in mostly silent substitutions compared to the 1st (15.1%) and 2nd (14.15%) position mutations resulting in nonsynonymous change.

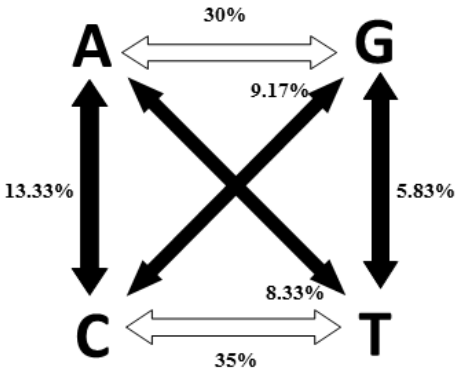

Figure 2.1. Percentages of transitions and transversions found in SNPs.

Eighteen insertion/deletion (indels) polymorphisms were found dispersed in ten genes and were all located in the non-coding intron region. There were no indel polymorphisms found in the coding region. Synonymous substitutions were found to be mostly transitions rather than transversions. Approximately 67 % of synonymous substitutions (71/106) were found in the coding-region while 33% were nonsynonymous (35/106) (Table 2.2). All SNPs identified in this study were unique SNPs between *Cx. pipiens* f. pipiens and *Cx. pipiens* f. molestus aligned against each other. Transition (Ts) and transversion (Tv) polymorphisms for the two *Culex pipiens* complex species are shown in Supplementary Table 2.1.

Supplemental Table 2.1. Nucleotide polymorphism in *Culex pipiens* gene (f. *pipiens* vs. f. *molestus*)

		Coding																		Non-Coding					
			Codon Polymorphic Position																						
			Transition				Transversion				# Polymorphism Types				Nucleotide Diversity				Polymorphism					Nucleotide Diversity
Gene ID	nHap	L (bp)	1st	2nd	3rd	Total	1st	2nd	3rd	Total	Syn	Nonsyn	Indel	Total	π	πn	Ks	Ka	L (bp)	Ts	Tv	Indel	Total	π
CPIJ000123	2	283	0	0	0	0	0	0	0	0	0	0	0	0	0.0000	0.0000	0.0000	0.0000	80	2	0	1	3	0.0253
CPIJ000290	2	159	0	0	2	2	1	1	2	4	3	3	0	6	0.0440	0.0266	0.0731	0.0271	134	3	2	0	5	0.0299
CPIJ000408	2	91	0	0	0	0	0	0	0	0	0	0	0	0	0.0000	0.0000	0.0000	0.0000	57	0	1	0	1	0.0175
CPIJ000494	2	215	0	0	0	0	1	0	0	1	0	1	0	1	0.0000	0.0063	0.0000	0.0063	52	1	2	0	3	0.0769
CPIJ000595	2	64	0	0	0	0	0	0	1	1	1	0	0	1	0.0156	0.0000	0.0751	0.0000	46	1	1	0	2	0.0435
CPIJ000874	2	134	0	0	2	2	0	0	1	1	3	0	0	3	0.0224	0.0000	0.1051	0.0000	41	7	8	1	16	0.4000
CPIJ000778	2	214	1	0	4	5	1	0	2	3	5	3	0	8	0.0374	0.0160	0.1102	0.0162	79	0	0	0	0	0.0000
CPIJ001132	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0000	0.0000	0.0000	0.0000	240	4	5	2	11	0.0556
CPIJ001573	2	171	0	1	0	1	0	0	1	1	1	1	0	2	0.0117	0.0077	0.0267	0.0077	47	0	1	0	1	0.0213
CPIJ001336	2	326	2	0	4	6	0	1	4	5	9	2	0	11	0.0337	0.0083	0.1185	0.0083	27	1	2	0	3	0.1111
CPIJ001674	2	289	0	0	6	6	0	0	0	0	6	0	0	6	0.0208	0.0000	0.0883	0.0000	37	0	1	0	1	0.0270
CPIJ002017	2	206	1	0	6	7	0	1	2	3	7	3	0	10	0.0485	0.0130	0.1793	0.0131	65	9	5	0	14	0.2154
CPIJ801721	2	156	0	0	0	0	0	0	0	0	0	0	0	0	0.0000	0.0000	0.0000	0.0000	93	2	0	0	2	0.0215
CPIJ801769	2	236	0	0	0	0	0	0	0	0	0	0	0	0	0.0000	0.0000	0.0000	0.0000	56	0	1	0	1	0.0179
CPIJ801866	2	279	0	0	4	4	1	0	1	2	6	0	0	6	0.0215	0.0000	0.0922	0.0000	58	6	4	0	10	0.1724
CPIJ002361	2	286	1	0	0	1	0	0	0	0	0	1	0	1	0.0000	0.0047	0.0000	0.0047	63	0	0	0	0	0.0000
CPIJ003251	2	266	0	0	1	1	0	1	1	2	2	1	0	3	0.0113	0.0050	0.0328	0.0050	0	0	0	0	0	0.0000
CPIJ003297	2	85	0	0	0	0	0	0	0	0	0	0	0	0	0.0000	0.0000	0.0000	0.0000	19	0	1	0	1	0.0526
CPIJ003806	2	281	0	0	2	2	0	0	1	1	3	0	0	3	0.0356	0.0111	0.0887	0.0112	22	0	0	0	0	0.0000
CPIJ003693	2	102	0	0	1	1	0	0	1	1	1	1	0	2	0.0196	0.0129	0.0480	0.0130	49	1	1	0	2	0.0408
CPIJ004052	2	17	0	0	0	0	0	0	0	0	0	0	0	0	0.0000	0.000	0.0000	0.0000	95	1	6	1	8	0.1290
CPIJ004085	2	109	0	0	0	0	0	0	0	0	0	0	0	0	0.0000	0.0000	0.0000	0.0000	233	7	5	2	14	0.0606
CPIJ004120	2	288	2	3	5	10	3	3	4	10	9	11	0	20	0.0694	0.0500	0.1526	0.0518	64	3	6	1	10	0.1429
CPIJ802176	2	20	0	0	0	0	0	0	0	0	0	0	0	0	0.0000	0.0000	0.0000	0.0000	116	3	2	0	5	0.0517
CPIJ802210	2	290	1	1	2	4	1	2	2	5	2	7	0	9	0.0310	0.0266	0.0494	0.0271	65	1	1	1	3	0.0313
CPIJ004636	2	270	0	1	3	4	0	0	0	0	3	1	0	4	0.0148	0.0049	0.0478	0.0049	7	0	0	0	0	0.0000
CPIJ802260	2	256	0	0	5	5	0	0	1	1	6	0	0	6	0.0234	0.0000	0.0976	0.0000	72	7	6	5	18	0.1940
CPIJ005102	2	292	0	0	2	2	0	0	0	0	2	0	0	2	0.0069	0.0000	0.0287	0.0000	74	1	4	1	6	0.0685
CPIJ005487	2	54	0	0	1	1	0	0	0	0	1	0	0	1	0.0370	0.0000	0.2012	0.0000	65	1	3	3	7	0.0656
CPIJ005773	2	207	0	0	1	1	0	0	0	0	1	0	0	1	0.0145	0.0000	0.0195	0.0000	76	0	0	0	0	0.0000
Total:		5646	8	6	51	65	8	9	24	41	71	35	0	106					2132	61	68	18	147	

Nucleotide Diversity

Nucleotide diversity was calculated for both coding and non-coding regions separately (Supplementary Table 2.1). The average nucleotide diversity (π) in the coding region was 0.0506 and in the non-coding region was 0.0691. The nucleotide diversity for the non-coding region was slightly higher than the coding region. The rate of synonymous substitution per synonymous site (Ks) and the rate of non-synonymous nucleotide substitution per nonsynonymous site (Ka) were calculated for the SNPs identified for *Cx. pipiens* (Supplementary Table 2.1). The overall average Ks for the coding region was 0.0545 and the average Ka was 0.0066. The Ka value was much lower than the Ks.

Discussion

This study provides valuable information to address the current lack of high-quality genomic sequences for a *Cx. pipiens* complex mosquito vector that is fully integrated with genetic and physical maps. This is one of the hurdles to the translation of genomic data into solutions for the control of vector-borne diseases. In this discussion we point out the notable differences and similarities in type of polymorphism, genetic diversity, and clustering patterns among the mosquito species including *Cx. pipiens* complex, *An. gambiae*, *Ae. Aegypti* as well as *D. melongaster*. All biotype-specific SNPs have been submitted to the NCBI SNP Database (ssSNP accession nos: ss947848635 - ss947848878).

The frequency of transitions in the coding region was 64.16% and non-coding was 40.49%. SNP transitions were more commonly found in the coding regions rather than non-coding regions (Table 2.2). The difference between transition polymorphisms in

the coding region compared to the non-coding region is significantly different (60.7% vs 47.3%, $\chi^2 = 4.26$, P=0.02). This might be due to the degeneracy of the genetic code and stronger selective pressure for synonymous substitutions to maintain proper gene function (Holliday and Grigg 1993; Moriyama and Powell 1996). The similar frequency of transversions at fourfold degenerate codon positions and at non-coding regions implies the fourfold degenerate codon is under neutral selection (Table 2.3). Although some functions of the genes are currently unknown, others are involved in well-known functions such as translation elongation, cell signaling, cell adhesion and catalytic enzymes that are all highly conserved in most organisms and can be potential markers for the genetic studies (Table 2.1).

Synonymous substitutions were mostly transitions rather than transversions. Roughly 67% of coding-region SNPs were synonymous substitutions (71/106) and the other 33% were nonsynonymous (35/106) which suggest a strong selection in the coding region. With a higher number of synonymous sites to nonsynonymous sites, one might assume selection strength is the only factor connected with the ratio. However, aside from positive selection and biased gene conversion, saturation may also be associated with this uneven ratio (Lartillot 2013). In contrast to previous speculation, synonymous sites themselves showed strong selective constraints which correlate with gene expression and are also important for developmental processes (Lawrie, Messer et al. 2013).

The comparison of Ka/Ks ratio shows purifying selection efficiency against deleterious mutations assuming that most coding areas have strong selective criterions for non-synonymous changes compared to synonymous ones. The rate of non-synonymous nucleotide substitution per non-synonymous site (Ka) was expected to be smaller than the

rate of synonymous substitution per synonymous site (Ks) in the coding region since most synonymous mutations are silent or selectively neutral (King and Jukes 1969). In the coding region, the Ka/Ks ratio between *Cx. pipiens* form pipiens *and Cx. pipiens* form molestus was 0.121. This was lower than the Ka/Ks ratios found in other mosquitoes like *An. gambiae* 0.192 (Morlais, Poncon et al. 2004) and *Ae. aegypti* 0.204 (Morlais and Severson 2003) but slightly higher than *D. melanogaster* 0.115 (Moriyama and Powell 1996). The Ka/Ks ratio of the biotypes compared to other Dipteran insects suggests similar purifying selection patterns.

The frequency of indels between the two *Culex* forms, 7%, was lower than those of *Ae. aegypti* 24% (Morlais and Severson 2003) and *An. gambiae* 25% (Morlais, Poncon et al. 2004). Despite the lower percentage in *Culex* species, these indels can be utilized as alternative genetic markers to SNPs for characterization of Culex genomes. Indels are important because of purifying selection acting on them like SNPs in coding regions (Montgomery, Goode et al. 2013). The frequency of indels between f. molestus vs. *Cx. quinquefasciatus* and f. pipiens vs. *Cx. quinquefasciatus* were 13% and 25% respectively (Supplementary Table 2 and 3). These two figures were closer to the percentages found for *An. gambiae* and *Ae. Aegypti* compared to the frequency of indels between the two *Culex* forms. This points to a similar selective pressure for indels to occur in the *Culex* mosquito genome. Although indels and their precise interaction with known SNP loci and their characteristics is not well understood, studies have shown that indels found in the protein coding regions may have a causal relationship and thus should not be disregarded (Montgomery, Goode et al. 2013).

The average nucleotide diversity for the noncoding regions was higher than the average for the synonymous sites of the coding region (0.0691 average nucleotide diversity and 0.0545 for the synonymous sites, $P < 0.01$). A different trend was observed in *An. gambiae*, *Ae. aegypti* and *Drosophila,* where it might indicate a greater purifying selection for the coding region compared to the noncoding region. However, it is noteworthy that the noncoding regions could be involved in gene regulation resulting in purifying selection (Shen, Basilion et al. 1999).

Recent mitotic chromosome based physical mapping on the southern house mosquito (Naumenko, Timoshevskiy et al. 2015) allowed for SNP loci present in this experiment to be added (Figure 2.2). The larger two chromosomes provide interesting comparisons between the three major types of mosquitoes: *Culex, Aedes* and *Anopheles*. Comparative linkage maps show whole arm translocation of chromosome 2 and 3 in *Aedes* and *Culex* mosquitoes (Mori, Severson et al. 1999). Markers in these areas have potential benefits to chromosome homology and evolution studies between species. Unfortunately, the most conserved chromosome between mosquito species, chromosome 1, had no added SNP loci.

The SNPs identified in this manuscript provide informative molecular markers in *Culex pipiens* complex. The two biotypes show unique phenotypic differences relevant to disease transmission such as diapause, stenogamy/eurygamy and anautogeny/autogeny. Thus, new sets of markers based on biotype-specific SNPs in *Cx. pipiens* complex mosquitoes are valuable for future genetic studies.

References

Arthofer, W., L. Bertini, et al. (2015). "Genomic Resources Notes accepted 1 February 2015 - 31 March 2015." Molecular ecology resources 15(4): 1014-1015.

Bahnck, C. M. and D. M. Fonseca (2006). "Rapid assay to identify the two genetic forms of Culex (Culex) pipiens L. (Diptera: Culicidae) and hybrid populations." The American journal of tropical medicine and hygiene 75(2): 251-255.

Byrne, K. and R. A. Nichols (1999). "Culex pipiens in London Underground tunnels: differentiation between surface and subterranean populations." Heredity 82 (Pt 1): 7-15.

Edwards, A. C. and T. F. C. Mackay (2009). "Quantitative Trait Loci for Aggressive Behavior in Drosophila melanogaster." Genetics 182(3): 889-897.

Engdahl, C., P. Larsson, et al. (2013). "Identification of Swedish mosquitoes based on molecular barcoding of the COI gene and SNP analysis." Molecular ecology resources.

Fonseca, D. M., N. Keyghobadi, et al. (2004). "Emerging vectors in the Culex pipiens complex." Science 303(5663): 1535-1538.

Hamer, G. L., U. D. Kitron, et al. (2008). "Culex pipiens (Diptera: Culicidae): a bridge vector of West Nile virus to humans." Journal of medical entomology 45(1): 125-128.

Harbach, R. E., C. Dahl, et al. (1985). "Culex (Culex) Pipiens-Linnaeus (Diptera, Culicidae) - Concepts, Type Designations, and Description." Proceedings of the Entomological Society of Washington 87(1): 1-24.

Harbach, R. E., B. A. Harrison, et al. (1984). "Culex (Culex) Molestus Forskal (Diptera, Culicidae) - Neotype Designation, Description, Variation, and Taxonomic Status." Proceedings of the Entomological Society of Washington 86(3): 521-542.

Hickner, P. V., B. Debruyn, et al. (2010). "Genome-based microsatellite development in the Culex pipiens complex and comparative microsatellite frequency with Aedes aegypti and Anopheles gambiae." PloS one 5(9).

Hickner, P. V., A. Mori, et al. (2013). "Composite Linkage Map and Enhanced Genome Map for Culex pipiens Complex Mosquitoes." Journal of Heredity 104(5): 649-655.

Hinomoto, N., T. Higaki, et al. (2006). "Genetic diversity in field and commercial populations of Orius strigicollis (Poppius) (Heteroptera : Anthocoridae) measured by microsatellite markers." Applied Entomology and Zoology 41(3): 499-506.

Holliday, R. and G. W. Grigg (1993). "DNA methylation and mutation." Mutation research 285(1): 61-67.

Huang, S. M., G. Molaei, et al. (2008). "Genetic insights into the population structure of Culex pipiens (Diptera : Culicidae) in the northeastern United States by using microsatellite analysis." American Journal of Tropical Medicine and Hygiene 79(4): 518-527.

Kang, D. and C. Sim (2013). "Identification of Culex complex species using SNP markers based on high-resolution melting analysis." Molecular ecology resources 13(3): 369-376.

King, J. L. and T. H. Jukes (1969). "Non-Darwinian evolution." Science 164(3881): 788-798.

Lartillot, N. (2013). "Interaction between selection and biased gene conversion in mammalian protein-coding sequence evolution revealed by a phylogenetic covariance analysis." Molecular biology and evolution 30(2): 356-368.

Lawrie, D. S., P. W. Messer, et al. (2013). "Strong purifying selection at synonymous sites in D. melanogaster." PLoS genetics 9(5): e1003527.

Lee, Y., S. N. Seifert, et al. (2012). "High Degree of Single Nucleotide Polymorphisms in California Culex pipiens (Diptera: Culicidae) sensu lato." Journal of Medical Entomology 49(2): 299-306.

Librado, P. and J. Rozas (2009). "DnaSP v5: a software for comprehensive analysis of DNA polymorphism data." Bioinformatics 25(11): 1451-1452.

Mattingly, P. F. (1967). "The systematics of the Culex pipiens complex." Bulletin of the World Health Organization 37(2): 257-261.

Monath, T. P. (1988). "Japanese encephalitis--a plague of the Orient." The New England Journal of Medicine 319(10): 641-643.

Montgomery, S. B., D. L. Goode, et al. (2013). "The origin, evolution, and functional impact of short insertion-deletion variants identified in 179 human genomes." Genome research 23(5): 749-761.

Mori, A., D. W. Severson, et al. (1999). "Comparative linkage maps for the mosquitoes (Culex pipiens and Aedes aegypti) based on common RFLP loci." The Journal of Heredity 90(1): 160-164.

Moriyama, E. N. and J. R. Powell (1996). "Intraspecific nuclear DNA variation in Drosophila." Molecular biology and evolution 13(1): 261-277.

Morlais, I., N. Poncon, et al. (2004). "Intraspecific nucleotide variation in Anopheles gambiae: new insights into the biology of malaria vectors." The American journal of tropical medicine and hygiene 71(6): 795-802.

Morlais, I. and D. W. Severson (2003). "Intraspecific DNA variation in nuclear genes of the mosquito Aedes aegypti." Insect molecular biology 12(6): 631-639.

Nakahara, S., Y. Kobashigawa, et al. (2008). "Genetic variations among and within populations of the Oriental fruit fly, Bactrocera dorsalis (Diptera; Tephritidae), detected by PCR-RFLP of the mitochondrial control region." Applied Entomology and Zoology 43(3): 457-+.

Naumenko, A. N., V. A. Timoshevskiy, et al. (2015). "Mitotic-Chromosome-Based Physical Mapping of the Culex quinquefasciatus Genome (vol 10, e0115737, 2015)." PloS one 10(6).

Saavedra-Rodriguez, K., C. Strode, et al. (2008). "Quantitative trait loci mapping of genome regions controlling permethrin resistance in the mosquito Aedes aegypti." Genetics 180(2): 1137-1152.

Savage, H. M., D. Aggarwal, et al. (2007). "Host choice and West Nile virus infection rates in blood-fed mosquitoes, including members of the Culex pipiens complex, from Memphis and Shelby County, Tennessee, 2002-2003." Vector borne and zoonotic diseases 7(3): 365-386.

Savage, H. M. and L. Kothera (2012). "The Culex pipiens complex in the Mississippi River basin: identification, distribution, and bloodmeal hosts." Journal of the American Mosquito Control Association 28(4 Suppl): 93-99.

Shen, L. X., J. P. Basilion, et al. (1999). "Single-nucleotide polymorphisms can cause different structural folds of mRNA." Proceedings of the National Academy of Sciences of the United States of America 96(14): 7871-7876.

Spielman, A. (1967). "Population structure in the Culex pipiens complex of mosquitos." Bulletin of the World Health Organization 37(2): 271-276.

Veyrieras, J. B., S. Kudaravalli, et al. (2008). "High-resolution mapping of expression-QTLs yields insight into human gene regulation." PLoS genetics 4(10): e1000214.

Data Accessibility

NCBI SNP Database (dbSNP accession no. ss539004799)

CHAPTER THREE

New SNP Markers to Identify *Culex* Complex Species (Diptera: Culicidae) by High-Resolution DNA Melting Analysis

This chapter is in press as Kim S, Lee R, and Sim C (2018) New SNP markers to identify C*ulex* complex species (Diptera: Culicidae) by high-resolution DNA melting analysis. Journal of Medical Entomology

Abstract

The *Culex pipiens* complex is the primary vector of many arboviruses including West Nile virus and Eastern equine encephalitis in the United States. Within *Cx. pipiens* complex there are three biotypes that differ largely in habitat, blood meal preference, mating behavior and overwintering strategy. The three biotypes are *Cx. pipiens* form molestus F., *Cx. pipiens* f. pipiens L. and *Cx. pipiens quinquefasciatus* (aka *Cx. quinquefasciatus*). Since the low number of genetic markers is the key obstacle in performing genetic studies in the *Cx. pipiens* complex, distinct single nucleotide polymorphisms (SNPs) were identified from the *Cx. pipiens* complex species. Genomic DNA was extracted from adult females of form pipiens and f. molestus and was amplified by PCR. Thirty loci out of 100 primer pairs showed amplification and were used for SNP identification. All thirty loci contained biotype-specific SNPs: 10 loci were located in the genetic map of *Cx. pipiens* complex from previous genetic studies. We also tested a high-resolution DNA melting analysis as a biotype identification method by examining the SNPs in the two loci (CPIJ005487 and CPIJ002074). Our method provides a high confidence for biotype determination among the three *Cx. pipiens* complex mosquitoes.

Keywords: *Culex pipiens* f. molestus, *Culex pipiens* f. pipiens, *Culex quinquefasciatus*, single nucleotide polymorphisms, a high-resolution melting analysis.

Introduction

The *Culex pipiens* complex mosquitoes are among the most widespread and important vectors of West Nile Virus, St. Louis encephalitis virus and other diseases (Hamer et al., 2008, Fonseca et al., 2004, Monath, 1988). The *Cx. pipiens* complex includes *Cx. pipiens* f. pipiens, *Cx. pipiens* f. molestus and *Cx. quinquefasciatus*. These biotypes are not reliably distinguishable through morphological differences but exhibit unique behavioral, physiological and reproductive traits (Spielman, 1967, Mattingly, 1967). These traits are important factors that may determine the outcome of disease transmission to the human population (Savage et al., 2007). For example, *Cx. pipiens* f. pipiens goes through an overwintering stage called diapause. *Cx. pipiens* f. molestus, however, does not undergo diapause and lays eggs without a blood meal (autogenous). In contrast with form molestus, *Cx. pipiens* f. pipiens and *Cx. quinquefasciatus* lays eggs only after a blood meal (anautogenous) but f. pipiens is known to prefer avian blood while *Cx. quinquefasciatus* is more opportunistic between avian and mammalian blood. *Cx. pipiens* f. molestus can mate in confined spaces (stenogamous) while, *Cx. pipiens* f. pipiens and *Cx. quinquefasciatus* needs extensive space for mating (eurygamous) (Harbach et al., 1985, Harbach et al., 1984). Also, *Cx. pipiens* f. molestus mostly inhabits underground areas in cities while *Cx. quinquefasciatus* and *Cx. pipiens* f. pipiens resides above ground creating distinct ecological niches (Byrne and Nichols, 1999, Huang et al., 2008). Additionally, a host-feeding study of *Cx. pipiens* complex showing preference for various species including human blood, emphasizes the need for accurate methods to not

only separate each biotype, but to further study their distinctive traits (Savage and Kothera, 2012).

In the past, *Cx. pipiens* complex mosquitoes were identified by morphological characteristics, mainly by male genitalia (phallosoma). This has been unreliable and time consuming due to various factors that affect accurate differentiation such as rearing temperature and presence of hybrids. Specifically, rearing temperature is shown to affect the (DV/D) ratio method of identification due to overall effect in stages of development (Dodson et al., 2012, Rueda et al., 1990). This ratio shows the distance of the ventral arm of the phallosoma extending from the dorsal arm (DV) and the distance between the dorsal arms (D). This method was mainly used to differentiate between *Cx. pipiens* and *Cx. quinquefasciatus* in the past, while leaving others in the same genus undistinguished.

Advances in molecular methods such as restriction fragment length polymorphisms and microsatellites have been used to differentiate within the *Cx. pipiens* complex (Mori et al., 1999, Fonseca et al., 2004). Unfortunately, conclusions from these methods show varied results, and thus it requires an alternative method such as the high-resolution melting (HRM) assay that can distinctively identify each biotype with high sensitivity. Additionally, this challenge also warrants the need to develop reliable molecular markers. Although many genetic markers such as microsatellites and RFLPs in different mosquitoes have been studied (Lovin et al., 2009, Severson et al., 1995), there has been relatively little work done on the development of molecular markers based on single nucleotide polymorphisms (SNPs).

In this study, we identified and characterized SNPs from 30 loci obtained from three biotypes in *Cx. pipiens* complex mosquitoes. Two of the investigated markers were

selected and tested for their ability to identify *Cx. pipiens* complex at the biotype level. Moreover, we developed a HRM analysis to screen and genotype SNP markers in the *Cx. pipiens* complex mosquitoes and aligned SNP markers to three supercontigs of the *Culex* genome assembly. Our results support the effectiveness of HRM assays by using two novel SNP loci to discriminate biotypes of the *Cx. pipiens* complex.

Materials and Methods

Mosquito Colony

The insectary for *Cx. pipiens* complex species was set at 26°C with 75% relative humidity under a 12 h light: 12 h dark (L:D) daily rotation cycle. Larvae were given Tetramin fish food (Tetra holding Inc., Blacksburg, VA, USA) and were kept in de-chlorinated water through their pupae stage. Adults were fed diluted honey absorbed by cotton balls and were kept in large screened cages with dimensions 22×26×24 inches. The adult females were fed chicken blood using a Hemotek feeding apparatus for 1 h (Discovery Workshops, Lancashire, UK). The colony was established in 2015, from Columbus, Ohio.

But, the larvae of *Cx. pipiens* f. molestus were fed Tetramin fish food mixed in with liver powder (MP Biomedicals, LLC, Solon, OH, USA) in a 4:1 ratio. The colony was kept in smaller screened cages with dimensions 10×14×12 inches to mimic its original underground population from the Calumet Water Reclamation Plant in Chicago. Although it originated from Chicago, the main colony of *Cx. pipiens* f. molestus was provided by the Centers for Disease Control and Prevention Division of Vector-Borne Infectious Diseases in Fort Collins, Colorado.

Individual field samples of *Cx. quinquefasciatus* were trapped from September 21, 2016 to July 1, 2017, at the 10 collection sites in the greater Waco, TX area. The Biogents® Sentinel (BGS) traps were placed at the sites during the late afternoon and retrieved the following morning. Date, time, humidity and temperature were recorded upon collection. Adult mosquitoes were preserved in 95% ethanol and kept chilled until identification on a stereomicroscope.

Morphological Characterization

Members of the *Cx. pipiens* complex were examined for key morphological characteristics such as a M-shaped banding on the abdomen and a distinct wing fringe pattern with the palp shorter than the proboscis (Smith and Fonseca, 2004). Thirty adult females from each biotype of *Cx. pipiens* complex species were taken for wing and abdomen measurements. Twelve males from the each biotype were used for the calculation of phallosome DV/D ratio as previously described (Sundararaman, 1949). Although similar, DV/D ratio of <0.2 is considered *Cx. pipiens* f. pipiens, larger than 0.4 is *Cx. quinquefasciatus* and in between 0.2-0.4 is considered a hybrid. Measurements of wing and abdomen lengths were averaged and standard deviation were calculated. Significance was determined by ANOVA with a p value of <0.05.

SNP Discovery and Validation

Based on the genome of *Cx. quinquefasciatus*, candidate genes for SNP selection were picked from among the largest 100 supercontigs. The supercontigs for *Cx. quinquefasciatus* were selected by size that ranged from 3.08Mb to 939Kb. These

candidate genes were obtained from Vectorbase (http://cquinquefasciatus.vectorbase.org/) and Primer3 (http://frodo.wi.mit.edu/primer3/) was used to design primers for each candidate gene.

Genomic DNA was extracted from 10 females of each biotype, *Cx. pipiens* f. pipiens, *Cx. pipiens* f. molestus and *Cx. quinquefasciatus*, using the DNeasy Blood and Tissue Kit following the protocol (Qiagen, Hilden, Germany). Once the purity of the genomic DNA was measured against the elution buffer on a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) at 260/280 nm, samples were amplified by polymerase chain reactions (PCR) using the Taq PCR Kit (New England BioLabs, Ipswich, MA). 50 µl PCR amplifications were assayed utilizing 200 µM dNTPs, 10 x PCR buffer, 2 mM $MgSO_4$, 10 mM primers and 10 ng of gDNA. Amplifications were then performed on a T100 thermal cycler (Bio-Rad, Hercules, CA) with an initial denaturation at 94°C for 2 minutes, followed by 39 cycles at 94°C for 15 seconds, 50°C for 15 seconds, 72° for 45 seconds, then one cycle of 72°C for 5 minutes. PCR products and a negative control were then visualized on 2% agarose with a 100 bp molecular weight ladder (Invitrogen, Carlsbad, CA). Amplified PCR products were purified using two different cleaning protocols, including ExoSAP-IT (GE Healthcare, Little Chalfont, UK) or the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) removing unincorporated primers, nucleotides and salts before being sent to MacrogenUSA Inc. (Rockville, Maryland, US) for direct DNA sequencing. The SNPs found were further validated by resequencing using reverse primers.

SNP marker selection

SNPs were identified by analyzing sequences of the two biotypes first aligned with *Cx. quinquefasciatus* by using the assemble sequences function of CLC Main Workbench 6 (CLC bio, Aarhus, Denmark). Once assembled, the sequences of the two biotypes aligned against each other were then manually checked for SNPs to validate each SNP location. Candidate SNP positions were examined between members of *Cx. pipiens* complex. These validated SNPs were also positioned on the relative locations of each chromosome, which is estimated from the genetic map of *Cx. pipiens* constructed in previous studies (Naumenko et al., 2015).

Quantitative PCR and High-Resolution-Melting Analysis

Among the validated SNPs of the candidate genes, two primer pairs were designed to amplify each of candidate polymorphic loci on chromosome 2 and 3, and the primer sequences for these genes as follows: CPIJ005487, F 5'-CAACCAGATTGGGTTCGAGT -3', R 5'- GGTCATCCACACGATCATG A -3'; CPIJ002074, F 5'- TGTACGTGGAGCACAAGAGC -3', R 5'- CTGGTCTCGGTCT ACTCGGA -3'. Amplicon size ranged from 178-194 bp flanked by conserved regions to insure optimal specific melting curves during HRM analysis. Oligo-Calc online oligonucleotide properties calculator was used to analyze the potential primers used in order to avoid dimerization and hairpin formations (Kibbe, 2007).

PCR amplification prior to standard resolution melting curve analysis further optimized and confirmed calculated Tm to ensure appropriate test conditions. Each reaction consisted of genomic DNA which was then subjected to pre-amplification and high-resolution-melting (HRM) analysis utilizing the iQ^{TM} SYBR Green Supermix (Bio-

Rad, Hercules, CA, USA) on the Rotor-Gene Q real-time thermal cycler (Qiagen, Hilden, Germany). The reaction mixture consisted of 10 µL SYBR Green Supermix, 1 pmol of each HRM forward and reverse primers and 20 ng/µL of gDNA. Pre-amplification was performed with an initial denaturation at 95°C for 5 minutes, followed by 45 cycles at 95°C for 15 seconds, 54°C for 30 seconds, and 72°C for 15 seconds. After a 90 second pre-melt hold dissociation was performed by ramping temperatures at 0.1°C increments every 2 second holding periods from 75°C to 95°C. HRM data melting curves were then inspected visually and normalized by Rotor-Gene software.

Results

Morphological Characteristics

In the past, *Culex* mosquitoes were identified by the females' unique wing fringing and venation pattern and the W-shaped band on the dorsal side of the abdomen. However, the three *Cx. pipiens* complex mosquitoes examined in our study show almost identical morphology (Fig 3.1). Wing and abdomen lengths showed no significant variation (Table 3.1). *Cx. quinquefasciatus* and *Cx. pipiens* f. pipiens and f. molestus also showed no significant differences with wing ($F=2.82388$, $p=0.07156$, $p>0.05$) and abdomen ($F=2.54546$, $p=0.097108$, $p>0.05$) measurements. The male DV/D ratio identification method outlined by Sundararaman in the identification of *Cx. pipiens* complex did not consistently correspond with our measurements (Fig 3.1 and Table 3.1).

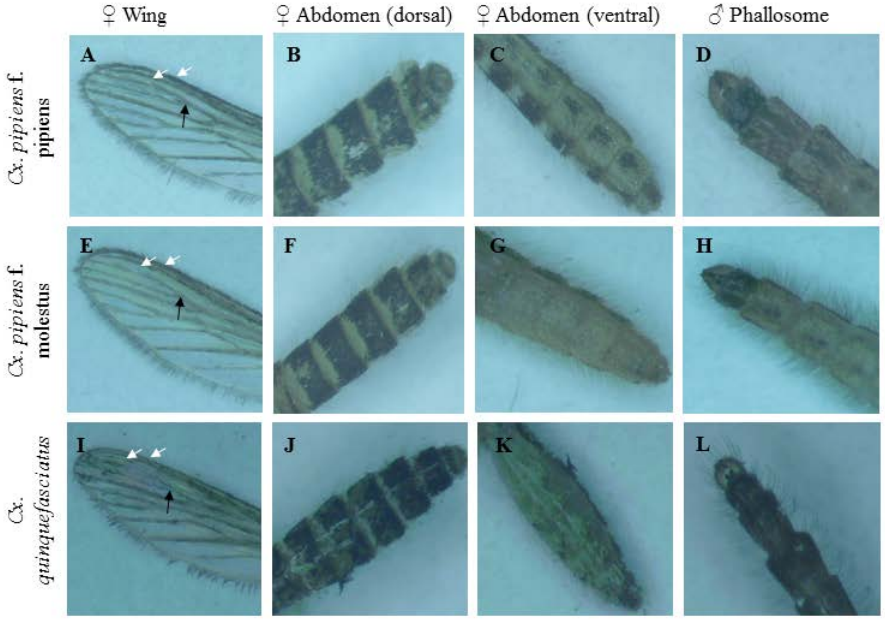

Figure 3.1. Morphological characteristics of adult females and males of the *Culex* complex species. Morphological keys were inconstant for identification of *Culex* complex species because of occurring variations among a sister species. (venation pattern of wings, "M" banding patterns in dorsal abdomen, male genitalia and DV/D ratio). (A-D) *Cx. pipiens* f. pipiens, (E-H) *Cx. pipiens* f. molestus, (I-L) *Cx. quinquefasciatus*. Costal and subcostal veins (white arrows) spread beyond the branching point of the radius veins (R2+3, black arrows) in all three *Culex* complex members.

Table. 3.1. Mean (standard deviation) of female wing and abdomen lengths and DV/D ratios of male phallosome for three members of the *Cx. pipiens* complex.

	Length (mm)		DV/D ratio
	♀ Wing	♀ Abdomen	♂ Phallosome
Cx. pipiens f. pipiens	3.8 (±0.28)	3.3(±0.34)	<0.2
Cx. pipiens f. molestus	3.6 (±0.26)	3.2 (±0.32)	0.2-0.4
Cx. quinquefasciatus	3.7 (±0.20)	3.4 (±0.32)	0.3-0.4

Gene Amplification

Thirty loci out of 100 primer pairs that were tested by PCR showed amplification and were used for SNP identification. The PCR amplicon location information from the reference genome of *Cx. quinquefasciastus* is shown in Table 3.2. The sizes of PCR products from the thirty-selected primer pairs in the study were 220-498 bp. There was a total of 5,646 bp of coding region and 2,132 bp of non-coding region to make up of a total of 7,778 bp sequenced. Out of the 7,778 bp sequenced, 253 SNPs were identified ranging from 1-30 SNPs per amplicon. Candidate SNP positions were examined for fixation within members of the *Cx. pipiens* complex and variability between members of the different forms. SNP data were submitted to the NCBI SNP database (dbSNP accession no ss947848635 - ss947848878).

Table 3.2. PCR amplicon location information from the *Culex* genome

Gene ID	Accession no	Function	SNPs
CPIJ000123	947848635-947848637	pyrroline-5-carboxylate reductase	3
CPIJ000290	947848638-947848648	hypothetical protein	11
CPIJ000408	947848649	hypothetical protein	1
CPIJ000494	947848650-947848652	hypothetical protein	3
CPIJ000595	947848653-947848656	hypothetical protein	4
CPIJ000874	947848657-947848674	carbohydrate sulfotransferase	18
CPIJ000778	947848675-947848683	phospholipase c	9
CPIJ001132	947848684-947848692	elongation factor 2	11
CPIJ001573	947848693-947848695	hypothetical protein	3
CPIJ001336	947848696-947848708	rRNA large subunit methyltransferase J	13
CPIJ001674	947848709-947848715	exocyst complex component 2	7
CPIJ002017	947848716-947848738	cell adhesion molecule	23
CPIJ801721	947848739-947848740	-	2
CPIJ801769	947848741-947848742	-	2
CPIJ801866	947848743-947848759	-	17
CPIJ002361	947848760	sodium/solute symporter, putative	1
CPIJ003251	947848761-947848764	hypothetical protein	4
CPIJ003297	947848765-947848765	bhlhzip transcription factor max/bigmax	1
CPIJ003806	947848766-947848768	hypothetical protein	3
CPIJ003693	947848769-947848772	glucosyl/glucuronosyl transferases	4

CPIJ004052	947848773-947848780	prospero	8
CPIJ004085	947848781-947848794	adenylate cyclase, putative	14
CPIJ004120	947848795-947848824	huntingtin	30
CPIJ802176	947848825-947848829	-	5
CPIJ802210	947848830-947848841	-	12
CPIJ004636	947848842-947848845	para-nitrobenzyl esterase	4
CPIJ802260	947848846-947848866	-	22
CPIJ005102	947848867-947848869	voltage-dependent p/q type calcium channel	3
CPIJ005487	947848870-947848877	tubulin gamma-1 chain	8
CPIJ005773	947848878	actin binding	1

Biotype-specific SNP markers were positioned on physical map of Cx. quinquefasciatus genome.

According to a recent progress of integrating physical mapping of 37 genomic supercontigs and the 58 genetic markers (Naumenko et al., 2015), the smallest chromosome was designated as Ch. 1, largest as Ch. 2, and intermediate as Ch. 3 (Figure 3.2). SNP markers identified in this study were positioned in this integrated physical map of *Cx. quinquefasciatus.* In total, the majority of the eight SNP markers was located on the largest Ch. 2, and two SNP markers were found on the intermediate-sized Ch.3, and none were identified on Ch.1.

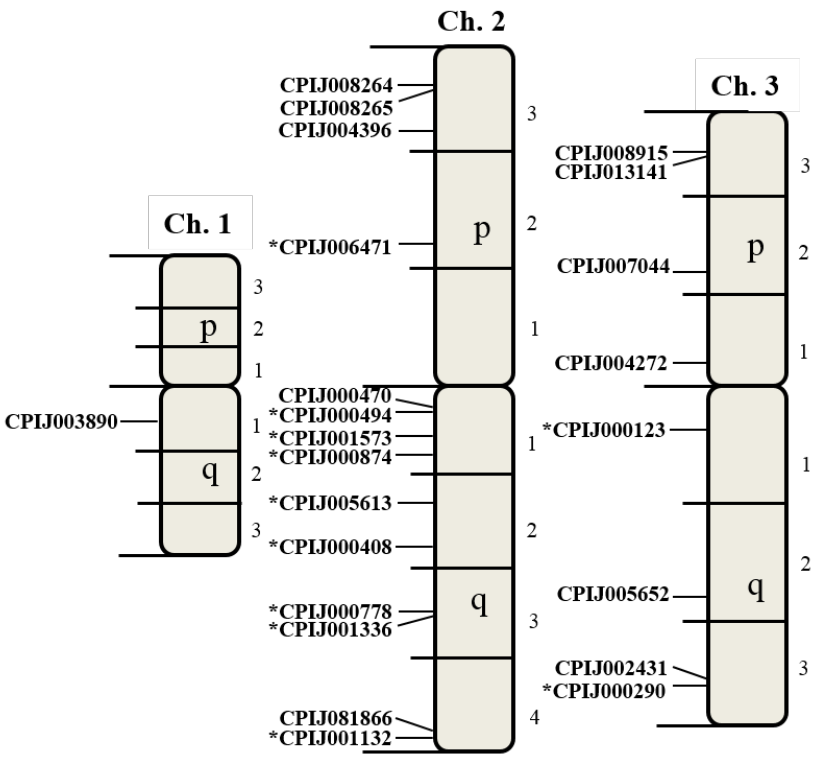

Figure 3.2. Location of SNP markers along with gene IDs mapped to the chromosomes of *Cx. quinquefasciatus*. Ch. 1, 2, and 3 indicate the chromosome numbers. "p" stands for the longer arm of the chromosome and "q" for the shorter one. Twenty-four SNP markers are shown with *Culex* gene IDs; ten new SNP markers are indicated by asterisks.

High Resolution Melting Analysis

We found HRM analysis to show distinct differences between the three *Cx. pipiens* complex species for genes CPIJ005487 and CPIJ002074 (Figure 3.3). Conditions for standard and normalized melting curves and temperature difference plots on the quantitative polymerase chain reactions were optimized to discriminate the members of *Cx. pipiens* complex. All HRM results for genes CPIJ005487 (Fig 3.4. A-C) and CPIJ002074 (Fig 3.4. D-F) can be used for future *Cx. pipiens* complex identification.

Figure 3.3. Single nucleotide polymorphisms (SNPs) across the *Culex* complex species. The High-Resolution Melting (HRM) primer sets were selected among the highly polymorphic regions of the genes CPIJ005487 and CPIJ002074. Portion of the polymorphic sites are shown boxed and colored gray. Each member of the *Culex pipiens* complex show clear single nucleotide differences.

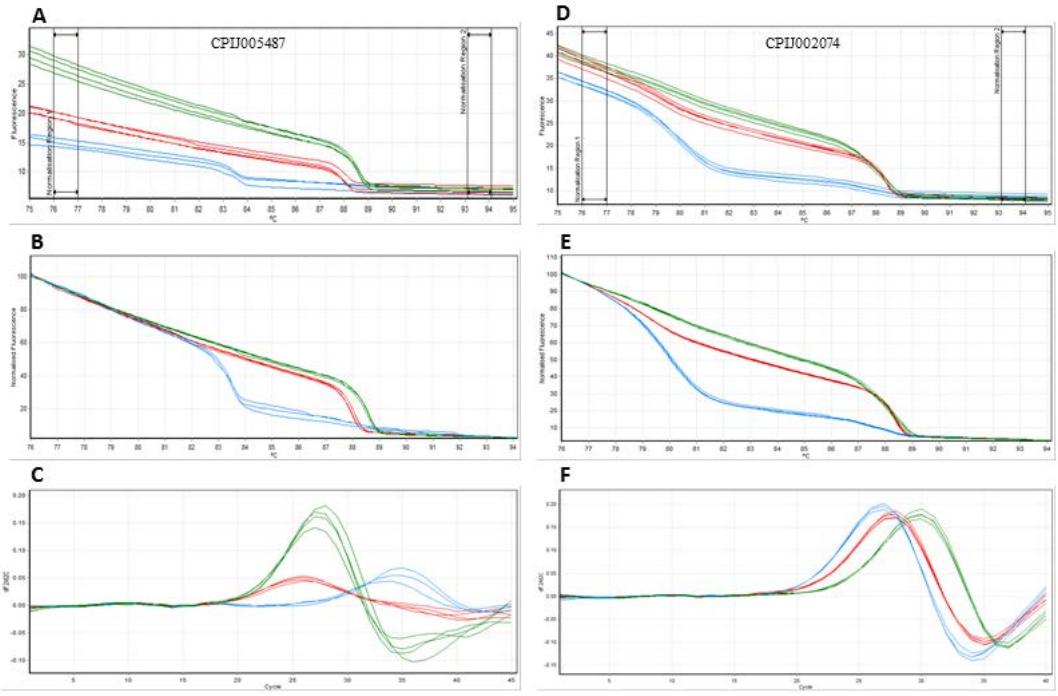

Figure 3.4. Discrimination of *Cx. pipiens* complex species by multiplex HRM analysis. (A, D) Raw melting profile of qPCR showing dissociation of double stranded DNA unique to each sister species of *Cx. pipiens*. (B-E) Raw melting data normalized to show further differentiation by using 2 normalization regions. (C, F) Genotype calling based on similarities in melting behavior at various temperatures. HRM assay performed using the primer pairs for the SNP markers in the gene CPIJ005487 (A-C) and in the gene CPIJ002074 (D-F). *Cx. pipiens* form pipiens is shown as red, *Cx. pipiens* form molestus and *Cx. quinquefasciatus* as blue and green, respectively.

Discussion

High resolution melting (HRM) assays generate DNA melting curve profiles that are both specific and highly sensitive to distinguish small sequence variations, enabling mutation scanning and genotyping. It is an established technique and has been effectively used to distinguish field-captured mosquitoes such as *Anopheles gambiae*, *An. arabiensis* and *Aedes aegypti* (Ajamma et al., 2016, Zianni et al., 2013, Kang and Sim, 2013). HRM is also used to discriminate the members of cryptic species which are morphologically inseparable but have distinctive eco-physiological differences. Our study intends to expedite the discrimination power of a sister species of the *Cx. pipiens* complex. Here, we have developed SNP markers from two loci in the genes (CPIJ005487 and CPIJ001674) of the *Cx. pipiens* complex mosquitoes and have assessed the performance of the HRM assay using the genomic DNA samples from laboratory and field-collected *Culex* mosquitoes.

It has been reported that different *Cx. pipiens* complex species exhibit distinguishable morphological features, which are utilized in morphometry to identify different biotypes (Kang and Sim, 2013). However, previously determined morphology standards are challenging to uphold due to various factors such as high genetic variations within biotype and effects of rearing temperatures to the mosquito morphology (Bahnck and Fonseca, 2006). In our study, the morphological differences within the biotypes of *Cx. pipiens* complex were consistently found in the laboratory and field-collected mosquitoes, which raise the question whether they can be distinguished by their morphology alone (Fig. 1). These morphological keys include venation pattern in the mosquito wing, banding marks on the abdomen of female mosquitoes as well as male

genetalia. The DV/D ratios of male genetalia, a previous standard of identification in *Cx. pipiens* complex mosquitoes, was also found inconsistent in our study. In contrast, the HRM assay using two selected SNP markers demonstrated a high discriminative power in the sister species of *Cx. pipiens* complex mosquitoes. Although adult males of *Cx. pipiens* f. pipiens and *Cx. quinquefasciatus* can be discriminated by the DV/D ratios, the HRM assay differentiate them with higher precision. Also, unlike the previous molecular and morphometry methods, this assay can sample male and female adults including all stages of development accurately and cost effectively.

In our previous, HRM assay using single nucleotide polymorphisms in the gene (*ace-2*) encoding the acetylcholinesterase-2 protein, we found that even the rarest of the SNPs of class IV can be used to differentiate between *Cx. pipiens* f. pipiens and *Cx. quinquefasciatus* (Kang and Sim, 2013). The class IV SNP (A/T) is the most challenging to resolve since homozygous genotypes differ least in their Tm by approximately only 0.2 °C. It thus requires a HRM instrument with sufficient thermal precision to resolve class IV SNP homozygotes and unique dsDNA intercalating dyes that can be used at high concentrations. In this study, two novel SNP markers were developed within the loci including a high level of single nucleotide polymorphisms. First, the amplicon sequencing of the locus in the gene CIPJ005487 revealed three class IV (A/T) transitions and a single class II (A/C) transition between *Cx. pipiens* f. pipiens and *Cx. pipiens* f. molestus, and two deletions and a single class II (A/C) transition when either was compared with *Cx. quinquefasciatus*. Second, the genomic SNPs of the locus in the gene CIPJ002074 also revealed a single class I (A/G) transition between *Cx. pipiens* f. pipiens and *Cx. pipiens* f. molestus, and a high level of polymorphisms when compared with *Cx. quinquefasciatus*.

HRM biotyping by using these SNP makers has provided compelling evidence of much greater discrepancy power than previous genetic makers.

Many mosquito strains, including *Cx. pipiens,* have been sequenced in pursuit of SNP-based identification methods to help clarify evolutionary mechanisms (Engdahl et al., 2013). Although sequencing is still the standard for genotyping, HRM can be a simple alternative that can save time and costs that can accurately identify mosquitoes that are hard to differentiate (Ewing et al., 1998). The advances in the overall HRM workflow which includes: saturating DNA binding dyes, real-time PCR instruments and HRM compatible analysis software has become a standard genotyping method. It uses both low amount of reagent and sample solution sizes and creates little waste compared to other techniques and the results can be analyzed right after each run. Optimization is required but can be easily modified to fit within a range that works best for the *Cx. pipiens* complex as supported here in our study.

We suggest that HRM is distinctively suited to analyze SNPs at a high resolution as an alternative to traditional methods of *Cx. pipiens* complex species identification such as PCR or morphology. The assay that we have developed for accurately identifying members of the *Cx. pipiens* complex will improve the understanding of distribution and unique biological traits of each biotype, which in turn will help to reduce the spread of diseases by limiting mosquito activity.

References

Ajamma, Y. U., Mararo, E., Omondi, D., Onchuru, T., Muigai, A. W., Masiga, D. & Villinger, J. 2016. Rapid and high throughput molecular identification of diverse mosquito species by high resolution melting analysis. *F1000Research,* 5**,** 1949.

Bahnck, C. M. & Fonseca, D. M. 2006. Rapid assay to identify the two genetic forms of *Culex* (*Culex*) *pipiens* L. (Diptera : Culicidae) and hybrid populations. *American Journal of Tropical Medicine and Hygiene,* 75**,** 251-255.

Byrne, K. & Nichols, R. A. 1999. *Culex pipiens* in London underground tunnels: differentiation between surface and subterranean populations. *Heredity,* 82 (Pt 1)**,** 7-15.

Dodson, B. L., Kramer, L. D. & Rasgon, J. L. 2012. Effects of larval rearing temperature on immature development and West Nile virus vector competence of *Culex tarsalis*. *Parasites & Vectors,* 5**,** 199.

Engdahl, C., Larsson, P., Naslund, J., Bravo, M., Evander, M., Lundstrom, J. O., Ahlm, C. & Bucht, G. 2013. Identification of Swedish mosquitoes based on molecular barcoding of the COI gene and SNP analysis. *Molecular Ecology Resources*.

Ewing, B., Hillier, L., Wendl, M. C. & Green, P. 1998. Base-calling of automated sequencer traces using phred. I. accuracy assessment. *Genome Research,* 8**,** 175-185.

Fonseca, D. M., Keyghobadi, N., Malcolm, C. A., Mehmet, C., Schaffner, F., Mogi, M., Fleischer, R. C. & Wilkerson, R. C. 2004. Emerging vectors in the *Culex pipiens* complex. *Science,* 303**,** 1535-8.

Hamer, G. L., Kitron, U. D., Brawn, J. D., Loss, S. R., Ruiz, M. O., Goldberg, T. L. & Walker, E. D. 2008. *Culex pipiens* (Diptera: Culicidae): a bridge vector of West Nile virus to humans. *Journal of Medical Entomology,* 45**,** 125-8.

Harbach, R. E., Dahl, C. & White, G. B. 1985. *Culex* (*Culex*) *pipiens*-Linnaeus (Diptera, Culicidae) - concepts, type designations, and description. *Proceedings of the Entomological Society of Washington,* 87**,** 1-24.

Harbach, R. E., Harrison, B. A. & Gad, A. M. 1984. *Culex* (*Culex*) *molestus* Forskal (Diptera, Culicidae) - neotype designation, description, variation, and taxonomic status. *Proceedings of the Entomological Society of Washington,* 86**,** 521-542.

Huang, S. M., Molaei, G. & Andreadis, T. G. 2008. Genetic insights into the population structure of *Culex pipiens* (Diptera : Culicidae) in the northeastern United States by using microsatellite analysis. *American Journal of Tropical Medicine and Hygiene,* 79**,** 518-527.

Kang, D. & Sim, C. 2013. Identification of *Culex* complex species using SNP markers based on high-resolution melting analysis. *Molecular Ecology Resources,* 13**,** 369-376.

Kibbe, W. A. 2007. OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Research,* 35**,** W43-W46.

Lovin, D. D., Washington, K. O., Debruyn, B., Hemme, R. R., Mori, A., Epstein, S. R., Harker, B. W., Streit, T. G. & Severson, D. W. 2009. Genome-based polymorphic microsatellite development and validation in the mosquito *Aedes aegypti* and application to population genetics in Haiti. *BMC Genomics,* 10.

Mattingly, P. F. 1967. The systematics of the *Culex pipiens* complex. *Bulletin of the World Health Organization,* 37**,** 257-61.

Monath, T. P. 1988. Japanese encephalitis--a plague of the Orient. *The New England Journal of Medicine,* 319**,** 641-3.

Mori, A., Severson, D. W. & Christensen, B. M. 1999. Comparative linkage maps for the mosquitoes (*Culex pipiens* and *Aedes aegypti*) based on common RFLP loci. *The Journal of Heredity,* 90**,** 160-4.

Naumenko, A. N., Timoshevskiy, V. A., Kinney, N. A., Kokhanenko, A. A., Debruyn, B. S., Lovin, D. D., Stegniy, V. N., Severson, D. W., Sharakhov, I. V. & Sharakhova, M. V. 2015. Mitotic-chromosome-based physical mapping of the *Culex quinquefasciatus* genome. *Plos One,* 10**,** e0115737.

Rueda, L. M., Patel, K. J., Axtell, R. C. & Stinner, R. E. 1990. Temperature-dependent development and survival rates of *Culex quinquefasciatus* and *Aedes aegypti* (Diptera: Culicidae). *Journal of Medical Entomology,* 27**,** 892-8.

Savage, H. M., Aggarwal, D., Apperson, C. S., Katholi, C. R., Gordon, E., Hassan, H. K., Anderson, M., Charnetzky, D., Mcmillen, L., Unnasch, E. A. & Unnasch, T. R. 2007. Host choice and West Nile virus infection rates in blood-fed mosquitoes, including members of the *Culex pipiens* complex, from Memphis and Shelby County, Tennessee, 2002-2003. *Vector Borne and Zoonotic Diseases,* 7**,** 365-86.

Savage, H. M. & Kothera, L. 2012. The *Culex pipiens* complex in the Mississippi River basin: identification, distribution, and bloodmeal hosts. *Journal of the American Mosquito Control Association,* 28**,** 93-9.

Severson, D. W., Thathy, V., Mori, A., Zhang, Y. & Christensen, B. M. 1995. Restriction fragment length polymorphism mapping of quantitative trait loci for malaria parasite susceptibility in the mosquito *Aedes aegypti*. *Genetics,* 139**,** 1711-7.

Smith, J. L. & Fonseca, D. M. 2004. Rapid assays for identification of members of the *Culex* (*Culex*) *pipiens* complex, their hybrids, and other sibling species (Diptera: Culicidae). *The American Journal of Tropical Medicine and Hygiene,* 70**,** 339-45.

Spielman, A. 1967. Population structure in the *Culex pipiens* complex of mosquitos. *Bulletin of the World Health Organization,* 37**,** 271-6.

Sundararaman, S. 1949. Biometrical studies on intergradation in the genitalia of certain populations of *Culex pipiens* and *Culex quinquefasciatus* in the United States. *American Journal of Hygiene,* 50**,** 307-14.

Zianni, M. R., Nikbakhtzadeh, M. R., Jackson, B. T., Panescu, J. & Foster, W. A. 2013. Rapid discrimination between *Anopheles gambiae* s.s. and *Anopheles arabiensis* by High-Resolution Melt (HRM) analysis. *Journal of Biomolecular Techniques : JBT,* 24**,** 1-7.

CHAPTER FOUR

Comparative Studies of Stenogamy Behavior in the Mosquito *Culex pipiens* Complex.

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Abstract

Understanding the processes of reproductive behavior in mosquitos is crucial for improving mating competitiveness and mating specificity for the sterile insect release program. *Culex pipiens* (Linneaus.) form pipiens and form molestus (Forskal.), two biotypes for *Cx. pipiens* complex, are vectors for West Nile Virus, St. Louis encephalitis virus, and lymphatic filariases. Hybridization of these biotypes is known to occur in nature, even though form pipiens mate above ground in larges spaces (eurygamy) and form molestus preferentially in small spaces (stenogamy) like sewage tunnels. Hybridization may allow gene flow of biotype-specific characteristics that are crucial in the disease transmission cycle. Here, we examined the mating behaviors, insemination rates, fecundity and fertility in parental and F1 hybrids between *Culex pipiens* form pipiens and *Cx. pipiens* f. molestus in stenogamy conditions. Unique mating behavior sequences were identified in the *Cx. pipiens* f. molestus including: tapping, mounting, co-flying and copulation. Despite the considerably high insemination rates from hybrid crosses, the fertility and fecundity rates were varied. This observation could suggest reproductive isolation in the hybrid zone. We also document a failure of heterospecific

males to produce fertile eggs in *Cx. pipiens* f. pipiens females, which may be due to gametic incompatibilities and an additional barrier to gene exchange.

Introduction

Culex pipiens complex mosquitoes are the most important vectors of West Nile Virus (WNV) and Eastern equine encephalitis virus in both birds and mammals. Geographically, the *Culex* mosquitoes are widespread and are found in almost every continent. Despite its importance in disease transmission, mating behavioral studies in *Culex* mosquitoes have been limited to date compared to extensive literature in *Aedes* and *Anopheles* mosquitoes (Oliva et al., 2012, Pitts et al., 2014, Pennetier et al., 2010, Helinski and Harrington, 2012). Within the *Culex pipiens* complex, the two biotypes': *Culex pipiens* (Linneaus.) f. pipiens and *Culex pipiens* f. molestus (Forskal.), morphological similarity but eco-physiological differences make it difficult to study their behavior. Morphologically speaking, the two biotypes are difficult to differentiate because of closely identical features such as male genitalia ratio and thorax banding. However, key eco-physiological characteristics such as mating behavior, oviposition, feeding habits and overwintering differentiate the two biotypes.

In North America, hybridization between the *Culex pipiens* complex mosquitoes including *Cx. pipiens*' two biotypes and *Cx. quinquefasciatus* is more common than previously thought. Although members of the *Cx. pipiens* complex have distinct niches, overlapping hybrid zones are found across the middle of the U.S. evidenced by mainly *Cx. pipiens* form pipiens and *Cx. quinquefasciatus* populations (Barr, 1957). Rare introgression of *Cx. pipiens* form molestus with form pipiens is found in other parts of the U.S. (Kent et al., 2007, Kothera et al., 2013). Hybrid forms exhibit increasing preference

for mammalian blood which may in turn contribute to the rise in disease transmission to humans (Kilpatrick et al., 2007, Hamer et al., 2008, Fritz et al., 2015). Indeed, the *Cx. pipiens* complex hybrids displayed stenogamy mating behavior, stayed primarily above ground, and favored mammalian blood over birds in parts of Europe (Rudolf et al., 2013, Reusken et al., 2010). However, in North America, empirical studies of key characteristics in sympatric populations between members of the *Cx. pipiens* complex are still lacking compared to Old World *Cx. pipiens* complex studies and is currently thought to rarely exist in the same environments (Kent et al., 2007).

Reducing mosquito-borne diseases rely heavily on the use of insecticides, but mosquito populations in various disease-endemic countries are developing insecticide resistance, which severly threatens the effectiveness of this approach (Roberts and Andre, 1994, Rivero et al., 2011). To combat insecticide resistance, sterile insect technology (SIT) has been proposed as an alternative strategy to vector control. This relies on the ability of sterile adult males to outcompete the wild males in order to mate with the females in nature. The success of such a program depends on the sexual competiveness of mass-reared sterile males. The mating competitiveness is in turn highly dependent on mating behavior of the target species (Koyama et al., 2004); therefore, the fundamental knowledge of courtship rituals of the adult mosquitoes would drastically improve sterile insect technology (Shaw et al., 2015).

In many organisms, a complex courtship ritual precedes copulation (Macnamara and Paterson, 1984). This complex system comprises of preferences involving communication signals leading to stimulation between potential mates and ultimately choice in a mate. Comparative studies that emphasize divergence of behaviors in sister

insect species, such as male courtship behavior, female contact pheromones and courtship song, are on the rise (Wijit et al., 2016, Taai et al., 2017). This comparative behavioral analysis can improve existing sterile insect technology or develop new tactics of vector control in mosquitoes by gauging mate competitiveness. For example, the mating ritual sequences such as the presence of each steps of mating ritual and the frequency of categorized behavior of sterile males, can be utilized to measure the mating success of those males after irradiation or genetic manipulation. Futhermore, these parameters in mating rituals among the members of criptic species or even the strains collected from differnt locations can be important factors to predict or simulate the effecitve population replacement in the field.

Specifically, in the *Cx. pipiens* complex, *Cx. pipiens* f. pipiens and f. molestus exhibit differences in mating behavior that is of interest. In general, mosquitoes including *Cx. pipiens* f. pipiens mate in swarms also known as “eurygamy,” which are aggregation of male mosquitoes over natural markers or bare ground in competition for female attention (Dabire et al., 2013, Fawaz et al., 2014, Service, 1994). However, the complexity of the swarming mating behavior makes the identification of such behavioral components a challenging problem. For example, a previous swarming study in *Culex quinquefasciatus* observed characteristic changes in overall flight speed and angle but described the patterns of flight to be random (Gibson, 1985). In *Anopheles* and *Aedes* show swarm size of tens to hundreds of mosquitoes per swarm (Butail et al., 2013, Tuten et al., 2013). Although this crucial mating behavior is common in mosquitoes, much is unknown about the underlying control mechanisms due to the difficulty in identifying individual mosquito activity in a swarm. In contrast, *Culex pipiens* f. molestus mate in

small spaces without nuptial flight. The term "stenogamy" is described as the ability of mosquitoes to mate in a small space, such as a small cage or tube. The mosquitoes of the *Cx. pipiens* f. molestus thus can exploit the small underground habitats (Becker et al., 2012).

In this study, we conducted a series of laboratory experiments aimed at determining the mating behavior sequences, insemination rates and fertility/fecundity in two biotypes of *Culex pipiens*, using reciprocal crosses and their F1 hybrids in stenogamy conditions. We predict that stenogamy conditions serve as a mating barrier that can be demonstrated by our experiments. Our results will provide a better understanding of the pre- and post- zygotic barriers present in the two biotypes leading to the development of competitive sterile *Culex pipiens* complex males.

Materials and Methods

Mosquito colonies

Experiments used two eurygamous colonies of *Cx. pipiens* form pipiens originating from larvae collected in Columbus, OH, in September 2000 or in the year of 2015. The insectary chamber conditions for *Cx. pipiens* f. pipiens were as follows: 25°C, 75% relative humidity with a daily light cycle of 15 hour light: 9 hour dark (L:D) rotation. Larvae were given Tetramin fish food (Tetra holding Inc., Blacksburg, VA, USA) and were kept in de-chlorinated water through their pupa stage. Adults were fed honey water and were kept in large screened cages with dimensions 55.8 × 66 × 60.9 cm. The adult females were fed chicken blood using a Hemotek feeding apparatus for an hour about once or twice a month (Discovery Workshops, Accrington, Lancashire, UK). The stenogamous colony of *Cx. pipiens* form molestus was collected from the Calumet Water

Reclamation Plant in Chicago (Mutebi & Savage 2009). Much like *Cx. pipiens* f. pipiens, the insectary chamber conditions for *Cx. pipiens* f. molestus were as follows: 25°C and 75% relative humidity under a 15 h light: 9 h dark (L:D) daily light cycle. Adults were fed honey water and were kept without a bloodmeal. The larvae were fed Tetramin fish food mixed with liver powder (MP Biomedicals, LLC, Solon, OH, USA) in a 4:1 ratio. The colony was kept in smaller screened cages with dimensions 25.4 × 35.5 × 30.4 cm to mimic its confined underground habitats.

Sex separation

Single pupa from both forms were picked and placed in individual 15 ml plastic tubes (VWR, Radnor, PA, USA) until adult emergence. The test tubes were labeled by date and placed in an incubator to maintain an environment of 25 ºC with 75% relative humidity. After the sex was determined for the adult mosquitoes, male and female virgins were separated into different 25.4 × 35.5 × 30.4 cm cages at 25 ºC with 75% relative humidity and left for another 2-5 days before mating behavior analysis.

Mating behavior analysis in small cage

In stenogamy mating, adult males and females (2-5 days after eclosion) were used for the experiment. Four replicates were set up with 30 females (♀) and 30 males (♂) in each reciprocal and homologous cage experiment as well as homologous F1 mating experiment. Crosses for mating behavior analysis were conducted as follows; 1) *Cx. pipiens* f. molestus ♀ with *Cx. pipiens* f. molestus ♂, 2) *Cx. pipiens* f. molestus ♀ with *Cx. pipiens* f. pipiens ♂, 3) *Cx. pipiens* f. pipiens ♀ with *Cx. pipiens* f. molestus ♂, 4) *Cx.*

pipiens f. pipiens ♀ with *Cx. pipiens* f. pipiens ♂. In addition, homologous mating from two F1 crosses; 5) ♀×♂ from F1 (*Cx. pipiens* f. molestus ♀ × *Cx. pipiens* f. pipiens ♂) and 6) ♀×♂ from F1 (*Cx. pipiens* f. pipiens ♀ × *Cx. pipiens* f. molestus ♂). The second experiment was repeated using different *Cx. pipiens* f. pipiens colony which were collected in the year of 2015. Thus, 240 total pairs of adult male and females were used for the mating behavior analysis.

For each trial of four replicates, thirty male and female mosquitoes were placed in a small glass cage measuring 27.5 × 17 × 20 cm (stenogamy condition). Mating activities were observed for an hour at simulated dusk (100-150 lux). In *Culex* mosquitoes, terminalia rotation completed in 19 hours (de Meillon et al., 1967). Then, distinctive mating behaviors were identified and the frequency and duration of each unique behavior was recorded. The behaviors defined are as follows: 1) tapping: male touches the female legs from all sides. 2) mounting: male climbs on top of a stationary female so that male ventral side is facing female dorsal side. 3) co-flying: male and female fly as a pair in contact with each other, usually ventral to ventral. 4) copulation: male and female genital contact that starts in the air and usually ends by landing. Mating success was determined by the percent copulated for each cross compared among groups with Chi-square analysis.

Insemination rate observation in stenogamy condition

Once the mating took place for 2-3 days, females were collected from each cross and were dissected to count successful insemination in the spermathecae (Rozeboom and Gilford, 1954, Jobling and Lewis, 1987). Briefly, female mosquitoes were anesthetized

by cold shock. Under a dissecting microscope, each female was placed ventral surface up on a glass slide. The terminalia of the female was removed with forceps separating from the rest of the body. Spermathecae were isolated from the terminalia and the presence or absence of sperm in spermathecae was recorded. For the analysis of insemination rate, a total of 180 adult females (60 females from the first trial and 120 females from the second one) were used to tally the sperm in the spermathecae. Comparison of insemination rates among groups was assessed by Chi-square analysis; a P-value less than 0.05 was considered to be a significant change in insemination rate.

Assessment of egg production and survival rate in stenogamy condition

For all crosses mated in stenogamy conditions, oviposition and survival rates to adulthood were measured to determine fertility and fecundity. For this analysis, a total of 180 adult females were collected for oviposition and were kept with 10% honey solution for 2 days. Autogenous females were kept with honey water for an additional 2-3 days for oviposition (Kassim et al., 2012). While anautogenous females were given access to chicken blood after a brief starvation period (6-12 hours). The blood-fed females were left in the cages with oviposition cups for 4-6 days (Richards et al., 2012). Egg rafts oviposited by each cross were counted and kept in separate tubs. This was carried out in the same conditions as the mating behavior experiment. Larvae, pupae and adulthood stages were recorded. Total egg production was considered as the sum of laid and retained eggs from the first gonotrophic cycle. Survival rate was measured as number of adult mosquitoes survived from 1st instar larvae. Comparisons of survival rates among

groups were assessed by Chi-square analysis; a P-value less than 0.05 was considered to be a significant change in survival rate.

Results

Sequence of stenogamy mating behaviors

Distinct mating behavior sequences were identified in stenogamy conditions (Figure 4.1). In spite of the rapid flight, it was still possible to distinguish four different stages in the courtship process. They were usually followed in order as follows:

(1) *Tapping*: Male actively searches for a female to mate with and is usually seen almost as soon as males and females are placed in the cage. Male makes initial physical contact with female primarily with its forelegs and start tapping female's hind legs (Fig. 4.1A).

(2) *Mounting*: Once the female is receptive to the male's tapping and does not fly away or kick him off, the male climbs on top of the female so that his ventral side is facing her dorsal side as described (Fig. 4.1B). At this point other males do not contact with the pairing male and female.

(3) *Co-flying*: Facing each other, male and female momentarily fly in a spiral pattern. Male initiates genital contact during the short flight and both begin to lose altitude (Fig. 4.1C).

(4) *Copulation*: Copulation is successful when male and female make firm genital contact. This stage usually starts midair, but successful copulation ends in a landing

position creating stability. Copulation on average lasts around 30-40 seconds (Fig. 4.1D and E).

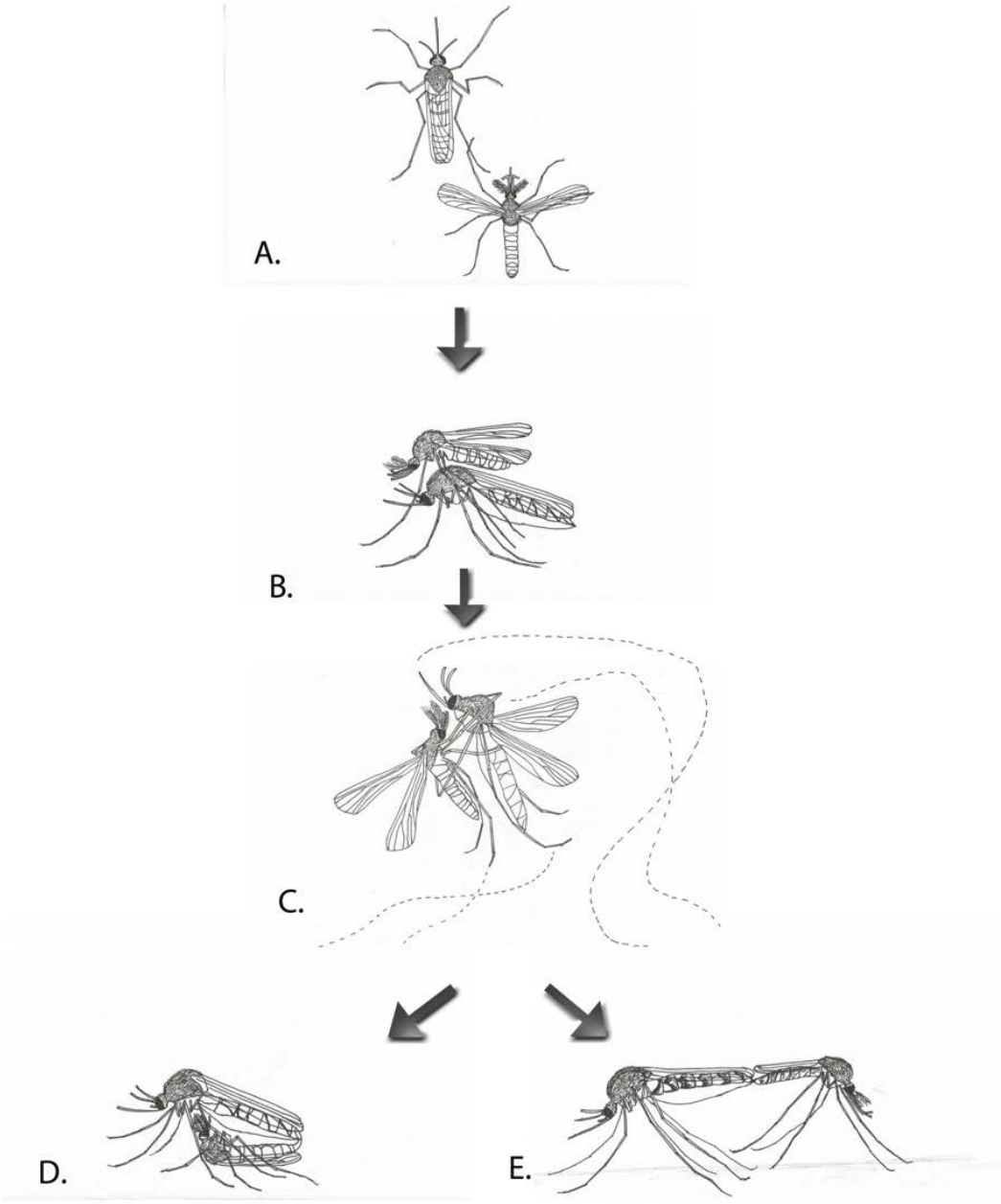


Figure 4.1. *Culex pipiens* biotypes mating behavior sequence in stenogamy conditions. Distinct mating behavior were observed and defined as follows: A) Tapping: Male touches the female leg area from all sides. B) Mounting: Male climbs on top of a stationary female so that his ventral side is facing her dorsal side. C) Co-flying: Male and female fly in contact with each other ventral to ventral. D) Copulation: male and female genital contact (ventral to ventral). E) Copulation: male and female genital contact (tail to tail).

Parent generation biotype combinations influenced copulation success rates (χ^2 = 0.3, $p < 0.0001$). As expected, the homologous parent cross (stenogamous *Cx. pipiens* f. molestus, M♀ × M♂) exhibited the higher copulation success rate of 90% compared to the other homologous parent cross (eurygamous *Cx. pipiens* f. pipiens, P♀ × P♂) which was only 3.3% (Table 4.1). Nevertheless, parent generation combination and reciprocal cross comparisons were not different in copulation success (χ^2 = 21.16, $p > 0.001$). Average copulation rates for combinations of f. molestus and f. pipiens (M♀ × P♂) and (P♀ × M♂) were low with 10% and 6.6%, respectively (Table 4.1).

Although defined courtship sequence events did occur in both intra- and inter-biotype crosses, stable copulation only occurred in pairs with *Cx. pipiens* f. molestus female. Inter biotype crosses resulted in low copulatory success due to female rejection behavior. *Cx. pipiens* f. molestus female actively eluded genital contact with f. pipiens male resulting in only 11% mating success. Likewise, *Cx. pipiens* f. pipiens female prevented copulation with *Cx. pipiens* f. molestus male (6.6%) by kicking with her hind legs or flying away (12 copulations observed over 180 total pairs). Both biotype males exhibited distinct mating sequences in inter-biotype crosses but females showed lack of interest. The homologous mating crosses from F1 population (M♀ × P♂) showed a high mating success rate of (83.3 - 90%) similar with those of homologous parent crosses (M♀ × M♂) in stenogamy conditions. In contrast, the other homologous mating crosses from F1 population (P♀ × M♂) exhibited the most activity but did not necessarily translate to successful copulations (40-43.3%) (Table 4.1).

Table 4.1. Stenogamy mating behavior analysis in homologous and reciprocal parent crosses (*Culex pipiens* f. pipiens and *Cx. pipiens* f. molestus) and in homologous mating crosses from two F1 populations (M♀ × P♂, P♀ × M♂).

	P♀ × P♂		M♀ × M♂		M♀ × P♂		P♀ × M♂		F1 (M♀ × P♂) F1♀ × F1♂		F1 (P♀ × M♂) F1♀ × F1♂	
Behavior	Occurrences											
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Tapping	16	33	213	639	58	258	60	160	252	522	324	424
Mounting	4	6	18	145	2	42	16	116	57	257	84	148
Co-Flying	6	11	94	340	6	61	6	26	171	271	27	111
Copulation (%)	3.3	3.3	90	90	10	20	6.6	10	90	83.3	40	43.3

P: *Cx. pipiens* form pipiens, M: *Cx. pipiens* form molestus

n = 30♀ × 30♂

Table 4.2. Insemination rate in homologous and reciprocal parent crosses (*Cx. pipiens* f. pipiens and *Cx. pipiens* f. molestus) and in homologous mating crosses from two F1 populations (M♀ × P♂, P♀ × M♂). Each experiment was conducted with (30♀ × 30♂) but a total number of female dissection for each combination was 120.

Crosses		Total No. Crosses	Insemination success	
♀	♂		*Trial 1*	*Trial 2*
f. molestus	f. molestus	180	48/60	98/120
f. molestus	f. pipiens	180	6/60	18/120
f. pipiens	f. pipiens	180	3/60	9/120
f. pipiens	f. molestus	180	3/60	11/120
F1 (M♀ × P♂)	F1 (M♀ × P♂)	180	48/60	94/120
F1 (P♀ × M♂)	F1 (P♀ × M♂)	180	25/60	63/120

Small cage mimics a stenogamy condition (27.5 × 17 × 20 cm).

Table 4.3. Egg production and survival rates in homologous and reciprocal parent crosses (*Cx. pipiens* f. pipiens and *Cx. pipiens* f. molestus) and in homologous mating crosses from two F1 populations (M♀ × P♂, P♀ × M♂).

	Parent crosses								F1 crosses			
	P♀ × P♂		M♀ × M♂		M♀ × P♂		P♀ × M♂		F1 (M♀ × P♂)		F1 (P♀ × M♂)	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Egg rafts	0	0	6	27	2	5	0	0	3	6	0	1
1st Instar	0	0	144	644	36	56	0	0	88	188	0	2
2nd Instar	0	0	135	535	15	55	0	0	84	184	0	0
3rd Instar	0	0	135	535	5	45	0	0	83	183	0	0
4th Instar	0	0	130	532	0	35	0	0	80	180	0	0
Pupae	0	0	130	520	0	25	0	0	74	174	0	0
Adult	0	0	126	502	0	23	0	0	71	171	0	0
Survival rate (%)	n.a.	n.a.	70.3	80	0	12	n.a.	n.a.	80.7	80	n.a.	n.a.

P: *Cx. pipiens* f. pipiens, M: *Cx. pipiens* f. molestus

Insemination rate

Insemination success was validated by presence of sperm in female spermathecae as shown in Figure 4.2. Between the *Cx. pipiens* f. pipiens and f. molestus parent crosses, insemination rates were significantly different ($\chi^2 = 8.6$, $p < 0.001$). *Cx. pipiens* f. pipiens males were not successful in transfer of sperm to f. pipiens female. It appears that *Cx. pipiens* f. pipiens females largely stayed uninseminated because of active prevention of genital contact as we observed in mating behavior analysis. Also, (M♀ × M♂) had an 80% insemination rate. Similarly, F1 (M♀ × P♂) resulted in high insemination rate of (80%). However, low sperm transfer occurred (4%) in both homologous (P♀ × P♂) and reciprocal parent crosses (P♀ × M♂) in which *Cx. pipiens* f. pipiens were crossed by either biotype, suggesting low receptivity of *Cx. pipiens* f. pipiens females (Table 4.2). In addition, *Cx. pipiens* f. pipiens females were unsuccessful in receiving sperm except for F1 (P ♀ × M♂) that showed about a 40% success rate. Reciprocal crosses showed the rejection behavior in which females avoid male copulation attempts, resulting in low insemination rates.

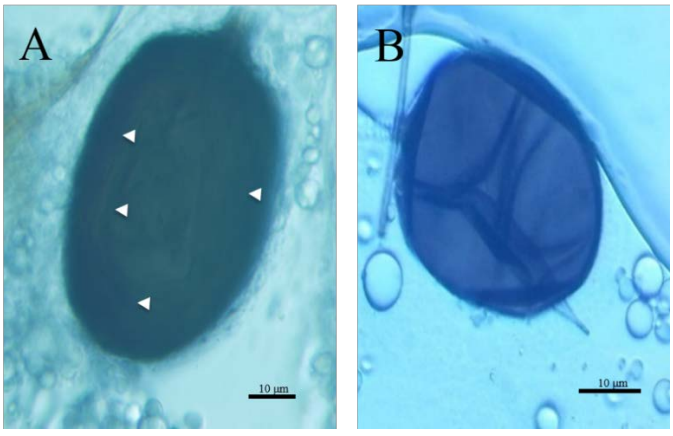


Figure 4.2. General view of *Culex pipiens* female spermathecae. A) The spermathecae reservoir from an inseminated *Cx. pipiens* female mosquito. Spermathecae is filled with spermatozoa moving in circular motion as noted by arrows. B) The empty spermathecae of *Cx. pipiens* virgin female indicated by dark uniform spermathecae reservoir.

Egg production and survival rates

Homologous parent crosses of *Cx. pipiens* f. molestus (M♀ × M♂) produced viable egg rafts (5.6 eggs/ female) and showed high survival rates to adult stage (70.3 - 80%) (Table 4.3). Comparison between parent generation results were significantly different ($\chi^2 = 14.97$, $p < 0.001$). Homologous mating crosses from the F1 population (M♀ × P♂) cross also produced viable eggs resulting in both male and female adult eclosion similar to those of parent crosses (M♀ × M♂). In contrast, all other crosses including *Cx. pipiens* f. pipiens females produced no eggs (Table 4.3).

Discussion

Stenogamous mosquitoes can mate with a single male and female in small spaces like pipes and underground sewage areas. In contrast to the swarming behavior of mosquitoes that makes the identification of such behavioral components a challenging problem, stenogamy allows for a meaningful segmentation of behavior sequences involved in mosquito mating. In this study, we demonstrate possible pre-zygotic mating barriers related to the stereotypic sequences in stenogamy traits from the homologous and reciprocal crosses of *Culex pipiens* f. pipiens and f. molestus.

Cx. pipiens f. pipiens were not successful (3.3%) at exhibiting the mating sequence seen in *Cx. pipens* f. *molestus* (90%) in a small cage (Table 4.1). Males of *Cx. pipiens* f. pipiens were active, trying to create a flying pattern to mate with conspecifics and even with non-conspecific females but the females were not responsive to their efforts. Also, interestingly, both reciprocal parental crosses of the two forms of *Culex pipiens* showed low copulation success rate. This evidence suggests at least partial

reproductive isolation between *Cx. pipiens* f. pipiens and f. molestus in stenogamy conditions. Although both males and females participate in mating sequences in stenogamy conditions, the female of *Cx. pipiens* f. molestus seems to be the determining factor for accepting the advances of either male form, while *Cx. pipiens* f. pipiens females exhibited rejecting characteristics as shown in F1 hybrid studies (Table 4.1).

Despite previous studies on the topic of mating in insects, much is unclear as to which characteristics effect mating behavior in mosquitoes especially regarding stenogamy in *Culex pipiens* f. molestus. For example, the *Culex pipiens* complex biotypes exhibited tapping the most compared to the other behaviors in the mating sequence (Table 4.1) suggesting that olfactory and or gustatory mechanisms could be the primary recognition mechanism for mate preference. Aggregation pheromones are produced by males that modulate mating behavior with conspecifics in the *Culex pipiens* complex (Gjullin, 1967) which could explain the proactive male behavior in presence of females. Studies in cuticular hydrocarbons in *Drosophila, Anopheles* and *Aedes* revealed their importance in mating rituals (Diabate and Tripet, 2015, Polerstock et al., 2002, Yamamoto and Koganezawa, 2013), and could be the case as well in the *Culex pipiens* complex.

Interestingly, stenogamy conditions did not prevent the *Culex pipiens* males and females to have a brief flight together (co-fly) before copulation which suggests auditory mechanisms as part of the elaborate stenogamy mating behavior pattern. This idea was supported in swarming behavior in *Aedes, Anopheles* and *Culex* mosquitoes, where both male and female exhibit synchronized frequencies of wing beats that aid in mate recognition (Warren et al., 2009). Sound generated by the beating of female wings

consists of a harmonious series that provides acoustic energy which is proportional to wing size, wing-beat frequency and ambient temperature (Brogdon, 1994). However, the brief duration of flight suggests auditory mechanisms as a minor factor, understandably due to compact space.

Although the behavioral sequences leading to copulation were distinct, the frequencies of each behavior were varied due to the receptivity of females. For example, in some cases, mounting was not obvious, while other sequences such as tapping and co-flying occurred repetitively. This result is consistent with the fundamental idea that the ability of males to gain access to females is a key determinant of mating systems. Other experimental mating systems show similar findings in which females exert more influence over mating than males due to their higher reproductive investment, potentially causing the males to exhibit repetition of certain patterns of behavior (Table 4.1) before successful copulation (Bateman, 1948). Thus, mounting behavior of males can possibly be explained by a period of male competition at different stages of courting that is a form of pre-copulatory guarding rather than directly a part of the mating sequence (Choe and Crespi, 1997). Despite its stereotypical pattern, mating behavior exhibits plasticity as often described in other insects (Crowder et al., 2010).

Successful copulation in the *Culex pipiens* biotypes also showed successful insemination. Although mating behavior exhibits plasticity, in general, presence of mating sequences are linked to successful insemination, fecundity and fertility. The mating sequence data was also consistent with the reciprocal crosses that show reduced sperm counts (Table 4.2). A previous study comparing stenogamy and eurygamy mating behavior between *Anopheles* mosquitoes also suggest similar comparisons between

mating behavior and insemination rate (Wijit et al., 2016). The differences in mosquito mating behavior may be a link to the morphological variations of male genitalia, in regards to frequency of clasper movement. Overall body and wing size could also contribute to differences in mating behavior and insemination outcome as previously reported (Sallum et al., 2005, Wijit et al., 2016).

F1 hybrids showed higher mating activities as well as increased insemination rates compared to those of the homologous parent cross (*Cx. pipiens* f. pipiens) and both reciprocal parent crosses (Table 4.1 and 4.2). Recently, a number of studies have explored sexual competitiveness of sterilized males (Hamady et al., 2013) and heterosis (Ekechukwu et al., 2015). In *Anopheles coluzzii*, F1 hybrids also showed increased fitness and insemination rates compared to those of the homologous crosses. Male and female F1 hybrids were generally larger (4.42-4.52 mm) than their parent generation (3.39-3.87 mm), which are correlated with increased frequencies of fertility and fecundity in F1 hybrids. Previous studies also found similar patterns that show that relatively lager males store more sperm and such females are also more receptive of mating advances (Helinski and Harrington, 2011, Packer and Corbet, 1989, Blay and Yuval, 1997, Ameneshewa and Service, 1996).

Despite the varied sequence (Table 4.1), complexity of mating behavior and F1 fitness in the *Cx. pipiens* complex, potential mechanisms including auditory, olfactory and gustatory signals may be important in the success of the SIT. Most male mosquitoes are known to locate their mates by sound in swarms. Among swarming studies, one of the best-studied mechanisms in mosquito mating behavior concerns flight tone (Brogdon, 1994, Wekesa et al., 1998, Warren et al., 2009). Flight tone from male and female wing

beats allows species to recognize their partners. However, our stenogamy-mating results showed short durations of flight from both *Cx. pipiens* f. pipiens and f. molestus, suggesting characteristics other than flight tone taking precedence for mating preference and perhaps species recognition in the *Cx. pipiens* f. molestus.

Unlike auditory signals, olfactory and gustatory mechanisms may be heavily relied upon for successful mating in *Culex pipiens* mosquitoes mating in stenogamy conditions. Mosquito antennae are known to host the majority of chemoreceptors that allows adult females to identify and discriminate appropriate oviposition sites and hosts (McIver, 1982). Thus, chemoreceptors in antennal sensilla could be linked with mate choice and stenogamy mating behavior since it affects most olfaction-driven behaviors. In *Drosophila* for example, olfactory pheromones (cVA) show decrease in sexual activity in females (Jallon, 1984, Billeter et al., 2009) and gustatory pheromones show male-predominant hydrocarbons that attract females (Everaerts et al., 2010). In this study, male mosquitoes directed their bodies behind females and often touched the female's hind legs with their forelegs. This tapping behavior may suggest that the male uses gustatory receptors in his forelegs to detect mating cues.

The majority of studies on *Culex* reproductive behavior focus on insemination ability of males and oviposition characteristics of females (Michaelakis et al., 2005). Our results (Table 3) support previous studies showing females' complex preference for oviposition. Female reproductive cost is much higher than males and is likely the reason for their choosey behavior (Long et al., 2009, Hiss and Fuchs, 1972). Results showed that females paired with reciprocal males produced fewer eggs and sometimes none at all. We speculate that frequent courtship behavior could interfere with successful insemination

and cause a reduction in fecundity in reciprocal crosses. Additionally, a previous study in *Aedes* showed that an inability to store sperm after insemination could be a barrier to producing viable eggs (Carrasquilla and Lounibos, 2015).

At large, insects have distinct mate recognition systems that determine their ability to distinguish conspecific mates for successful copulation. Previous studies in *Aedes* and *Anopheles* have focused on areas such as auditory, olfactory and gustatory systems in mate recognition and mating behavior. Much like the *Aedes* and *Anopheles* mosquitoes, both sexes of the *Culex pipiens* biotypes also utilize auditory, olfactory and gustatory systems in their mating ritual. The extent to which the mechanisms are used in stenogamy conditions for the *Culex pipiens* biotypes are still poorly understood. This study is the first to demonstrate biotype-specific mating behavioral sequences in *Culex pipiens* complex, including female-dependent stenogamy. Furthermore, our results can be a starting point to unravel the molecular mechanisms linked to mosquito mating sequences. Behavioral observational studies are crucial to optimize mass reared males that outperform wild males, thus this study provides insight for the success of SIT program relevant to control of disease vectors and agricultural pests. However, further studies surrounding the actual courtship events must be conducted before fully understanding their function.

References

Ameneshewa, B. & Service, M. W. (1996) The relationship between female body size and survival rate of the malaria vector *Anopheles arabiensis* in Ethiopia. *Medical and Veterinary Entomology,* 10**,** 170-172.

Barr, A. R. (1957) The distribution of *Culex pipiens pipiens* and *Cx. quinquefasciatus* in North America. *American Journal of Tropical Medicine and Hygiene,* 6**,** 153-165.

Bateman, A. J. (1948) Intra-sexual selection in *Drosophila melanogaster*. *Heredity,* 2**,** 277-277.

Becker, N., Jost, A. & Weitzel, T. (2012) The *Culex pipiens* complex in Europe. *Journal of the American Mosquito Control Association,* 28**,** 53-67.

Billeter, J. C., Atallah, J., Krupp, J. J., Millar, J. G. & Levine, J. D. (2009) Specialized cells tag sexual and species identity in *Drosophila melanogaster*. *Nature,* 461**,** 987-91.

Blay, S. & Yuval, B. (1997) Nutritional correlates of reproductive success of male Mediterranean fruit flies (Diptera: Tephritidae). *Animal Behaviour,* 54**,** 59-66.

Brogdon, W. G. (1994) Measurement of flight tone differences between female *Aedes aegypti* and *Ae. albopictus* (Diptera, Culicidae). *Journal of Medical Entomology,* 31**,** 700-703.

Butail, S., Manoukis, N. C., Diallo, M., Ribeiro, J. M. & Paley, D. A. (2013) The dance of male Anopheles gambiae in wild mating swarms. *Journal of Medical Entomology,* 50**,** 552-9.

Carrasquilla, M. C. & Lounibos, L. P. (2015) Satyrization without evidence of successful insemination from interspecific mating between invasive mosquitoes. *Biology letters,* 11.

Choe, J. C. & Crespi, B. J. (1997) *The evolution of mating systems in insects and arachnids,* Cambridge ; New York, Cambridge University Press.

Crowder, D. W., Sitvarin, M. I. & Carriere, Y. (2010) Plasticity in mating behaviour drives asymmetric reproductive interference in whiteflies. *Animal Behaviour,* 79**,** 579-587.

Dabire, K. R., Sawadodgo, S., Diabate, A., Toe, K. H., Kengne, P., Ouari, A., Costantini, C., Gouagna, C., Simard, F., Baldet, T., Lehmann, T. & Gibson, G. (2013) Assortative mating in mixed swarms of the mosquito *Anopheles gambiae* s.s. M and S molecular forms, in Burkina Faso, West Africa. *Medical and Veterinary Entomology,* 27**,** 298-312.

De Meillon, B., Sebastian, A. & Khan, Z. H. (1967) Exodus from a breeding place and the time of emergence from the pupa of *Culex pipiens fatigans*. *Bulletin of the World Health Organization,* 36**,** 163-7.

Diabate, A. & Tripet, F. (2015) Targeting male mosquito mating behaviour for malaria control. *Parasites & vectors,* 8.

Ekechukwu, N. E., Baeshen, R., Traore, S. F., Coulibaly, M., Diabate, A., Catteruccia, F. & Tripet, F. (2015) Heterosis increases fertility, fecundity, and survival of laboratory-produced F1 hybrid males of the malaria mosquito *Anopheles coluzzii*. *G3,* 5**,** 2693-709.

Everaerts, C., Farine, J. P., Cobb, M. & Ferveur, J. F. (2010) *Drosophila* cuticular hydrocarbons revisited: mating status alters cuticular profiles. *Plos One,* 5**,** e9607.

Fawaz, E. Y., Allan, S. A., Bernier, U. R., Obenauer, P. J. & Diclaro, J. W. (2014) Swarming mechanisms in the yellow fever mosquito: aggregation pheromones are involved in the mating behavior of *Aedes aegypti. Journal of Vector Ecology,* 39**,** 347-354.

Fritz, M. L., Walker, E. D., Miller, J. R., Severson, D. W. & Dworkin, I. (2015) Divergent host preferences of above- and below-ground *Culex pipiens* mosquitoes and their hybrid offspring. *Medical and Veterinary Entomology,* 29**,** 115-123.

Gibson, G. (1985) Swarming behavior of the mosquito *Culex pipiens quinquefasciatus* - a quantitative analysis. *Physiological Entomology,* 10**,** 283-296.

Gjullin, C. M. W., T. L.; Buckley, J.F. (1967) Male pheromones of *Culex quinquefasciatus, Cx. tarsalis* and *Cx. pipiens* that attract females of these species. *Mosquito News,* 27**,** 382-387.

Hamady, D., Ruslan, N. B., Ahmad, A. H., Rawi, C. S., Ahmad, H., Satho, T., Miake, F., Zuharah, W. F., Fukumitsu, Y., Saad, A. R., Rajasaygar, S., Vargas, R. E., Majid, A. H., Fadzly, N., Ghani, I. A. & Abubakar, S. (2013) Colonized *Aedes albopictus* and its sexual performance in the wild: implications for SIT technology and containment. *Parasites & vectors,* 6**,** 206.

Hamer, G. L., Kitron, U. D., Brawn, J. D., Loss, S. R., Ruiz, M. O., Goldberg, T. L. & Walker, E. D. (2008) Culex pipiens (Diptera : Culicidae): A bridge vector of West Nile virus to humans. *Journal of Medical Entomology,* 45**,** 125-128.

Helinski, M. E. H. & Harrington, L. C. (2011) Male mating history and body size influence female fecundity and longevity of the dengue vector *Aedes aegypti. Journal of Medical Entomology,* 48**,** 202-211.

Helinski, M. E. H. & Harrington, L. C. (2012) The role of male harassment on female fitness for the dengue vector mosquito *Aedes aegypti*. *Behavioral Ecology and Sociobiology,* 66**,** 1131-1140.

Hiss, E. A. & Fuchs, M. S. (1972) Effect of matrone on oviposition in mosquito, *Aedes aegypti*. *Journal of insect physiology,* 18**,** 2217-&.

Jallon, J. M. (1984) A few chemical words exchanged by *Drosophila* during courtship and mating. *Behavior genetics,* 14**,** 441-78.

Jobling, B. & Lewis, D. (1987) Anatomical drawings of biting flies. *British Museum of Natural History* [Online]. [Accessed February 11, 2015 2015].

Kassim, N. F. A., Webb, C. E. & Russell, R. C. (2012) Is the expression of autogeny by *Culex molestus* Forskal (Diptera: Culicidae) influenced by larval nutrition or by adult mating, sugar feeding, or blood feeding? *Journal of Vector Ecology,* 37**,** 162-171.

Kent, R. J., Harrington, L. C. & NORRIS, D. E. (2007) Genetic differences between *Culex pipiens* f. molestus and *Culex pipiens* pipiens (Diptera : Culicidae) in New York. *Journal of Medical Entomology,* 44**,** 50-59.

Kilpatrick, A. M., Kramer, L. D., Jones, M. J., Marra, P. P., Daszak, P. & Fonseca, D. M. (2007) Genetic influences on mosquito feeding behavior and the emergence of zoonotic pathogens. *American Journal of Tropical Medicine and Hygiene,* 77**,** 667-671.

Kothera, L., Nelms, B. M., Reisen, W. K. & Savage, H. M. (2013) Population genetic and admixture analyses of *Culex pipiens* complex (Diptera: Culicidae) populations in California, United States. *American Journal of Tropical Medicine and Hygiene,* 89**,** 1154-1167.

Koyama, J., Kakinohana, H. & Miyatake, T. (2004) Eradication of the melon fly, *Bactrocera cucurbitae,* in Japan: Importance of behavior, ecology, genetics, and evolution. *Annual Review of Entomology,* 49**,** 331-349.

Long, T. A. F., Pischedda, A., Stewart, A. D. & Rice, W. R. (2009) A cost of sexual attractiveness to high-fitness females. *Plos Biology,* 7.

Macnamara, M. & Paterson, H. E. H. (1984) The recognition concept of species. *South African Journal of Science,* 80**,** 312-318.

Mciver, S. B. (1982) Sensilla mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology,* 19**,** 489-535.

Michaelakis, A., Mihou, A. P., Couladouros, E. A., Zounos, A. K. & Koliopoulos, G. (2005) Oviposition responses of *Culex pipiens* to a synthetic racemic *Culex quinquefasciatus* oviposition aggregation pheromone. *Journal of Agricultural and Food Chemistry,* 53**,** 5225-5229.

Oliva, C. F., Jacquet, M., Gilles, J., Lemperiere, G., Maquart, P. O., Quilici, S., Schooneman, F., Vreysen, M. J. & Boyer, S. (2012) The sterile insect technique for controlling populations of *Aedes albopictus* (Diptera: Culicidae) on Reunion Island: mating vigour of sterilized males. *Plos One,* 7**,** e49414.

Packer, M. J. & Corbet, P. S. (1989) Size variation and reproductive success of female *Aedes punctor* (Diptera, Culicidae). *Ecological Entomology,* 14**,** 297-309.

Pennetier, C., Warren, B., Dabire, K. R., Russell, I. J. & Gibson, G. (2010) "Singing on the wing" as a mechanism for species recognition in the malarial mosquito *Anopheles gambiae. Current biology : CB,* 20**,** 131-6.

Pitts, R. J., Mozuraitis, R., Gauvin-Bialecki, A. & Lemperiere, G. (2014) The roles of kairomones, synomones and pheromones in the chemically-mediated behaviour of male mosquitoes. *Acta tropica,* 132 Suppl**,** S26-34.

Polerstock, A. R., Eigenbrode, S. D. & Klowden, M. J. (2002) Mating alters the cuticular hydrocarbons of female *Anopheles gambiae* sensu stricto and *Aedes aegypti* (Diptera: Culicidae). *Journal of Medical Entomology,* 39**,** 545-52.

Reusken, C. B., De Vries, A., Buijs, J., Braks, M. A., Den Hartog, W. & Scholte, E. J. (2010) First evidence for presence of *Culex pipiens* biotype molestus in the Netherlands, and of hybrid biotype pipiens and molestus in northern Europe. *Journal of vector ecology : journal of the Society for Vector Ecology,* 35**,** 210-2.

Richards, S. L., Anderson, S. L. & Yost, S. A. (2012) Effects of blood meal source on the reproduction of *Culex pipiens quinquefasciatus* (Diptera: Culicidae). *Journal of Vector Ecology,* 37**,** 1-7.

Rivero, A., Magaud, A., Nicot, A. & Vezilier, J. (2011) Energetic cost of insecticide resistance in *Culex pipiens* mosquitoes. *Journal of Medical Entomology,* 48**,** 694-700.

Roberts, D. R. & Andre, R. G. (1994) Insecticide resistance issues in vector-borne disease-control. *American Journal of Tropical Medicine and Hygiene,* 50**,** 21-34.

Rozeboom, L. E. & Gilford, B. N. (1954) Sexual isolation between populations of the *Culex pipiens* complex in North America. *The Journal of parasitology,* 40**,** 237-44.

Rudolf, M., Czajka, C., Borstler, J., Melaun, C., Jost, H., Von Thien, H., Badusche, M., Becker, N., Schmidt-Chanasit, J., Kruger, A., Tannich, E. & Becker, S. (2013) First nationwide surveillance of *Culex pipiens* complex and *Culex torrentium* mosquitoes demonstrated the presence of *Culex pipiens* biotype pipiens/molestus hybrids in Germany. *Plos One,* 8**,** e71832.

Sallum, M. A. M., Peyton, E. L. & Wilkerson, R. C. (2005) Six new species of the *Anopheles leucosphyrus* group, reinterpretation of *An. elegans* and vector implications. *Medical and Veterinary Entomology,* 19**,** 158-199.

Service, M. W. (1994) Male swarming of the mosquito *Culex (Cx.) torrentium* in England. *Medical and Veterinary Entomology,* 8**,** 95-98.

Shaw, W. R., Attardo, G. M., Aksoy, S. & Catteruccia, F. (2015) A comparative analysis of reproductive biology of insect vectors of human disease. *Current Opinion in Insect Science,* 10**,** 142-148.

Taai, K., Harbach, R. E., Aupalee, K., Srisuka, W., Yasanga, T., Otsuka, Y. & Saeung, A. (2017) An effective method for the identification and separation of *Anopheles minimus*, the primary malaria vector in Thailand, and its sister species *Anopheles harrisoni*, with a comparison of their mating behaviors. *Parasites & vectors,* 10**,** 97.

Tuten, H. C., Stone, C. M. & Dobson, S. L. (2013) Swarming behavior of *Aedes polynesiensis* (Diptera: Culicidae) and characterization of swarm markers in American Samoa. *Journal of Medical Entomology,* 50**,** 740-7.

Warren, B., Gibson, G. & Russell, I. J. (2009) Sex recognition through midflight mating duets in *Culex* mosquitoes is mediated by acoustic distortion. *Current Biology,* 19**,** 485-491.

Wekesa, J. W., Brogdon, W. G., Hawley, W. A. & Besansky, N. J. (1998) Flight tone of field-collected populations of *Anopheles gambiae* and *An. arabiensis* (Diptera : Culicidae). *Physiological Entomology,* 23**,** 289-294.

Wijit, A., Taai, K., Dedkhad, W., Hempolchom, C., Thongsahuan, S., Srisuka, W., Otsuka, Y., Fukuda, M. & Saeung, A. (2016) Comparative studies on the stenogamous and eurygamous behavior of eight *Anopheles* species of the hyrcanus group (Diptera: Culicidae) in Thailand. *Insects,* 7.

Yamamoto, D. & Koganezawa, M. (2013) Genes and circuits of courtship behaviour in *Drosophila* males. *Nature reviews. Neuroscience,* 14**,** 681-92.

CHAPTER FIVE

RNA-Seq Reveals Significant Genetic Variants between Adult Females of *Culex pipiens* f. pipiens and *Culex pipiens* f. molestus.

Abstract

The *Culex pipiens* complex mosquitoes are known to transmit diseases such as West Nile virus, St. Louis encephalitis virus, Rift Valley fever virus, and lymphatic filariases. Members of the *Cx. pipiens* complex are highly conserved morphologically but have distinct differences in ecological and physiological traits that require further development for distinction between the biotypes of this complex species. Single nucleotide polymorphisms (SNPs) have become the marker of choice for evolutionary genetics studies in many species. High-throughput sequencing of RNA was developed primarily to analyze global gene expression and is also an efficient way to discover SNPs from the expressed genes. In this study, we conducted transcriptome sequencing of the two biotypes of *Cx. pipiens* using Illumina HiSeq2000 platform to identify gene-associated SNPs from the two biotypes (form pipiens vs. form molestus). These SNPs were located in 19,363 expressed genes and 3,171 scaffolds of *Cx. quinquefasciatus* genome. The results suggest that RNA-Seq is an efficient and cost-effective approach to discover gene-associated SNPs in non-model organisms. This data will be a useful resource for and allele specific expression and functional analysis.

Keywords: *Culex pipiens* f. molestus, *Culex pipiens* f. pipiens, *Culex quinquefasciatus*, single nucleotide polymorphisms, RNA-seq, transcriptome

Introduction

Mosquitoes have been successful at spreading infectious diseases all over the world, posing risk of epidemics (Lounibos, 2002). Among the different types of mosquitoes, the genus *Culex* is one of the most widespread and is a known vector of West Nile Virus (WNV), St. Louis encephalitis, and other diseases (Farajollahi et al., 2011, Turell et al., 2010, Reinsen 2013). Generally believed to first evolve in Africa, the *Culex pipiens* complex have become commonplace in almost every continent due to human activities and transport (Harbach, 2012). Recently, the *Culex pipiens* complex have become a point of interest for a WNV outbreak in North America (Turell, 2002) supporting *Culex pipiens* complex' expansion as a vector.

Culex pipiens complex mosquitoes have two biotypes namely, *Cx. pipiens* form pipiens and *Cx. pipiens* f. molestus. They are well adapted in different environments following the micro-evolutionary divergences of eco-physiologically relevant traits. These adaptations are also associated with a complicated transmission cycle of human pathogens, which include variations of winter diapause, habitat selections, mating patterns, and host preferences. Hybridization between forms of *Culex pipiens* complex is found in North America and can serve to be a bridge between non-human biters to human biters (Fonseca et al., 2004a). Once hybridization possibilities are also considered, this complicated transmission cycle becomes even more complex for disease vector control. Thus, developing biotype-specific molecular markers is important to understand evolutionary dynamics of the eco-physiological traits and transmission cycle of vector-borne diseases.

Informative molecular markers are needed for future studies in disease vector control and for the assembly of *Culex* genomes which is still fragmented (Naumenko et al., 2015). Although fragmented, a reference genome from a sister species (*Cx. quinqufasciatus*) is available for genomic studies in the *Cx. pipiens* complex. Likewise, other mosquito genomes such as *Anopheles gambiae* and *Aedes aegypti* have relatively well assembled supercontigs and have been successfully utilized for functional studies to further understand their traits related to vectoral capacity (Juneja et al., 2014, Li et al., 2013). Many have chosen single nucleotide polymorphisms (SNPs) as their molecular marker of choice due to their abundance and usefulness in a wide range of studies (Tormey et al., 2015, David et al., 2014, Bonizzoni et al., 2013, Cohuet et al., 2008).

Further progress in next generation sequencing allows for genome-wide and transcriptome-wide studies to fully utilize genetic markers for studying loci that link to a trait relevant to disease transmission (Ozsolak and Milos, 2011, Metzker, 2010). One of the sequencing techniques, RNA-Seq, generates sequences on a large scale with low cost in comparison to the conventional Sanger sequencing and microarray (Sanger et al., 1977). RNA-seq has been utilized to identify genome-wide SNPs from various species (Chepelev et al., 2009, Cirulli et al., 2010, Cloonan et al., 2008, Morin et al., 2008) and can also be useful for identification of candidate genes in *Cx. pipiens* complex mosquitoes for future functional studies (Vasemagi et al., 2005). The aim of this study was to utilize RNA-seq for identifying biotype-specific SNP markers from the *Cx. pipiens* complex mosquitoes for future functional genetic studies to elucidate the causative link to stenogamy mating behavior in the mosquito *Culex pipiens* f. molestus.

Materials and Methods

Mosquito Rearing

Culex pipiens form pipiens and *Culex pipiens* form molestus specimens are raised under 75% relative humidity, 25 ^{o}C, and a 15 hour light: 9 hour dark (L:D) daily light cycle. Tetramin fish food is fed to larvae (Tetra holding inc., Blacksburg, VA), while adults are maintained on honey-soaked sponges. *Cx pipiens* f. pipiens colony was established in September 2000 from larvae collected in Columbus, OH, and additional field-collected mosquitoes were added to the laboratory colony in 2009 (Meuti et al 2015). *Cx. pipiens* f. molestus colony were provided by Dr. Linda Kothera at the Centers for Disease Control and Prevention Division of Vector Borne Infectious Diseases at Fort Collins, Colorado and originated from the Calumet Water Reclamation Plant in Chicago, Illinois (Mutebi and Savage 2009).

RNA sample preparation

Fifteen females were collected a week after adult eclosion from each form of *Culex pipiens* mosquitoes. Total RNA samples were extracted by using TRIzol Reagent (Life Technologies) with phase lock gel-heavy. A mix of 1mL of TRIzol Reagent per 50-100 mg of tissue was homogenized and total RNA was extracted using the TRIzol Reagent via the manufacturer's protocol. These prepared samples were kept in a -80 °C for storage until they were sent off to be used for transcriptome SNP analysis.

Library construction and sequencing

The standard Illumina RNA-seq sample collection kit was used followed by the protocol provided by the company (Illumina Inc., San Francisco, CA, USA). The mosquito RNA samples were purified by using polyA mRNA selection using magnetic beads with poly-T oligo-attached. This step was soon followed by a second round of poly-A RNA purification ensuing to RNA fragmentation for cDNA synthesis preparation. Utilizing random primers and reverse transcriptase, the RNA fragments are transcribed into the first strand of cDNA. The RNA template is replaced by the second strand cDNA changing the once single stranded cDNA to double strand. End Repair (ERP) was used to correct overhangs for both 5' and 3' UTR regions. After the final step of cDNA preparation, which is the ligating adapters to the ends of the fragments, PCR was run in order to amplify the DNA with adapter ends. This process was then validated and went through a quality control process.

Illumina data analysis

Reads from the RNA-Seq data were aligned to the *Culex quinquefasciatus* reference genome utilizing Bowtie (Langmead, 2010) a short read alignment tool. After alignment, it was then analyzed for splice junctions in the intronic areas using TopHat v1.3.3 (Trapnell et al., 2014). Cufflinks (Trapnell et al., 2014) was also used to assemble transcripts as it figures the transcript repeats or abundance to the main alignment. The RNA-Seq reads went through quality control using the manufacturer's guide (Illumina Inc., San Francisco, CA, USA).

Transcript based variant discovery and annotation

Cleaned and trimmed HiSeq reads were aligned to the genome of *Culex quinquefasciatus* (Johannesburg strain version 2.2) with TopHat.\Freebayes (Garrison & Marth, 2012), a haplotype based variant detector, was utilized in the identification of SNP variation. SNP and indel discovery as well as genotyping between two samples was performed simultaneously using hard filtering parameters appropriate for RNA-seq data. Prior to variant discovery, reads in regions identified as possible indels were realigned in Freebayes as recommended (Garrison & Marth, 2012).

To increase the confidence of variant calls, we filtered out low-quality variants with a DP < 10 and QUAL < 40 using VCFannotate and VCFfilter (Danecek et al. 2011). DP combines depth across samples and QUAL is scales PHRED probability of variation (REF/ALT). Thus, combining the two filter sets is expected to produce high quality results for subsequent analysis.

The localization of SNPs/indels in both genic regions and intergenic regions was based on annotation of gene models provided by the *Culex quinquefasciatus* genome database (Johannesburg strain version 2.2). The putative SNPs/indels were tagged and scored as coding/non-coding, silent/missense with strand positions and ontology via SnpEff (Cingolani et al. 2012) and SnpSift (Cingolani et al. 2012). Genes with significantly differential expression between biotypes were given careful consideration while testing for SNPs/indels. The localization of SNPs/indels were categorized based on coding-regions, 5'-upstream or 3'downstream regions. The bioinformatics tools used study FPKM and the putative SNPs/indels were summarized in the flowchart (Fig. 1).

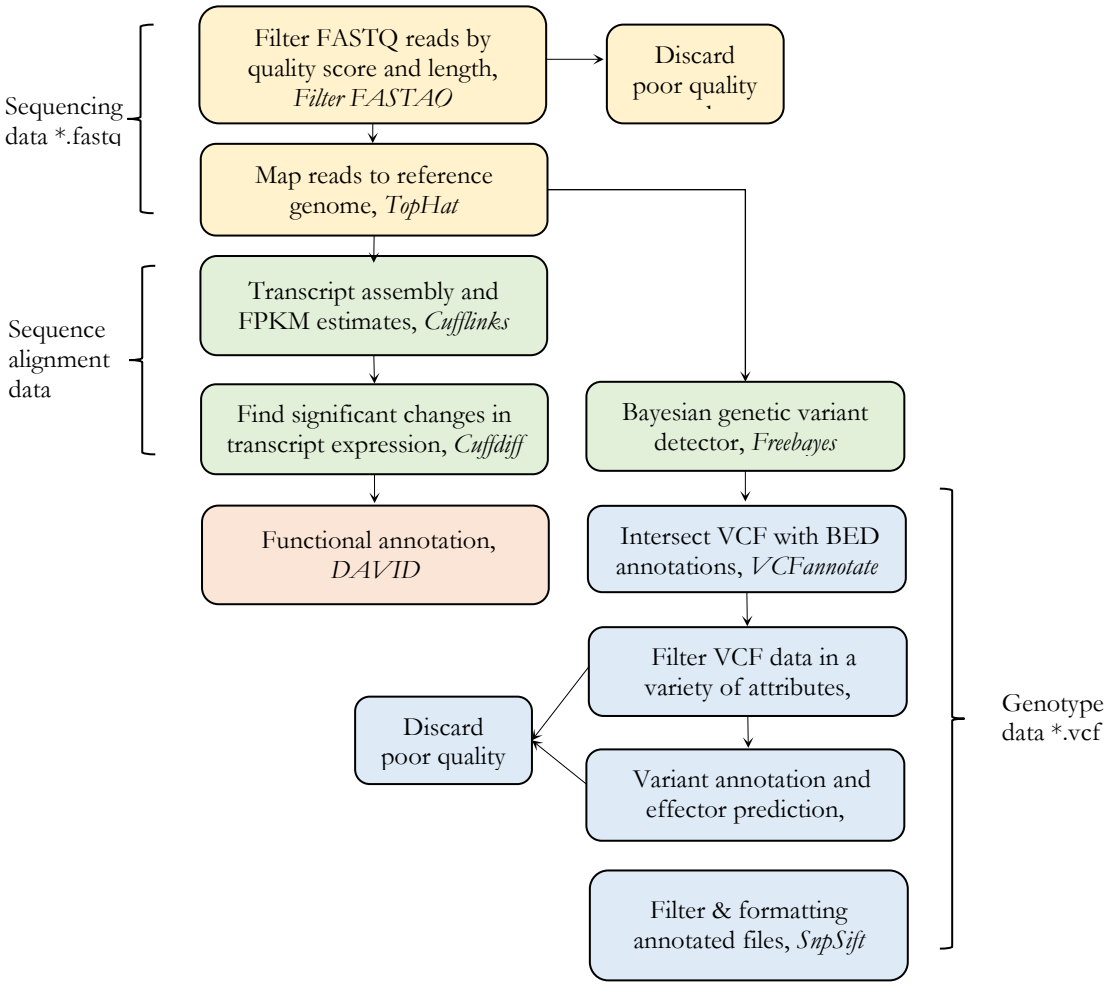


Figure 5.1. Flow diagram for the RNA-sequencing data analysis. Data file types are indicate at the either side (*). Each group of node is also colored by file type. Software platforms are in typed in italics.

The putative SNPs/indels among the five validated transcripts by qRT-PCR

Five genes with high levels of transcripts were selected in previous study based on fold change, statistical significance, and relevant ontologies as targets for vector control (Kang 2015). An iQ5 real-time PCR detection system (Bio-Rad, Hercules, CA) was then used for qRT-PCR validation with ribosomal protein L19 (RpL19). These genes include: adult cuticle protein 1 precursor, hexamerin 1.1 precursor, juvenile hormone esterase, cathepsin C, and an odorant receptor. The results were then subjected to a Student's t-test to examine statistical significance.

The putative SNPs/indels were identified among these five genes which show differential expression between two biotypes. The localization of SNPs/indels were tagged and by coding/non-coding regions and silent/missense/nonsense mutations.

Results

Analysis of genetic variants from RNA-seq reads.

A total of 73,568 and 73,375 putative SNPs was discovered from *Cx. pipiens* f. pipiens and f. molestus, respectively. These SNPs were located in 19,363 genes and 3,171 scaffolds of reference genome *Cx. quinquefasciatus*. SNPs were then divided into the categories include: 5'-UTR, 3'UTR, CDS, intergenic, splice sites, upstream regions and downstream regions (Table 5.1 and Suppl. file 3 and 4). Form pipiens showed 681, 2,319, 42,874 and 513 variants in 5'-UTR, 3'-UTR, coding and splice sites along with other variant effects, respectively and f. molestus showed 534, 2,030, 49,811 and 591, respectively (Table 5.1). As expected, both forms showed the highest number of

sequence variants in the silent effect category followed by missense mutation. *Cx. pipiens* f. pipiens showed 36,113 silent effects and Form molestus had 43,330 (Suppl. file 3 and 4).

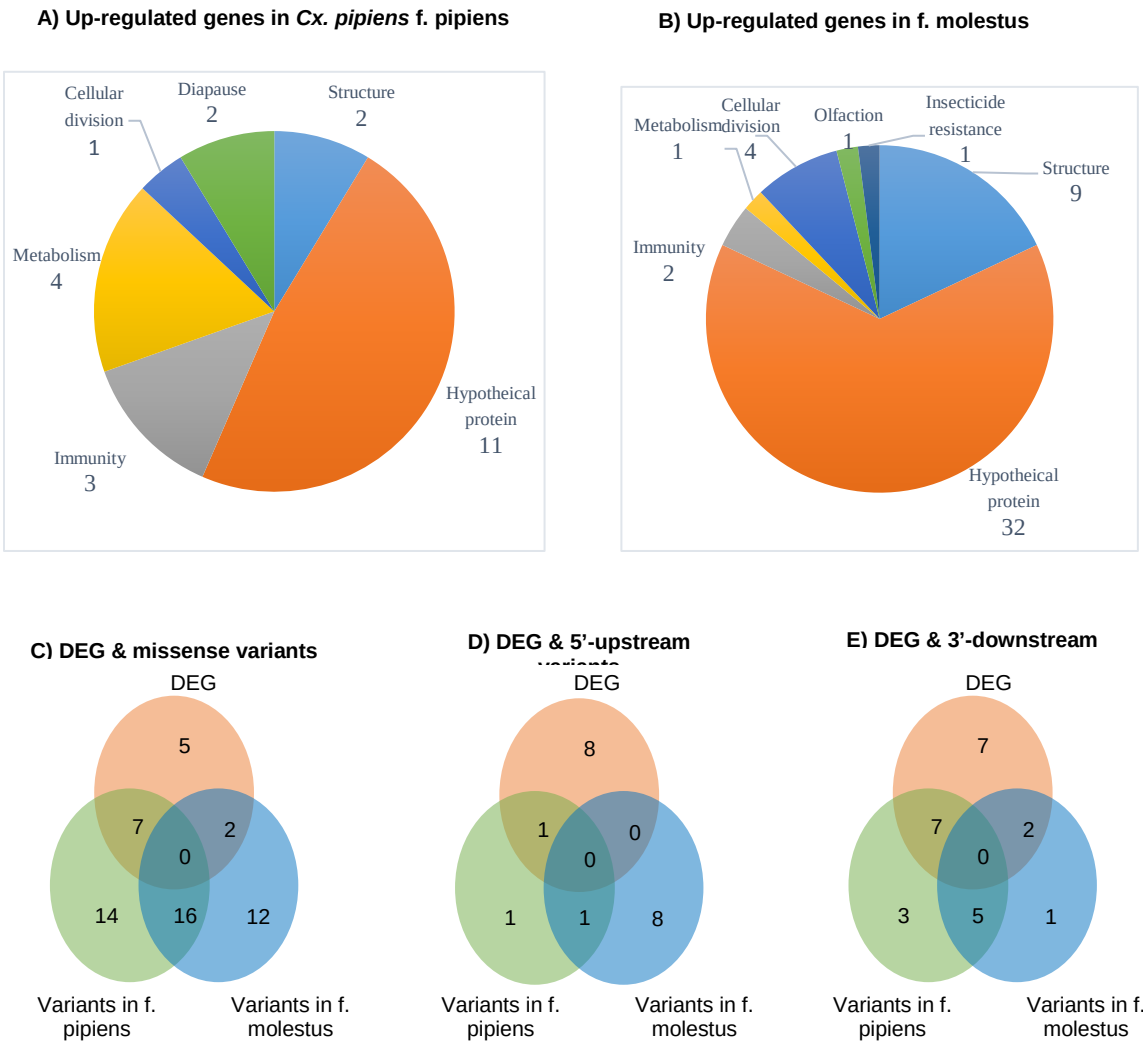


Figure 5.2. Distribution of the Gene Ontology (GO) functional categories and transcriptome-wide sequence polymorphisms. The Differentially Expressed Genes (DEGs) identified using RNA-Seq of the *Cx. pipiens* form molestus (A) and f. pipiens (B) were classified into GO categories based on biological process, molecular function and cellular component. Venn diagram depicting overlap between Differentially Expressed Genes (DEGs) identified using RNA-Seq and missense variants (D), variants in 5'-UTR (D), and variants in 3'-UTR (E) using SnpEff and SnpSift.

Table 5.1. Number of variant effects by genomic region.

Genomic region	Variant count (%)	
	f. pipiens	f. molestus
5'-UTR	681 (0.5)	534 (0.4)
3'-UTR	2,319 (1.6)	2,030 (1.5)
CDS	42,874 (30.4)	49,811 (28.2)
INTERGENIC	25,210 (17.9)	19,038 (14.0)
INTRON	2,941 (2.1)	2,511 (1.8)
SPLICE SITES	513 (0.3)	591 (0.4)
UPSTREAM (up 5Kb)	24,297 (17.2)	23,429 (17.2)
DOWNSTREM (down to 5Kb)	42,288 (30.0)	38,497 (28.2)
Total	73,568	73,375

To identify putative SNPs/Indels that can modify the functions or transcription levels, genetic variants were further analyzed among the genes that showed significantly different transcript abundance between the two biotypes (Kang, 2015). Then, these genetic variants are compared and divided into three categories include: missense, 5'-UTR and 3'UTR. The missense variants were found 7 and 25 among the differentially expressed genes between *Cx. pipiens* f. pipiens and f. molestus, respectively (Fig. 5.3 and Suppl. file 5). Among the variants found in 5'-UTR, only 1 SNP was found in f. pipiens. Among the variants found in 3'-UTR, 7 and 2 among the differentially expressed genes between *Cx. pipiens* f. pipiens and f. molestus, respectively (Fig. 5.3).

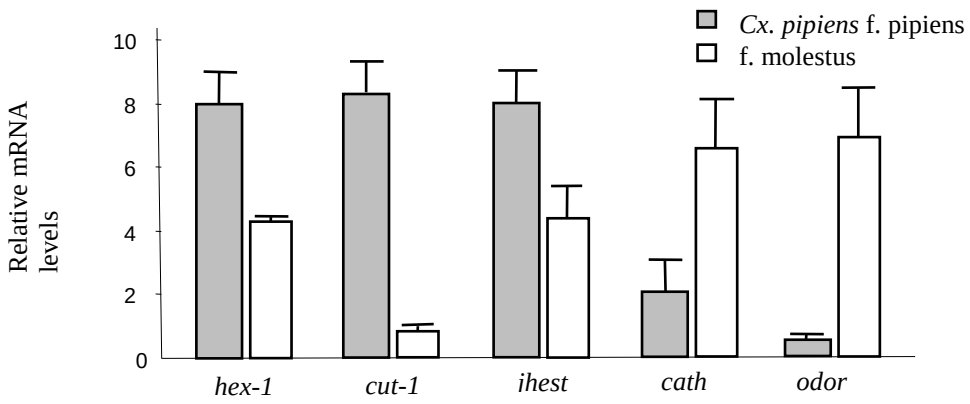
Analysis of genetic variants among the candidate genes validated by qRT-PCR.

The five genes showed significantly different transcript abundance between adult, females of *Cx. pipiens* f. pipiens and f. molestus. To identify the putative SNPs/Indels in the five genes, we compared the sequences of *Cx. pipiens* f. pipiens and f. molestus with

the reference genome of *Cx. quinquefasciatus*. With the exception of *cut-1*, all transcripts include a high number or SNPs either in the coding and/or noncoding regions.

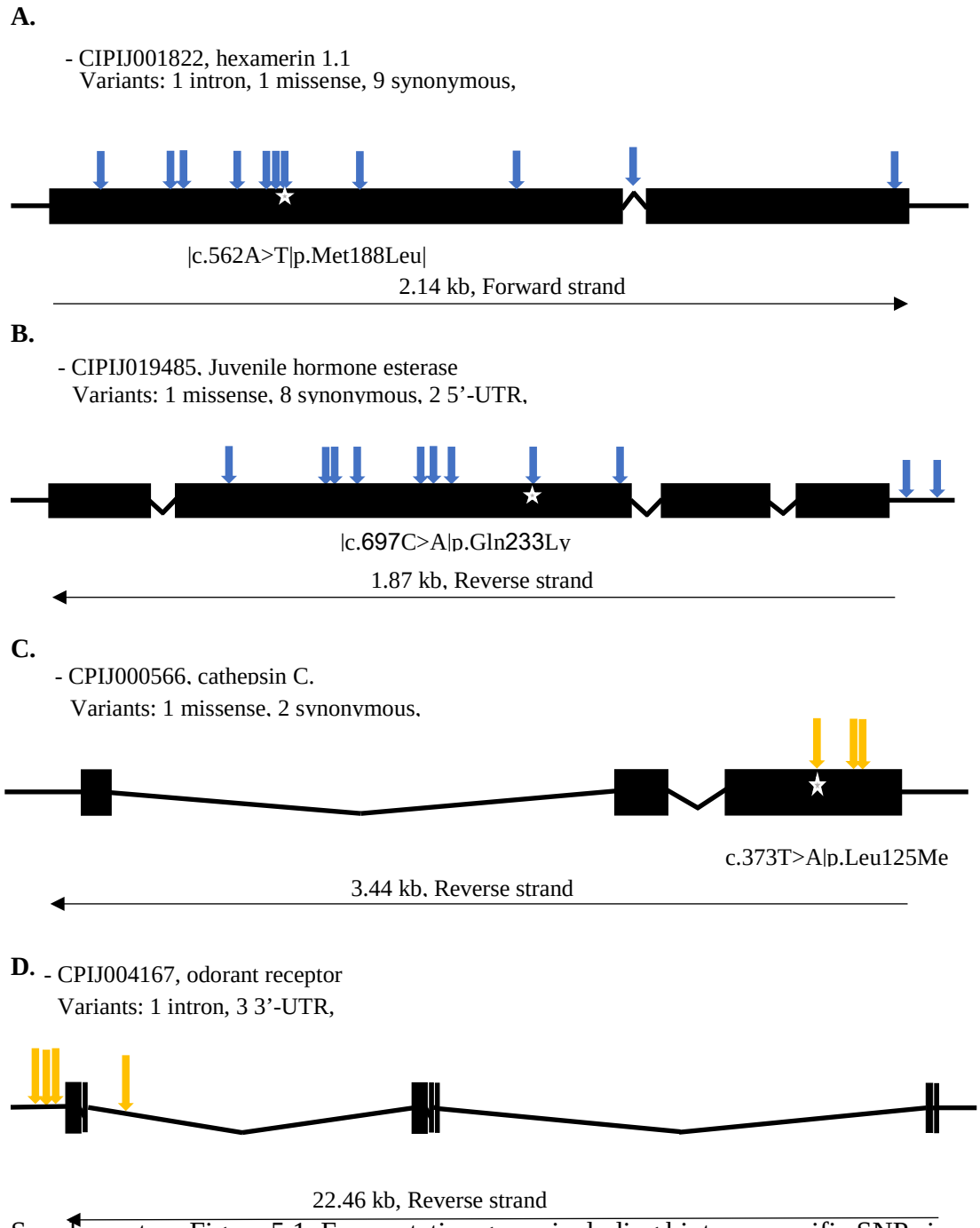
In *Cx. pipiens* f. pipiens, the gene (*hex-1*, CPIJ001822) encoding hexamerin precursor includes 1 missense and 9 silent mutations in the coding region and 1 splice site, but no variation was detected in f. molestus (Fig. 5.3 and Fig. S5.1). The missense mutation is a transition (562 A>G) that replaces methionine at position 188 with leucine (Fig. 5.3 and Fig. S5.1). The gene (*jhest*, CPIJ0019485) encoding juvenile hormone esterase also includes 1 missense and 8 silent mutations in the coding region and 2 in 5'-UTR, with no variation detected in f. molestus. The missense mutation is a transversion (697 A>T) that replaces glutamine at position 188 with lysine (Fig. 5.3 and Fig. S5.1).

In f. molestus, the gene (*cath*, CIPJ000566) encoding cathepsin C includes 1 missense and 2 silent mutations in the coding region, with no variation detected in f. pipiens. The missense mutation is a transversion (373 T>A) that replaces leucine at position 125 with methionine (Fig. 5.3 and Fig. S5.1). The gene (*odor*, CPIJ004167) encoding putative odorant receptor includes 1 SNP in splice site and 3 SNPs in 5'-UTR, with no variation detected in *Cx. pipiens* f. pipiens (Fig. 5.3 and Fig. S5.1). This gene showed significantly upregulation in adult females of f. molestus compared to those of *Cx. pipiens* f. pipiens.



	hex-1		*cut-1*		*ihest*		*cath*		*odor*	
Total SNPs	11	0	0	0	11	0	0	3	0	4
Noncoding	1	0	0	0	2	0	0	0	0	4
Silent	9	0	0	0	8	0	0	2	0	0
Missense	1	0	0	0	1	0	0	1	0	0
Indels	0	0	0	0	0	0	0	0	0	0

Figure 5.3. Expression abundance of female *Cx. pipiens* form molestus and f. pipiens at 7 days after adult eclosion via quantitative real-time PCR found in previous study (Kang 2015) Ribosomal protein large subunit 19 (RpL19) was used as a loading control. Error bars represent standard error. Total number of SNPs, noncoding, silent, missense mutations and indels were shown based on the respective genes and biotypes.



Supplementary Figure 5.1. Four putative genes including biotype-specific SNPs in females of *Cx. pipiens* form pipiens versus *Cx. pipiens* f. molestus. Blue and orange arrows are variants for f. pipiens and f. molestus respectively. Asterisks show missense mutations.

Discussion

To elucidate adaptive alleles, the process of adaptation, and the functionality of adaptive changes among the biotypes of *Cx. pipiens* complex, dense molecular markers are indispensable tools in marker-assisted selection, QTL mapping, allele-specific expression analysis, and genome-wide association study (Gompert et al., 2014, Zou et al., 2015, Shen et al., 2012, McCarthy et al., 2008). SNPs are utilized for this reason because of the recent advance of next-generation sequencing technology leading to low sequencing cost and relatively high accuracy in sequence readouts (Shendure and Ji, 2008, Ozsolak and Milos, 2011, Metzker, 2010)

We utilized RNA-seq to identify SNP markers in the transcriptome for two biotypes in *Cx. pipiens* complex. These fine-scale molecular markers can be utilized to link the genes to the traits relevant to disease transmission in the mosquitoes. In this study, we identified a total of 73,568 and 73,375 putative SNPs in *Cx. pipiens* f. pipiens and f. molestus, respectively. These SNPs were located in 19,363 genes and 3,171 scaffolds of reference genome *Cx. quinquefasciatus*. For future functional analysis, we then focused on the five candidate genes which are differentially expressed between females of the two biotypes from previous RNA-seq study (Kang 2015). The five genes include: adult cuticle protein 1 precursor, hexamerin 1.1 precursor, juvenile hormone esterase, cathepsin C, and an odorant receptor.

Adult cuticle protein 1 precursor (CPIJ003488) was the only gene of the five that did not have any variant for both f. molestus and f. pipiens. Despite the high expression patterns, adult cuticle protein is highly conserved in the biotypes. This is interesting since adult cuticle protein is needed for *Cx. pipiens* for deltamethrin resistance, a form of stress tolerance where the cuticle thickens providing a protective barrier (Fang, Wang et al.

2015, Li and Denlinger 2009). Likewise, cuticle thickening is attributed to slower rates of insecticide absorption (Wood et al 2010). Since *Culex* are highly resistant to contaminated environments, the lack of polymorphism in the biotypes is interesting and needs further investigation. Aside from metabolic detoxification, the reason for no SNP detection might be for functions that do not require much variation such as growth rings found in *Aedes* that help predict age (Wood et al 2010). For this gene, other trans-activating factors may play a significant role in gene regulation of the cuticle protein family.

In mosquitoes, hexamerins (CPIJ001822) are known to synthesize during the final stage of larval development in the fat body, secreted into the hemolymph and stored by the fat body. Indeed, in *Anopheles gambiae*, hexamerin 1.1 (*hex-1*) was found to show high expression during the fourth instar phase and then dropped during the pupal stage (Zakharkin et al 1997). This initiation of fat storage is later used in the mosquito's lifetime, particularly metamorphosis and reproduction (Totten, Vuong et al. 2013). Female mosquitoes require a vast number of amino acids for the production of egg proteins in vitellogenesis as well as other female specific reproduction factors. Autogenic females were shown to utilize fat storage from this stage for the development of egg protein without a blood meal (Zakharkin et al 2001). Although hexamerins are found in both males and females, some are sex specific as shown in *Aedes*. Indeed, *hex* genes in *Aedes* is thought to work similarly to the yolk protein of *Drosophila* and was found to have almost identical binding sites as the two male and female specific DSX proteins. Previous study shows the link between female specific larval hexamerin with autogeny which might be supported in the two biotypes (Zakharkin et al 2001). Although there was

a missense mutation (562 A>G) identified here, the impact is considered minimal. However, the role of each mutation in regulating the gene expression and in altering protein structure of hexamerin warrants further investigation. The broad usage of hexamerins in the mosquito life cycle make useful targets for vector control.

Similarly, with hexamerins, juvenile hormone esterase (*jhest*), serves many functions that are crucial for all stages of the mosquito life cycle (Lassiter, Apperson et al. 1995; Schomburg and Stephan 1998). In early development, JH hormone esterase modulates fat body competence which is crucial for larval and pupae stage but is also important for the number of blood feedings needed to complete the first gonotrophic cycle, increasing the likelihood for host-vector contact (Shiao, Hansen et al. 2008). JH esterase and JH III responds to blood meals of *Aedes* females while in males, JH III and I are synthesized by the male accessory glands (MAG) indicating a distinct JH III biosynthetic pathway in mosquito reproduction (Borovsky, Carlson et al. 1994). In *Drosophila* high levels of JH were found in females after copulation, triggering the process of gut remodeling and could be the case for mosquitoes after the delivery of the male mating plug (Rahman et al. 2017). JH regulates the fluctuations in the fat body during the pre-vitellogenic preparation phase and signals the fat body to be responsive to steroid hormone 20 E which is crucial for both mating behavior and egg formation. In the gene (*jhest*, CPIJ019485) encoding juvenile hormone esterase, 2 SNPs occur in 5'-UTR of the *jhest* gene in *Cx. pipiens* f. pipiens that were absent in f. molestus. Since SNPs in the 5' UTR can change the dynamics of regulatory factor interactions to the target elements (Hernandez-Garcia and Finer, 2014), it is interesting to see differences at the 5'

upstream regulatory region, indicating that these SNPs may be related to the differential expression level of *jhest* between the two biotypes.

Although much is unknown of Cathepsin C (*cath*, CPIJ000566) also known as DPP-I in insects, it is thought to initiate serine proteinases cascades that are involved in diverse immune related mechanisms (Turk, Janjić et al. 2001). In *Aedes*, serine proteases are known to show a direct link to digestive physiology targeting the midgut's ability to absorb and filter out needed nutrients (Isoe, Rascon et al. 2009). Specifically, studies showed DPP I's role in hemoglobin degradation (Hola-Jamriska, King et al. 2000) potentially supporting the differences in blood feeding patterns in the two biotypes. As expected, the autogenous form molestus females exhibited more than 3-fold upregulation of *cath*, which unsurprisingly coincided with an upregulation of serine proteases. Contrary to the *cut-1* and *jhest* genes, the single missense mutation in *cath* was unique to f. molestus.

In this study, only f. molestus harbors 4 SNPs in noncoding regions of the gene *odor*, (CPIJ004167) encoding putative odorant receptor absent in *Cx. pipiens* f. pipiens. This is interesting as odor receptors are key to many fundamental mechanisms. For example, females possess a wide array of odorant receptors mostly on their antennae which aid in detecting blood hosts, selecting oviposition sites and mate recognition (McIver, 1971, Bentley and Day 1989, Pennetier, Warren 2010). A recent study of stenogamy behavior in the *Cx. pipiens* f. molestus showed signs of mating preference that is thought to result from odorant and gustatory factors. Since the olfactory sensory neurons are dependent on olfactory receptors (Hallem et al., 2004, Kwon, Lu et al 2006), unsurprisingly, odor gene would particularity be an excellent target regarding stenogamy

mating behavior in *Cx. pipiens* f. molestus. However, much is still unknown regarding *Culex* mating behavior in general and thus requires further examination.

Most scientists distinguish the *Culex pipiens* complex biotypes by their distinctive physiological traits. However, the development of SNPs will contribute to the genetic background knowledge of these unique traits for further understanding. Here we have five potential candidate genes with unique biotype specific SNPs that can be utilized for future functional analysis and methods of vector control in the *Cx pipiens* complex.

References

Bentley, M. D. and J. F. Day (1989). "Chemical ecology and behavioral aspects of mosquito oviposition." Annual Review of Entomology **34**: 401-421.

Bonizzoni, M., M. Britton, et al. (2013). "Probing functional polymorphisms in the dengue vector, Aedes aegypti." BMC genomics **14**: 739.

Borovsky, D., D. A. Carlson, et al. (1994). "De-Novo Biosynthesis of Juvenile Hormone-Iii and Hormone-I by the Accessory-Glands of the Male Mosquito." Insect Biochemistry and Molecular Biology **24**(5): 437-444.

Chepelev, I., G. Wei, et al. (2009). "Detection of single nucleotide variations in expressed exons of the human genome using RNA-Seq." Nucleic acids research **37**(16): e106.

Cingolani, P., A. Platts, et al. (2012). "A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3." Fly **6**(2): 80-92.

Cirulli, E. T., A. Singh, et al. (2010). "Screening the human exome: a comparison of whole genome and whole transcriptome sequencing." Genome biology **11**(5): R57.

Cloonan, N., A. R. Forrest, et al. (2008). "Stem cell transcriptome profiling via massive-scale mRNA sequencing." Nature methods **5**(7): 613-619.

Cohuet, A., S. Krishnakumar, et al. (2008). "SNP discovery and molecular evolution in Anopheles gambiae, with special emphasis on innate immune system." BMC genomics **9**: 227.

Danecek, P., A. Auton, et al. (2011). "The variant call format and VCFtools." Bioinformatics **27**(15): 2156-2158.

David, J. P., F. Faucon, et al. (2014). "Comparative analysis of response to selection with three insecticides in the dengue mosquito Aedes aegypti using mRNA sequencing." BMC genomics **15**: 174.

Fang, F. J., W. J. Wang, et al. (2015). "The cuticle proteins: a putative role for deltamethrin resistance in Culex pipiens pallens." Parasitology research **114**(12): 4421-4429.

Farajollahi, A., D. M. Fonseca, et al. (2011). ""Bird biting" mosquitoes and human disease: A review of the role of Culex pipiens complex mosquitoes in epidemiology." Infection Genetics and Evolution **11**(7): 1577-1585.

Fonseca, D. M., N. Keyghobadi, et al. (2004). "Emerging vectors in the Culex pipiens complex." Science **303**(5663): 1535-1538.

Garrison, E. & Marth, G. (2012) Haplotype-based variant detection from short-read sequencing. Preprint at arXiv:1207.3907v2 [q-bio.GN].

Gompert, Z., A. A. Comeault, et al. (2014). "Experimental evidence for ecological selection on genome variation in the wild." Ecology letters **17**(3): 369-379.

Hallem, E. A., A. Nicole Fox, et al. (2004). "Olfaction: mosquito receptor for human-sweat odorant." Nature **427**(6971): 212-213.

Harbach, R. E. (2012). "Culex pipiens: species versus species complex taxonomic history and perspective." Journal of the American Mosquito Control Association **28**(4 Suppl): 10-23.

Hernandez-Garcia, C. M. and J. J. Finer (2014). "Identification and validation of promoters and cis-acting regulatory elements." Plant science : an international journal of experimental plant biology **217-218**: 109-119.

Hola-Jamriska, L., L. T. King, et al. (2000). "Functional expression of dipeptidyl peptidase I (cathepsin C) of the oriental blood fluke Schistosoma japonicum in Trichoplusia ni insect cells." Protein Expression and Purification **19**(3): 384-392.

Isoe, J., A. A. Rascon, et al. (2009). "Molecular genetic analysis of midgut serine proteases in Aedes aegypti mosquitoes." Insect Biochemistry and Molecular Biology **39**(12): 903-912.

Juneja, P., J. Osei-Poku, et al. (2014). "Assembly of the genome of the disease vector Aedes aegypti onto a genetic linkage map allows mapping of genes affecting disease transmission." PLoS neglected tropical diseases **8**(1): e2652.

Kang, David S. “Genomic Analysis of the Diapause Program in the West Nile Virus Vector Culex Pipiens.” *BEARdocs Home, Baylor University*, 1 Dec. 2015, baylor-ir.tdl.org/baylor-ir/handle/2104/9560.

Kwon, H. W., T. Lu, et al. (2006). "Olfactory responses in a gustatory organ of the malaria vector mosquito Anopheles gambiae." Proceedings of the National Academy of Sciences of the United States of America **103**(36): 13526-13531.

Langmead, B. (2010). "Aligning short sequencing reads with Bowtie." Current protocols in bioinformatics / editoral board, Andreas D. Baxevanis ... [et al.] **Chapter 11**: Unit 11 17.

Lassiter, M. T., C. S. Apperson, et al. (1995). "Juvenile hormone metabolism during the fourth stadium and pupal stage of the southern house mosquito, Culex quinquefasciatus Say." Journal of Insect Physiology **41**(10): 869-876.

Li, J., X. Wang, et al. (2013). "Genome-block expression-assisted association studies discover malaria resistance genes in Anopheles gambiae." Proceedings of the National Academy of Sciences of the United States of America **110**(51): 20675-20680.

Lounibos, L. P. (2002). "Invasions by insect vectors of human disease." Annual Review of Entomology **47**: 233-266.

McCarthy, M. I., G. R. Abecasis, et al. (2008). "Genome-wide association studies for complex traits: consensus, uncertainty and challenges." Nature reviews. Genetics **9**(5): 356-369.

McIver, S. (1971). "Comparative studies on the sense organs on the antennae and maxillary palps of selected male culicine mosquitoes." Canadian journal of zoology **49**(2): 235-239.

Metzker, M. L. (2010). "Sequencing technologies - the next generation." Nature reviews. Genetics **11**(1): 31-46.

Meuti, M. E., M. Stone, et al. (2015). "Functional circadian clock genes are essential for the overwintering diapause of the Northern house mosquito, Culex pipiens." Journal of Experimental Biology **218**(3): 412-422.

Morin, R. D., M. D. O'Connor, et al. (2008). "Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells." Genome research **18**(4): 610-621.

Mutebi, J. P. and H. M. Savage (2009). "Discovery of Culex pipiens pipiens form molestus in Chicago." J Am Mosq Control Assoc **25**(4): 500-503.

Naumenko, A. N., V. A. Timoshevskiy, et al. (2015). "Mitotic-Chromosome-Based Physical Mapping of the Culex quinquefasciatus Genome (vol 10, e0115737, 2015)." PloS one **10**(6).

Ozsolak, F. and P. M. Milos (2011). "RNA sequencing: advances, challenges and opportunities." Nature reviews. Genetics **12**(2): 87-98.

Pennetier, C., B. Warren, et al. (2010). ""Singing on the wing" as a mechanism for species recognition in the malarial mosquito Anopheles gambiae." Current biology : CB **20**(2): 131-136.

Rahman, M. M., X. Franch-Marro, et al. (2017). "Local Juvenile Hormone activity regulates gut homeostasis and tumor growth in adult Drosophila." Scientific Reports **7**(1): 11677.

Reisen, W. K., M. M. Milby, et al. (1992). "Ecology of Mosquitos and St-Louis Encephalitis-Virus in the Los-Angeles Basin of California, 1987-1990." Journal of Medical Entomology **29**(4): 582-598.

Sanger, F., S. Nicklen, et al. (1977). "DNA Sequencing with Chain-Terminating Inhibitors." Proceedings of the National Academy of Sciences of the United States of America **74**(12): 5463-5467.

Schomburg, D. and D. Stephan (1998). Juvenile-hormone esterase. Enzyme Handbook 15. D. Schomburg and D. Stephan, Springer Berlin Heidelberg**:** 21-26.

Shen, Y., J. Catchen, et al. (2012). "Identification of transcriptome SNPs between Xiphophorus lines and species for assessing allele specific gene expression within F(1) interspecies hybrids." Comparative biochemistry and physiology. Toxicology & pharmacology : CBP **155**(1): 102-108.

Shendure, J. and H. Ji (2008). "Next-generation DNA sequencing." Nature biotechnology **26**(10): 1135-1145.

Shiao, S. H., I. A. Hansen, et al. (2008). "Juvenile hormone connects larval nutrition with target of rapamycin signaling in the mosquito Aedes aegypti." Journal of insect physiology **54**(1): 231-239.

Tormey, D., J. K. Colbourne, et al. (2015). "Evolutionary divergence of core and post-translational circadian clock genes in the pitcher-plant mosquito, Wyeomyia smithii." BMC genomics **16**(1): 754.

Totten, D. C., M. Vuong, et al. (2013). "Targeting gene expression to the female larval fat body of transgenic Aedes aegypti mosquitoes." Insect Molecular Biology **22**(1): 18-30.

Trapnell, C., A. Roberts, et al. (2014). "Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks (vol 7, pg 562, 2012)." Nature Protocols **9**(10): 2513-2513.

Turell, M. J., M. R. Sardelis, et al. (2002). "Potential vectors of West Nile virus in North America." Current topics in microbiology and immunology **267**: 241-252.

Turell, M. J., W. C. Wilson, et al. (2010). "Potential for North American Mosquitoes (Diptera: Culicidae) to Transmit Rift Valley Fever Virus." Journal of Medical Entomology **47**(5): 884-889.

Turk, D., V. Janjić, et al. (2001). "Structure of human dipeptidyl peptidase I (cathepsin C): exclusion domain added to an endopeptidase framework creates the machine for activation of granular serine proteases." The EMBO Journal **20**(23): 6570-6582.

Vasemagi, A., J. Nilsson, et al. (2005). "Expressed sequence tag-linked microsatellites as a source of gene-associated polymorphisms for detecting signatures of divergent selection in atlantic salmon (Salmo salar L.)." Molecular biology and evolution **22**(4): 1067-1076.

Wood, O., S. Hanrahan, et al. (2010). "Cuticle thickening associated with pyrethroid resistance in the major malaria vector Anopheles funestus." Parasites & vectors **3**: 67.

Zakharkin, S. O., A. V. Gordadze, et al. (1997). "Molecular cloning and expression of a hexamerin cDNA from the malaria mosquito, Anopheles gambiae." European journal of biochemistry **246**(3): 719-726.

Zakharkin, S. O., V. V. Headley, et al. (2001). "Female-specific expression of a hexamerin gene in larvae of an autogenous mosquito." European journal of biochemistry **268**(22): 5713-5722.

Zhou, X., M. R. Tarver, et al. (2007). "Hexamerin-based regulation of juvenile hormone-dependent gene expression underlies phenotypic plasticity in a social insect." Development **134**(3): 601-610.

Zou, F., C. Chen, et al. (2015). "Identification of QTLs Conferring Resistance to Deltamethrin in Culex pipiens pallens." PloS one **10**(10): e0140923.

COMPILED REFERENCES

Ajamma, Y. U., Mararo, E., Omondi, D., Onchuru, T., Muigai, A. W., Masiga, D. & Villinger, J. 2016. Rapid and high throughput molecular identification of diverse mosquito species by high resolution melting analysis. *F1000Research,* 5, 1949.

Ameneshewa, B. & Service, M. W. (1996) The relationship between female body size and survival rate of the malaria vector *Anopheles arabiensis* in Ethiopia. *Medical and Veterinary Entomology,* 10, 170-172.

Arthofer, W., L. Bertini, et al. (2015). "Genomic Resources Notes accepted 1 February 2015 - 31 March 2015." Molecular ecology resources 15(4): 1014-1015.

Bahnck, C. M. & Fonseca, D. M. 2006. Rapid assay to identify the two genetic forms of *Culex* (*Culex*) *pipiens* L. (Diptera : Culicidae) and hybrid populations. *American Journal of Tropical Medicine and Hygiene,* 75, 251-255.

Barr, A. R. (1957) The distribution of *Culex pipiens pipiens* and *Cx. quinquefasciatus* in North America. *American Journal of Tropical Medicine and Hygiene,* 6, 153-165.

Bateman, A. J. (1948) Intra-sexual selection in *Drosophila melanogaster*. *Heredity,* 2, 277-277.

Becker, N., D. Petric, et al. (2010). "Mosquitoes and Their Control, Second Edition." Mosquitoes and Their Control, Second Edition: 1-577.

Becker, N., Jost, A. & Weitzel, T. (2012) The *Culex pipiens* complex in Europe. *Journal of the American Mosquito Control Association,* 28, 53-67.

Bentley, M. D. and J. F. Day (1989). "Chemical ecology and behavioral aspects of mosquito oviposition." Annual Review of Entomology 34: 401-421.

Billeter, J. C., Atallah, J., Krupp, J. J., Millar, J. G. & Levine, J. D. (2009) Specialized cells tag sexual and species identity in *Drosophila melanogaster*. *Nature,* 461, 987-91.

Blay, S. & Yuval, B. (1997) Nutritional correlates of reproductive success of male Mediterranean fruit flies (Diptera: Tephritidae). *Animal Behaviour,* 54, 59-66.

Bonizzoni, M., M. Britton, et al. (2013). "Probing functional polymorphisms in the dengue vector, Aedes aegypti." BMC genomics 14: 739.Borovsky, D., D. A. Carlson, et al. (1994). "De-Novo Biosynthesis of Juvenile Hormone-Iii and Hormone-I by the Accessory-Glands of the Male Mosquito." Insect Biochemistry and Molecular Biology 24(5): 437-444.

Brogdon, W. G. (1994) Measurement of flight tone differences between female *Aedes aegypti* and *Ae. albopictus* (Diptera, Culicidae). *Journal of Medical Entomology,* 31, 700-703.

Butail, S., Manoukis, N. C., Diallo, M., Ribeiro, J. M. & Paley, D. A. (2013) The dance of male Anopheles gambiae in wild mating swarms. *Journal of Medical Entomology,* 50, 552-9.

Byrne, K. & Nichols, R. A. 1999. *Culex pipiens* in London underground tunnels: differentiation between surface and subterranean populations. *Heredity,* 82 (Pt 1), 7-15.

Carrasquilla, M. C. & Lounibos, L. P. (2015) Satyrization without evidence of successful insemination from interspecific mating between invasive mosquitoes. *Biology letters,* 11.

Charlwood, J. D. and M. D. R. Jones (1980). "Mating in the Mosquito, Anopheles-Gambiae S-1 .2. Swarming Behavior." Physiological Entomology 5(4): 315-320.

Chepelev, I., G. Wei, et al. (2009). "Detection of single nucleotide variations in expressed exons of the human genome using RNA-Seq." Nucleic acids research 37(16): e106.

Choe, J. C. & Crespi, B. J. (1997) *The evolution of mating systems in insects and arachnids,* Cambridge ; New York, Cambridge University Press.

Cingolani, P., A. Platts, et al. (2012). "A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila melanogaster strain w1118; iso-2; iso-3." Fly 6(2): 80-92.

Cirulli, E. T., A. Singh, et al. (2010). "Screening the human exome: a comparison of whole genome and whole transcriptome sequencing." Genome biology 11(5): R57.

Cloonan, N., A. R. Forrest, et al. (2008). "Stem cell transcriptome profiling via massive-scale mRNA sequencing." Nature methods 5(7): 613-619.

Cohuet, A., S. Krishnakumar, et al. (2008). "SNP discovery and molecular evolution in Anopheles gambiae, with special emphasis on innate immune system." BMC genomics 9: 227.

Cornel, A. J., R. D. McAbee, et al. (2003). "Differences in extent of genetic introgression between sympatric Culex pipiens and Culex quinquefasciatus (Diptera : Culicidae) in California and South Africa." Journal of Medical Entomology 40(1): 36-51.

Crowder, D. W., Sitvarin, M. I. & Carriere, Y. (2010) Plasticity in mating behaviour drives asymmetric reproductive interference in whiteflies. *Animal Behaviour,* 79, 579-587.

Dabire, K. R., Sawadodgo, S., Diabate, A., Toe, K. H., Kengne, P., Ouari, A., Costantini, C., Gouagna, C., Simard, F., Baldet, T., Lehmann, T. & Gibson, G. (2013) Assortative mating in mixed swarms of the mosquito *Anopheles gambiae* s.s. M and S molecular forms, in Burkina Faso, West Africa. *Medical and Veterinary Entomology,* 27, 298-312.

Danecek, P., A. Auton, et al. (2011). "The variant call format and VCFtools." Bioinformatics 27(15): 2156-2158.

David, J. P., F. Faucon, et al. (2014). "Comparative analysis of response to selection with three insecticides in the dengue mosquito Aedes aegypti using mRNA sequencing." BMC genomics 15: 174.

De Meillon, B., Sebastian, A. & Khan, Z. H. (1967) Exodus from a breeding place and the time of emergence from the pupa of *Culex pipiens fatigans*. *Bulletin of the World Health Organization,* 36, 163-7.

Diabate, A. and F. Tripet (2015). "Targeting male mosquito mating behaviour for malaria control." Parasites & vectors 8.

Dodson, B. L., L. D. Kramer, et al. (2012). "Effects of larval rearing temperature on immature development and West Nile virus vector competence of Culex tarsalis." Parasites & vectors 5: 199.

Downes, J. A. (1969). "Swarming and Mating Flight of Diptera." Annual Review of Entomology 14: 271-+.

Edwards, A. C. and T. F. C. Mackay (2009). "Quantitative Trait Loci for Aggressive Behavior in Drosophila melanogaster." Genetics 182(3): 889-897.

Ekechukwu, N. E., Baeshen, R., Traore, S. F., Coulibaly, M., Diabate, A., Catteruccia, F. & Tripet, F. (2015) Heterosis increases fertility, fecundity, and survival of laboratory-produced F1 hybrid males of the malaria mosquito *Anopheles coluzzii*. *G3,* 5, 2693-709.

Engdahl, C., Larsson, P., Naslund, J., Bravo, M., Evander, M., Lundstrom, J. O., Ahlm, C. & Bucht, G. 2013. Identification of Swedish mosquitoes based on molecular barcoding of the COI gene and SNP analysis. *Molecular Ecology Resources*.

Everaerts, C., Farine, J. P., Cobb, M. & Ferveur, J. F. (2010) *Drosophila* cuticular hydrocarbons revisited: mating status alters cuticular profiles. *Plos One,* 5, e9607.

Ewing, B., Hillier, L., Wendl, M. C. & Green, P. 1998. Base-calling of automated sequencer traces using phred. I. accuracy assessment. *Genome Research,* 8, 175-185.

Fang, F. J., W. J. Wang, et al. (2015). "The cuticle proteins: a putative role for deltamethrin resistance in Culex pipiens pallens." Parasitology research 114(12): 4421-4429.

Farajollahi, A., D. M. Fonseca, et al. (2011). ""Bird biting" mosquitoes and human disease: A review of the role of Culex pipiens complex mosquitoes in epidemiology." Infection Genetics and Evolution 11(7): 1577-1585.

Fawaz, E. Y., Allan, S. A., Bernier, U. R., Obenauer, P. J. & Diclaro, J. W. (2014) Swarming mechanisms in the yellow fever mosquito: aggregation pheromones are involved in the mating behavior of *Aedes aegypti. Journal of Vector Ecology,* 39, 347-354.

Fonseca, D. M., Keyghobadi, N., Malcolm, C. A., Mehmet, C., Schaffner, F., Mogi, M., Fleischer, R. C. & Wilkerson, R. C. 2004. Emerging vectors in the *Culex pipiens* complex. *Science,* 303, 1535-8.

Fritz, M. L., Walker, E. D., Miller, J. R., Severson, D. W. & Dworkin, I. (2015) Divergent host preferences of above- and below-ground *Culex pipiens* mosquitoes and their hybrid offspring. *Medical and Veterinary Entomology,* 29, 115-123.

Gabrieli, P., E. G. Kakani, et al. (2014). "Sexual transfer of the steroid hormone 20E induces the postmating switch in Anopheles gambiae." Proceedings of the National Academy of Sciences of the United States of America 111(46): 16353-16358.

Garrison, E. & Marth, G. (2012) Haplotype based variant detection from short read sequencing. Preprint at arXiv:1207 3907v2 (q-bio.GN).

Gibson, G. (1985) Swarming behavior of the mosquito *Culex pipiens quinquefasciatus* - a quantitative analysis. *Physiological Entomology,* 10, 283-296.

Giglioli, M. E. and G. F. Mason (1966). "Mating Plug in Anopheline Mosquitoes." Proceedings of the Royal Entomological Society of London Series a-General Entomology 41: 123-&.

Gjullin, C. M. W., T. L.; Buckley, J.F. (1967) Male pheromones of *Culex quinquefasciatus, Cx. tarsalis* and *Cx. pipiens* that attract females of these species. *Mosquito News,* 27, 382-387.

Gompert, Z., A. A. Comeault, et al. (2014). "Experimental evidence for ecological selection on genome variation in the wild." Ecology letters 17(3): 369-379.

Hallem, E. A., A. Nicole Fox, et al. (2004). "Olfaction: mosquito receptor for human-sweat odorant." Nature 427(6971): 212-213.

Hamer, G. L., Kitron, U. D., Brawn, J. D., Loss, S. R., Ruiz, M. O., Goldberg, T. L. & Walker, E. D. (2008) Culex pipiens (Diptera : Culicidae): A bridge vector of West Nile virus to humans. *Journal of Medical Entomology,* 45, 125-128.

Hamer, G. L., U. D. Kitron, et al. (2008). "Culex pipiens (Diptera: Culicidae): a bridge vector of West Nile virus to humans." Journal of medical entomology 45(1): 125-128.

Hamady, D., Ruslan, N. B., Ahmad, A. H., Rawi, C. S., Ahmad, H., Satho, T., Miake, F., Zuharah, W. F., Fukumitsu, Y., Saad, A. R., Rajasaygar, S., Vargas, R. E., Majid, A. H., Fadzly, N., Ghani, I. A. & Abubakar, S. (2013) Colonized *Aedes albopictus* and its sexual performance in the wild: implications for SIT technology and containment. *Parasites & vectors,* 6, 206.

Harbach, R. E., Dahl, C. & White, G. B. 1985. *Culex* (*Culex*) *pipiens*-Linnaeus (Diptera, Culicidae) - concepts, type designations, and description. *Proceedings of the Entomological Society of Washington,* 87, 1-24.

Harbach, R. E., Harrison, B. A. & Gad, A. M. 1984. *Culex* (*Culex*) *molestus* Forskal (Diptera, Culicidae) - neotype designation, description, variation, and taxonomic status. *Proceedings of the Entomological Society of Washington,* 86, 521-542.

Helinski, M. E. H. & Harrington, L. C. (2011) Male mating history and body size influence female fecundity and longevity of the dengue vector *Aedes aegypti. Journal of Medical Entomology,* 48, 202-211.

Helinski, M. E. H. & Harrington, L. C. (2012) The role of male harassment on female fitness for the dengue vector mosquito *Aedes aegypti. Behavioral Ecology and Sociobiology,* 66, 1131-1140.

Hernandez-Garcia, C. M. and J. J. Finer (2014). "Identification and validation of promoters and cis-acting regulatory elements." Plant science : an international journal of experimental plant biology 217-218: 109-119.

Hickner, P. V., B. Debruyn, et al. (2010). "Genome-based microsatellite development in the Culex pipiens complex and comparative microsatellite frequency with Aedes aegypti and Anopheles gambiae." PloS one 5(9).

Hickner, P. V., A. Mori, et al. (2013). "Composite Linkage Map and Enhanced Genome Map for Culex pipiens Complex Mosquitoes." Journal of Heredity 104(5): 649-655.

Hinomoto, N., T. Higaki, et al. (2006). "Genetic diversity in field and commercial populations of Orius strigicollis (Poppius) (Heteroptera : Anthocoridae) measured by microsatellite markers." Applied Entomology and Zoology 41(3): 499-506.

Hiss, E. A. & Fuchs, M. S. (1972) Effect of matrone on oviposition in mosquito, *Aedes aegypti*. *Journal of insect physiology*, 18, 2217-&.

Hola-Jamriska, L., L. T. King, et al. (2000). "Functional expression of dipeptidyl peptidase I (cathepsin C) of the oriental blood fluke Schistosoma japonicum in Trichoplusia ni insect cells." Protein Expression and Purification 19(3): 384-392.

Holliday, R. and G. W. Grigg (1993). "DNA methylation and mutation." Mutation research 285(1): 61-67.

Huang, S. M., G. L. Hamer, et al. (2009). "Genetic Variation Associated with Mammalian Feeding in Culex pipiens from a West Nile Virus Epidemic Region in Chicago, Illinois." Vector-Borne and Zoonotic Diseases 9(6): 637-642.

Huang, S. M., Molaei, G. & Andreadis, T. G. 2008. Genetic insights into the population structure of *Culex pipiens* (Diptera : Culicidae) in the northeastern United States by using microsatellite analysis. *American Journal of Tropical Medicine and Hygiene,* 79, 518-527.

Isoe, J., A. A. Rascon, et al. (2009). "Molecular genetic analysis of midgut serine proteases in Aedes aegypti mosquitoes." Insect Biochemistry and Molecular Biology 39(12): 903-912.

Jallon, J. M. (1984) A few chemical words exchanged by *Drosophila* during courtship and mating. *Behavior genetics*, 14, 441-78.

Jobling, B. & Lewis, D. (1987) Anatomical drawings of biting flies. *British Museum of Natural History* [Online]. [Accessed February 11, 2015 2015].

Juneja, P., J. Osei-Poku, et al. (2014). "Assembly of the genome of the disease vector Aedes aegypti onto a genetic linkage map allows mapping of genes affecting disease transmission." PLoS neglected tropical diseases 8(1): e2652.

Kang, David S. Genomic analysis of the diapause program in the West Nile virus vector Culex pipiens. “Beardocs Home, Baylor University, 1 Dec. 2015, baylor-irtdl.org/baylor-ir/handle/2014/9560.

Kang, D. and C. Sim (2013). "Identification of Culex complex species using SNP markers based on high-resolution melting analysis." Molecular ecology resources 13(3): 369-376.

Kassim, N. F. A., Webb, C. E. & Russell, R. C. (2012) Is the expression of autogeny by *Culex molestus* Forskal (Diptera: Culicidae) influenced by larval nutrition or by adult mating, sugar feeding, or blood feeding? *Journal of Vector Ecology,* 37, 162-171.

Kent, R. J., Harrington, L. C. & NORRIS, D. E. (2007) Genetic differences between *Culex pipiens* f. molestus and *Culex pipiens* pipiens (Diptera : Culicidae) in New York. *Journal of Medical Entomology,* 44, 50-59.

Kibbe, W. A. 2007. OligoCalc: an online oligonucleotide properties calculator. *Nucleic Acids Research,* 35, W43-W46.

Kilpatrick, A. M., Kramer, L. D., Jones, M. J., Marra, P. P., Daszak, P. & Fonseca, D. M. (2007) Genetic influences on mosquito feeding behavior and the emergence of zoonotic pathogens. *American Journal of Tropical Medicine and Hygiene,* 77, 667-671.

Kilpatrick, A. M., L. D. Kramer, et al. (2006). "West Nile virus epidemics in North America are driven by shifts in mosquito feeding behavior." Plos Biology 4(4): 606-610.

King, J. L. and T. H. Jukes (1969). "Non-Darwinian evolution." Science 164(3881): 788-798.

Koyama, J., Kakinohana, H. & Miyatake, T. (2004) Eradication of the melon fly, *Bactrocera cucurbitae,* in Japan: Importance of behavior, ecology, genetics, and evolution. *Annual Review of Entomology,* 49, 331-349.

Koyama, J., H. Kakinohana, et al. (2004). "Eradication of the melon fly, Bactrocera cucurbitae, in Japan: Importance of behavior, ecology, genetics, and evolution." Annual Review of Entomology 49: 331-349.

Kwon, H. W., T. Lu, et al. (2006). "Olfactory responses in a gustatory organ of the malaria vector mosquito Anopheles gambiae." Proceedings of the National Academy of Sciences of the United States of America 103(36): 13526-13531.

Langmead, B. (2010). "Aligning short sequencing reads with Bowtie." Current protocols in bioinformatics / editoral board, Andreas D. Baxevanis ... [et al.] Chapter 11: Unit 11 17.

Lartillot, N. (2013). "Interaction between selection and biased gene conversion in mammalian protein-coding sequence evolution revealed by a phylogenetic covariance analysis." Molecular biology and evolution 30(2): 356-368.

Lassiter, M. T., C. S. Apperson, et al. (1995). "Juvenile hormone metabolism during the fourth stadium and pupal stage of the southern house mosquito, Culex quinquefasciatus Say." Journal of Insect Physiology 41(10): 869-876.

Lawrie, D. S., P. W. Messer, et al. (2013). "Strong purifying selection at synonymous sites in D. melanogaster." PLoS genetics 9(5): e1003527.

Lee, Y., S. N. Seifert, et al. (2012). "High Degree of Single Nucleotide Polymorphisms in California Culex pipiens (Diptera: Culicidae) sensu lato." Journal of Medical Entomology 49(2): 299-306.

Li, J., X. Wang, et al. (2013). "Genome-block expression-assisted association studies discover malaria resistance genes in Anopheles gambiae." Proceedings of the National Academy of Sciences of the United States of America 110(51): 20675-20680.

Librado, P. and J. Rozas (2009). "DnaSP v5: a software for comprehensive analysis of DNA polymorphism data." Bioinformatics 25(11): 1451-1452.

Long, T. A. F., Pischedda, A., Stewart, A. D. & Rice, W. R. (2009) A cost of sexual attractiveness to high-fitness females. *Plos Biology,* 7.

Lounibos, L. P. (2002). "Invasions by insect vectors of human disease." Annual Review of Entomology 47: 233-266.

Lovin, D. D., Washington, K. O., Debruyn, B., Hemme, R. R., Mori, A., Epstein, S. R., Harker, B. W., Streit, T. G. & Severson, D. W. 2009. Genome-based polymorphic microsatellite development and validation in the mosquito *Aedes aegypti* and application to population genetics in Haiti. *BMC Genomics,* 10

Macnamara, M. & Paterson, H. E. H. (1984) The recognition concept of species. *South African Journal of Science,* 80, 312-318.

Mattingly, P. F. (1967). "The systematics of the Culex pipiens complex." Bulletin of the World Health Organization 37(2): 257-261.

McCarthy, M. I., G. R. Abecasis, et al. (2008). "Genome-wide association studies for complex traits: consensus, uncertainty and challenges." Nature reviews. Genetics 9(5): 356-369.

McIver, S. (1971). "Comparative studies on the sense organs on the antennae and maxillary palps of selected male culicine mosquitoes." Canadian journal of zoology 49(2): 235-239.

Mciver, S. B. (1982) Sensilla mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology*, 19, 489-535.

Metzker, M. L. (2010). "Sequencing technologies - the next generation." Nature reviews. Genetics 11(1): 31-46.

Meuti, M. E., M. Stone, et al. (2015). "Functional circadian clock genes are essential for the overwintering diapause of the Northern house mosquito, Culex pipiens." Journal of Experimental Biology 218(3): 412-422.

Michaelakis, A., Mihou, A. P., Couladouros, E. A., Zounos, A. K. & Koliopoulos, G. (2005) Oviposition responses of *Culex pipiens* to a synthetic racemic *Culex quinquefasciatus* oviposition aggregation pheromone. *Journal of Agricultural and Food Chemistry*, 53, 5225-5229.

Monath, T. P. (1988). "Japanese encephalitis--a plague of the Orient." The New England journal of medicine 319(10): 641-643.

Montgomery, S. B., D. L. Goode, et al. (2013). "The origin, evolution, and functional impact of short insertion-deletion variants identified in 179 human genomes." Genome research 23(5): 749-761.

Mori, A., D. W. Severson, et al. (1999). "Comparative linkage maps for the mosquitoes (Culex pipiens and Aedes aegypti) based on common RFLP loci." The Journal of heredity 90(1): 160-164.

Morin, R. D., M. D. O'Connor, et al. (2008). "Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells." Genome research 18(4): 610-621.

Moriyama, E. N. and J. R. Powell (1996). "Intraspecific nuclear DNA variation in Drosophila." Molecular biology and evolution 13(1): 261-277.

Morlais, I., N. Poncon, et al. (2004). "Intraspecific nucleotide variation in Anopheles gambiae: new insights into the biology of malaria vectors." The American journal of tropical medicine and hygiene 71(6): 795-802.

Morlais, I. and D. W. Severson (2003). "Intraspecific DNA variation in nuclear genes of the mosquito Aedes aegypti." Insect molecular biology 12(6): 631-639.

Mutebi, J. P. and H. M. Savage (2009). "Discovery of Culex pipiens pipiens form molestus in Chicago." J Am Mosq Control Assoc 25(4): 500-503.

Nakahara, S., Y. Kobashigawa, et al. (2008). "Genetic variations among and within populations of the Oriental fruit fly, Bactrocera dorsalis (Diptera; Tephritidae), detected by PCR-RFLP of the mitochondrial control region." Applied Entomology and Zoology 43(3): 457-+.

Oliva, C. F., Jacquet, M., Gilles, J., Lemperiere, G., Maquart, P. O., Quilici, S., Schooneman, F., Vreysen, M. J. & Boyer, S. (2012) The sterile insect technique for controlling populations of *Aedes albopictus* (Diptera: Culicidae) on Reunion Island: mating vigour of sterilized males. *Plos One,* 7, e49414.

Ozsolak, F. and P. M. Milos (2011). "RNA sequencing: advances, challenges and opportunities." Nature reviews. Genetics 12(2): 87-98.

Packer, M. J. & Corbet, P. S. (1989) Size variation and reproductive success of female *Aedes punctor* (Diptera, Culicidae). *Ecological Entomology,* 14, 297-309.

Pennetier, C., B. Warren, et al. (2010). ""Singing on the wing" as a mechanism for species recognition in the malarial mosquito Anopheles gambiae." Current biology : CB 20(2): 131-136.

Pennetier, C., Warren, B., Dabire, K. R., Russell, I. J. & Gibson, G. (2010) "Singing on the wing" as a mechanism for species recognition in the malarial mosquito *Anopheles gambiae. Current biology : CB,* 20, 131-6.

Pitts, R. J., Mozuraitis, R., Gauvin-Bialecki, A. & Lemperiere, G. (2014) The roles of kairomones, synomones and pheromones in the chemically-mediated behaviour of male mosquitoes. *Acta tropica,* 132 Suppl, S26-34.

Polerstock, A. R., Eigenbrode, S. D. & Klowden, M. J. (2002) Mating alters the cuticular hydrocarbons of female *Anopheles gambiae* sensu stricto and *Aedes aegypti* (Diptera: Culicidae). *Journal of Medical Entomology,* 39, 545-52.

Polerstock, A. R., S. D. Eigenbrode, et al. (2002). "Mating alters the cuticular hydrocarbons of female Anopheles gambiae sensu stricto and aedes Aegypti (Diptera: Culicidae)." Journal of Medical Entomology 39(3): 545-552.

Ponlawat, A. and L. C. Harrington (2009). "Factors Associated with Male Mating Success of the Dengue Vector Mosquito, Aedes aegypti." American Journal of Tropical Medicine and Hygiene 80(3): 395-400.

Rahman, M. M., X. Franch-Marro, et al. (2017). "Local Juvenile Hormone activity regulates gut homeostasis and tumor growth in adult Drosophila." Scientific Reports 7(1): 11677.

Reisen, W. K., N. F. Knop, et al. (1985). "Swarming and Mating-Behavior of Laboratory and Field Strains of Culex-Tarsalis (Diptera, Culicidae)." Annals of the Entomological Society of America 78(5): 667-673.

Reisen, W. K., M. M. Milby, et al. (1992). "Ecology of Mosquitos and St-Louis Encephalitis-Virus in the Los-Angeles Basin of California, 1987-1990." Journal of Medical Entomology 29(4): 582-598.

Reusken, C. B., De Vries, A., Buijs, J., Braks, M. A., Den Hartog, W. & Scholte, E. J. (2010) First evidence for presence of *Culex pipiens* biotype molestus in the Netherlands, and of hybrid biotype pipiens and molestus in northern Europe. *Journal of vector ecology : journal of the Society for Vector Ecology,* 35, 210-2.

Richards, S. L., Anderson, S. L. & Yost, S. A. (2012) Effects of blood meal source on the reproduction of *Culex pipiens quinquefasciatus* (Diptera: Culicidae). *Journal of Vector Ecology,* 37, 1-7.

Rivero, A., Magaud, A., Nicot, A. & Vezilier, J. (2011) Energetic cost of insecticide resistance in *Culex pipiens* mosquitoes. *Journal of Medical Entomology,* 48, 694-700.

Roberts, D. R. & Andre, R. G. (1994) Insecticide resistance issues in vector-borne disease-control. *American Journal of Tropical Medicine and Hygiene,* 50, 21-34.

Rozeboom, L. E. & Gilford, B. N. (1954) Sexual isolation between populations of the *Culex pipiens* complex in North America. *The Journal of parasitology,* 40, 237-44.

Rudolf, M., Czajka, C., Borstler, J., Melaun, C., Jost, H., Von Thien, H., Badusche, M., Becker, N., Schmidt-Chanasit, J., Kruger, A., Tannich, E. & Becker, S. (2013) First nationwide surveillance of *Culex pipiens* complex and *Culex torrentium* mosquitoes demonstrated the presence of *Culex pipiens* biotype pipiens/molestus hybrids in Germany. *Plos One,* 8, e71832.

Rueda, L. M., K. J. Patel, et al. (1990). "Temperature-dependent development and survival rates of Culex quinquefasciatus and Aedes aegypti (Diptera: Culicidae)." Journal of Medical Entomology 27(5): 892-898.

Rueda, L. M., Patel, K. J., Axtell, R. C. & Stinner, R. E. 1990. Temperature-dependent development and survival rates of *Culex quinquefasciatus* and *Aedes aegypti* (Diptera: Culicidae). *Journal of Medical Entomology,* 27, 892-8.

.

Saavedra-Rodriguez, K., C. Strode, et al. (2008). "Quantitative trait loci mapping of genome regions controlling permethrin resistance in the mosquito Aedes aegypti." Genetics 180(2): 1137-1152.

Sallum, M. A. M., Peyton, E. L. & Wilkerson, R. C. (2005) Six new species of the *Anopheles leucosphyrus* group, reinterpretation of *An. elegans* and vector implications. *Medical and Veterinary Entomology*, 19, 158-199

Sanger, F., S. Nicklen, et al. (1977). "DNA Sequencing with Chain-Terminating Inhibitors." Proceedings of the National Academy of Sciences of the United States of America 74(12): 5463-5467.

Sanogo, Y. O., C. H. Kim, et al. (2008). "Identification of male specimens of the Culex pipiens complex (Diptera : Culicidae) in the hybrid zone using morphology and molecular techniques." Journal of Medical Entomology 45(2): 203-209.

Schomburg, D. and D. Stephan (1998). Juvenile-hormone esterase. Enzyme Handbook 15. D. Schomburg and D. Stephan, Springer Berlin Heidelberg: 21-26.

Service, M. W. (1994) Male swarming of the mosquito *Culex (Cx.) torrentium* in England. *Medical and Veterinary Entomology,* 8, 95-98.

Shaw, W. R., Attardo, G. M., Aksoy, S. & Catteruccia, F. (2015) A comparative analysis of reproductive biology of insect vectors of human disease. *Current Opinion in Insect Science,* 10, 142-148.

Shen, L. X., J. P. Basilion, et al. (1999). "Single-nucleotide polymorphisms can cause different structural folds of mRNA." Proceedings of the National Academy of Sciences of the United States of America 96(14): 7871-7876.

Shen, Y., J. Catchen, et al. (2012). "Identification of transcriptome SNPs between Xiphophorus lines and species for assessing allele specific gene expression within F(1) interspecies hybrids." Comparative biochemistry and physiology. Toxicology & pharmacology : CBP 155(1): 102-108.

Shendure, J. and H. Ji (2008). "Next-generation DNA sequencing." Nature biotechnology 26(10): 1135-1145.

Shiao, S. H., I. A. Hansen, et al. (2008). "Juvenile hormone connects larval nutrition with target of rapamycin signaling in the mosquito Aedes aegypti." Journal of insect physiology 54(1): 231-239.

Smith, J. L. and D. M. Fonseca (2004). "Rapid assays for identification of members of the Culex (Culex) pipiens complex, their hybrids, and other sibling species (Diptera : Culicidae)." American Journal of Tropical Medicine and Hygiene 70(4): 339-345

Smith, J. L. & Fonseca, D. M. 2004. Rapid assays for identification of members of the *Culex* (*Culex*) *pipiens* complex, their hybrids, and other sibling species (Diptera: Culicidae). *The American Journal of Tropical Medicine and Hygiene,* 70, 339-45.

Sundararaman, S. 1949. Biometrical studies on intergradation in the genitalia of certain populations of *Culex pipiens* and *Culex quinquefasciatus* in the United States. *American Journal of Hygiene,* 50, 307-14.

Taai, K., Harbach, R. E., Aupalee, K., Srisuka, W., Yasanga, T., Otsuka, Y. & Saeung, A. (2017) An effective method for the identification and separation of *Anopheles minimus*, the primary malaria vector in Thailand, and its sister species *Anopheles harrisoni*, with a comparison of their mating behaviors. *Parasites & vectors,* 10, 97.

Tormey, D., J. K. Colbourne, et al. (2015). "Evolutionary divergence of core and post-translational circadian clock genes in the pitcher-plant mosquito, Wyeomyia smithii." BMC genomics 16(1): 754.

Totten, D. C., M. Vuong, et al. (2013). "Targeting gene expression to the female larval fat body of transgenic Aedes aegypti mosquitoes." Insect Molecular Biology 22(1): 18-30.

Trapnell, C., A. Roberts, et al. (2014). "Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks (vol 7, pg 562, 2012)." Nature Protocols 9(10): 2513-2513.

Turell, M. J., M. R. Sardelis, et al. (2002). "Potential vectors of West Nile virus in North America." Current topics in microbiology and immunology 267: 241-252.

Turell, M. J., W. C. Wilson, et al. (2010). "Potential for North American Mosquitoes (Diptera: Culicidae) to Transmit Rift Valley Fever Virus." Journal of Medical Entomology 47(5): 884-889.

Turk, D., V. Janjić, et al. (2001). "Structure of human dipeptidyl peptidase I (cathepsin C): exclusion domain added to an endopeptidase framework creates the machine for activation of granular serine proteases." The EMBO Journal 20(23): 6570-6582.

Tuten, H. C., Stone, C. M. & Dobson, S. L. (2013) Swarming behavior of *Aedes polynesiensis* (Diptera: Culicidae) and characterization of swarm markers in American Samoa. *Journal of Medical Entomology,* 50, 740-7.

Vasemagi, A., J. Nilsson, et al. (2005). "Expressed sequence tag-linked microsatellites as a source of gene-associated polymorphisms for detecting signatures of divergent selection in atlantic salmon (Salmo salar L.)." Molecular biology and evolution 22(4): 1067-1076.

Veyrieras, J. B., S. Kudaravalli, et al. (2008). "High-resolution mapping of expression-QTLs yields insight into human gene regulation." PLoS genetics 4(10): e1000214.

Warren, B., Gibson, G. & Russell, I. J. (2009) Sex recognition through midflight mating duets in *Culex* mosquitoes is mediated by acoustic distortion. *Current Biology,* 19, 485-491.

Wekesa, J. W., Brogdon, W. G., Hawley, W. A. & Besansky, N. J. (1998) Flight tone of field-collected populations of *Anopheles gambiae* and *An. arabiensis* (Diptera : Culicidae). *Physiological Entomology*, 23, 289-294.

Wijit, A., Taai, K., Dedkhad, W., Hempolchom, C., Thongsahuan, S., Srisuka, W., Otsuka, Y., Fukuda, M. & Saeung, A. (2016) Comparative studies on the stenogamous and eurygamous behavior of eight *Anopheles* species of the hyrcanus group (Diptera: Culicidae) in Thailand. *Insects,* 7.

Wood, O., S. Hanrahan, et al. (2010). "Cuticle thickening associated with pyrethroid resistance in the major malaria vector Anopheles funestus." Parasites & vectors 3: 67.

Yamamoto, D. & Koganezawa, M. (2013) Genes and circuits of courtship behaviour in *Drosophila* males. *Nature reviews. Neuroscience,* 14, 681-92.

Yamamoto, D. and M. Koganezawa (2013). "Genes and circuits of courtship behaviour in Drosophila males." Nature reviews. Neuroscience 14(10): 681-692.

Zakharkin, S. O., A. V. Gordadze, et al. (1997). "Molecular cloning and expression of a hexamerin cDNA from the malaria mosquito, Anopheles gambiae." European journal of biochemistry 246(3): 719-726.

Zakharkin, S. O., V. V. Headley, et al. (2001). "Female-specific expression of a hexamerin gene in larvae of an autogenous mosquito." European journal of biochemistry 268(22): 5713-5722.

Zhou, X., M. R. Tarver, et al. (2007). "Hexamerin-based regulation of juvenile hormone-dependent gene expression underlies phenotypic plasticity in a social insect." Development 134(3): 601-610.

Zianni, M. R., Nikbakhtzadeh, M. R., Jackson, B. T., Panescu, J. & Foster, W. A. 2013. Rapid discrimination between *Anopheles gambiae* s.s. and *Anopheles arabiensis* by High-Resolution Melt (HRM) analysis. *Journal of Biomolecular Techniques : JBT*, 24, 1-7.

Zou, F., C. Chen, et al. (2015). "Identification of QTLs Conferring Resistance to Deltamethrin in Culex pipiens pallens." PloS one 10(10): e0140923.