

**A cytogenetic map for the genomic studies of the West Nile Virus vector *Culex tarsalis***

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**Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of**

**Master of Science**

**In**

**Entomology**

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**May 15, 2020**

**Blacksburg, Virginia**

**Keywords: mitotic chromosomes, genome mapping, mosquitoes, *Culex tarsalis***

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## ABSTRACT

*Culex tarsalis* is a major vector of West Nile Virus (WNV) in North America. Although the genome for this species was recently sequenced, the physical genome map has not developed. Unlike other *Culex* species, that have sex-determination locus on chromosome 1, the sex locus in *Cx. tarsalis* is located on chromosome 3, the longest chromosome. It is currently unknown if this difference is associated with chromosomal rearrangements. The objectives of this study were to develop a high-resolution map for the precise physical genome mapping in *Cx. tarsalis* and to compare mitotic chromosomes between three species of *Culicinae* mosquitoes. Using mitotic chromosomes from imaginal discs of 4<sup>th</sup> instar larvae of *Cx. tarsalis*, we developed idiograms based on morphology and proportions of the mitotic chromosomes. In addition, the physical mapping of ribosomal genes using fluorescence *in situ* hybridization was performed.

The comparative analysis of *Cx. tarsalis* to *Cx. pipiens* and *Cx. quinquefasciatus* chromosomes showed that the total chromosome length in *Cx. tarsalis* is longer than the other two species suggesting the bigger genome size in this mosquito. A comparison of the relative chromosome length between the species indicated no significant differences suggesting that no large chromosomal translocation occurred between the species. Comparisons of the centromeric indexes demonstrated a significant difference in chromosome 1 between *Cx. pipiens* and *Cx. quinquefasciatus*. This difference suggests the presence of pericentric inversion between the species or amplification of ribosomal

genes in *Cx. pipiens*. Studying mosquito chromosomes advances our understanding of *Culex* cytogenetics. Further comparative physical mapping of the three major mosquito genera will help us to understand the evolution of genus *Culex* better and to develop genome-based strategies for the vector control.

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GENERAL AUDIENCE ABSTRACT

West Nile Virus (WNV) is the most common virus transmitted to humans by mosquitoes in the United States. While many species of mosquitoes are known to carry WNV, *Culex tarsalis* is a major vector on the west coast of North America. However, previous research on *Cx. tarsalis* lack chromosome studies on this mosquito. Our study aims to develop a high-quality chromosome map for *Cx. tarsalis* and to compare the mitotic chromosomes of *Cx. tarsalis* and *Cx. quinquefasciatus* and *Cx. pipiens* in respect of chromosomal rearrangements. We used a fluorescent DNA probe to find the location of the ribosomal locus in the chromosomes of *Cx. tarsalis*. This study developed a cytogenetic tool for further genomic studies of *Cx. tarsalis* that will help to develop genome-based strategies for vector control. Comparing the physical mapping of the three major mosquito genera will help to understand the genome evolution in Culicinae mosquitoes better.

## **Dedication**

I dedicated this project to my parents, Adrienne and Victor, my family, and friends.

## **Acknowledgments**

I would like to thank Dr. Ed Smith, Dr. Randolph Grayson, Dr. Kring, and Dr. Maria Sharakhova, for allowing me the opportunity to continue my education at this great institution. I am very grateful to Dr. Maria Sharakhova for allowing me the opportunity to work in her lab on this project. I would also like to thank my committee members: Dr. Igor Sharakhov and Dr. Jonathan Auguste, for supporting me through every step of my studies.

I am thankful to my lab mates, Michael Hodge, and the not yet doctor Reem Marsi for teaching me how to perform FISH, their advice, company, and kindness. I would also like to thank Dr. Anastasia Naumenko for providing the data for *Cx. quinquefasciatus*.

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## **Introduction**

Mosquito-borne diseases have been flourishing worldwide for centuries, killing and disabling millions of people every year (Tolle 2009). *Culicinae* mosquitoes are efficient vectors of arthropod-borne viruses and parasitic nematodes such as the West Nile virus, dengue virus, and lymphatic filariasis. Recent reports strongly suggest that West Nile Virus (WNV) is among several re-emerging viruses that pose a significant public health threat. WNV is the leading cause of mosquito-borne diseases in the United States (Centers for Disease Control and Prevention, 2020). The Centers for Disease Control and Prevention (CDC) reported 2,544 cases of WNV, and 63% of these cases were classified as neuroinvasive disease (i.e., affecting the nervous system, likely resulting in encephalitis). Mosquitoes are important vectors of human diseases and can develop these viral infections with no apparent effects, despite the high levels of virus circulating within them.

Understanding the genomes of mosquitoes provides essential insight into genetic diversity and the evolution of mosquito-pathogen interactions. The genomes of *Anopheles gambiae* (Holt, Subramanian et al. 2002), *Aedes aegypti* (Nene, Wortman et al. 2007), and *Culex quinquefasciatus* (Arensburger, Megy et al. 2010) have been studied as well as sequenced. Recently, the first genome assembly was released for *Culex tarsalis* (Main, Brown et al. 2020). Physical mapping in *Culicinae* mosquitoes was challenging due to the poor quality of polytene chromosomes. Dr. Maria Sharakhova identified a reliable source for mitotic chromosomes for physical mapping. She identified that the location where legs develop in mosquito larvae called imaginal discs to show improved banding patterns (Sharakhova, Timoshevskiy et al. 2011). The development of cytogenetic maps, which illustrates chromosome structures and banding,

provides a basis for the genome analysis (Lewin, Larkin et al. 2009). However, such studies have never been performed for *Cx. tarsalis*.

Different organisms have a variation in the number and morphology of chromosomes. The size of chromosomes and genome sizes are different between species of mosquitoes. There are also differences in the karyotypes of closely related species, and males and females of the same species (Kitzmiller 1963). In contrast to malaria mosquitoes *Anophelinae* which have sex chromosome X and Y, in *Culicinae* mosquitoes, sex-determining chromosomes are homomorphic (Rai 1963) and male sex is determined by a single-sex locus (M locus) located on one of the autosomes (Severson 2008). Intriguingly, based on genetic mapping, the sex-determining locus was mapped on the shortest chromosome 1 in *Ae. aegypti* (Matthews, Dudchenko et al. 2018) and *Cx. pipiens* (Mori, Romero-Severson et al. 2007) mosquitoes but on the largest chromosome 2 in *Cx. tarsalis* (Venkatesan, Broman et al. 2009). This observation may suggest chromosomal rearrangement between chromosome 1 and chromosome 2 of *Cx. pipiens* and *Cx. tarsalis*.

The goals of this study are the following:

1. Develop a chromosome map for the genome mapping of *Cx. tarsalis* and optimize fluorescent in situ hybridization using 18S ribosomal genes.
2. Compare chromosome measurements in three species *Cx. tarsalis*, *Cx. quinquefasciatus* and *Cx. pipiens*. Our hypothesis is the re-positioning of the sex-determination locus from the shortest chromosome 1 to the longest chromosome in *Cx. tarsalis* is related to chromosome translocation between these two chromosomes. Such differences will be

reflected in chromosome proportions and also in orders of genes in *Cx. tarsalis* chromosomes.

## **Chapter 1: Literature Review**

### **1.1 Arthropod-borne viruses**

Understanding the evolutionary and ecological emergence of Arthropod-borne viruses or arboviruses and their vectors is critical for the control and prevention strategies for public health. The World Health Organization estimates that mosquito-borne diseases are among the leading causes of morbidity and mortality in developing countries (Tolle 2009, WHO 2012). *Culicidae* are significant vectors of arboviruses, malaria, and filariasis (Rai and Black 1999). However, a small number of mosquito species are genetically competent to transmit pathogens to vertebrate hosts. Among mosquitoes, species of the genus *Culex* the principal vectors of lymphatic filariasis worms and encephalitis viruses, including the WNV, the most common arboviral disease in the USA (Reisen 2013, Brown, Young et al. 2015). Control of the latter disease is challenging because its transmission involves multiple hosts and species of mosquitoes that are domestic in southern and northern, as well as urban and agricultural regions in the USA. Another challenge is that mosquitoes can develop these viral infections with no apparent effects, despite the high levels of virus circulating within them.

#### **1.1.1 West Nile Virus**

The most important disease transmitted by mosquitoes from genus *Culex* is West Nile fever (Reisen 2013, Brown, Young et al. 2015). The virus (WNV) is genetically and geographically diverse (Chancey, Grinev et al. 2015). WNV is the leading cause of mosquito-borne diseases in the United States (Moreno-Madriñán and Turell 2018). WNV belongs to the genus *Flavivirus* in

the family Flaviviridae, and it is also a member of the Japanese encephalitis serocomplex (Chancey, Grinev et al. 2015). WNV is broken into four genetic lineages based on the host that they infect. WNV is found in both tropical and temperate regions of the world. It was first introduced into the Western Hemisphere in the United States in 1999 and now became a significant public health concern in the United States since an endemic in 1999 (Chancey, Grinev et al. 2015).

In nature, the WNV is a zoonotic disease with a transmission cycle between birds and mosquitoes; also it can infect humans and other vertebrates and cause disease and death (Chancey, Grinev et al. 2015). The predominant and preferred host for WNV is birds. Humans and horses are considered a dead-end host of WNV; this is due to the levels of viremia that are low in mammals (Chancey, Grinev et al. 2015). Since the levels of viremia are low, the vector competence is not enough to transmit back to mosquitoes, resulting in the ending of the transmission cycle (Chancey, Grinev et al. 2015).

Since its discovery, WNV has spread to vast regions of the globe and is considered the most important causative agent of viral encephalitis worldwide (Chancey, Grinev et al. 2015). Humans that are infected with WNV, around 80% are asymptomatic, and symptomatic infection varies from flu-like sickness to serious neuroinvasive. Less than 1% of human infections progress to severe illness. Many different species of mosquito can acquire and transmit WNV. Over 65 mosquito species are infected by the WNV (Colpitts, Conway et al. 2012).

Globally, *Culex* mosquitoes are known as the critical bridge vector for this virus (Chancey, Grinev et al. 2015). Right now, in the United States, West Nile infection is a major public health concern among other diseases transmitted by mosquitoes, due to the virus causing recurring outbreaks.

### 1.1.2 Western Equine Encephalitis Virus

Western Equine Encephalitis Virus is an arbovirus in the family *Togaviridae*, genus *Alphavirus* (Bergren, Haller et al. 2020). Western equine encephalitis virus is a mosquito-transmitted infection that may progress to an acute inflammation of the brain (Western Equine encephalitis). It is spread primarily by the bite of *Culex* and *Aedes* mosquitoes; Western Equine encephalitis has been reported on the west coast of the United States and in Canada. The principle bridge vector for Western Equine Encephalitis Virus is *Culex tarsalis* (Bergren, Haller, et al. 2020). Similar to WNV, Western Equine Encephalitis Virus occurs both in humans and horses. Still, they are considered dead-end hosts because viremia does not reach sufficient levels to infect mosquito vectors (Bergren, Haller, et al. 2020).

### 1.2 Culicinae mosquitoes

Mosquitoes in the genus *Culex* are very diverse and have a highly opportunistic host choice compared to other vectors. The mosquitoes have different blood-meal host preferences, the ability to diapause, and some other physiological characteristics (Unger, Sharakhova, et al. 2015). Research has shown that *C. pipiens* may feed on mammals and humans instead of birds during the late summer and early fall (Vinogradova 2000, Farajollahi, Fonseca, et al. 2011).

Studies have shown that mosquitoes within the genus *Culex* (including *Culex quinquefasciatus*, *Culex pipiens*, and *Culex tarsalis*) are significant vectors of WNV because of their seasonal abundance, vector competence, and high infection rates (Venkatesan, Broman et al. 2009). *Cx. pipiens* are the bridge vector in the northeastern, northcentral, and mid-Atlantic, *Cx. quinquefasciatus* in the south and southwest, and *Cx. tarsalis* in the west (Chancey,

Grinev et al. 2015). Estimate that *Cx. tarsalis* diverged from *Cx. quinquefasciatus* approximately 13 million years ago based on full mitochondrial genome alignments (Main, Brown et al. 2020).

### 1.3 *Culex tarsalis*

*Cx. tarsalis* is an important vector of WNV and the Western Equine Encephalitis Virus in North America (Venkatesan, Broman et al. 2009). This species distribution spans from the west coast of the United States to the Mississippi River and extends to portions of Canada and Mexico. *Cx. tarsalis* can feed on a variety of avian and mammalian species. *Cx. tarsalis* mosquitoes primarily feed on birds during spring and early summer, but they can become opportunistic feeders and tend to switch from bird to mammals in the late summer (Reisen and Reeves, 1990). *Cx. tarsalis* exhibits high intra-species variation aiding in its ability to transmit arboviruses (Venkatesan, Broman et al. 2009). While the molecular tools for this mosquito have been challenging to investigate the relationship between phenotypic and genotypic variation. The phenotypic variation in *Cx. tarsalis* has been well-described. Being that *Cx. tarsalis* can transmit several arboviruses; it has been investigated for many years by arbovirologists and ecologists. Quantitative trait loci (QTL) associated with pathogen susceptibility transmission have been identified in many mosquitoes and arboviral systems (Venkatesan, Broman et al. 2009).

### 1.4 Mosquito genome

To facilitate new approaches for vector control, quality genome assemblies, based on next-generation technologies, have been developed for species from the *An. gambiae* complex (Neafsey, Waterhouse, et al. 2015), *Ae. aegypti* (Dudchenko, Batra et al. 2017), *Ae. albopictus*

(Chen, Jiang, et al. 2015), *Cx. quinquefasciatus* and *Cx. tarsalis*. The first genome assembly of *Cx. tarsalis* was just released (Main, Brown et al. 2020). Based on PacBio HiFi reads from a single adult male using the assembly of the Mitochondrial genome (Main, Brown, et al. 2020). The *Culex tarsalis* genome was sequenced at 793 Mb and is 37 percent larger than the *Cx. quinquefasciatus* genome at 578 Mb (Main, Brown, et al. 2020). Most of the differences between the two species appear to be transposable elements (Main, Brown et al., 2020).

In the past decade, essential insights into the genetic diversity of mosquitoes and the evolution of mosquito-pathogen interactions have been made (Ciota and Kramer, 2013). Mosquito-borne diseases can be controlled by genetic manipulation of the mosquito population to reduce their effectiveness as a disease vector (David W. Severson, Susan E. Brown, et al. 2001). The chromosomal mapping of the three major mosquito taxa *Anopheles gambiae*, *Aedes aegypti*, and recently *Cx. quinquefasciatus* provides important insights into genome evolution of the mosquitoes (Timoshevskiy, Kinney, et al. 2014, Neafsey, Waterhouse, et al. 2015). Cytogenetic genome mapping is the effort to construct physical maps of genomes using specific banding patterns of the chromosomes (Sharakhova, Xia et al. 2010, Sharakhova, Timoshevskiy et al. 2011). Physical mapping was an early stage of discovery of its genome organization (genetic and physical mapping) (David W. Severson, Susan E. Brown, et al. 2001). In further studies, chromosome homology and rearrangements have been examined between *Aedes aegypti* and *Anopheles gambiae* (Timoshevskiy, Kinney et al. 2014). A chromosomal rearrangement is an irregularity that results in a change in the structure of the native chromosome. These changes include several different events, such as deletions, duplications, inversions, and translocation. Speciation does not occur only by gene mutations, but also by chromosomal rearrangements (Unger, Sharakhova et al. 2015).

The recombination rates are known to vary in insects, including *Drosophila* species (Venkatesan, Broman et al. 2009). Research done on *Drosophila melanogaster* showed that positions of ectopic contacts in chromosomes are connected with the location of gene-poor and repeat-rich regions of intercalary heterochromatin (Unger, Sharakhova et al. 2015). This is shown in mosquitoes; there is an abundance of ectopic contacts between chromosomes; this correlates with the amount of repetitive DNA within their genomes (Unger, Sharakhova et al. 2015). The transposable elements equal to 16% for the genome of *Anopheles gambiae*, 29% *Culex quinquefasciatus*, and 47% *Aedes aegypti* (Unger, Sharakhova et al. 2015).

#### **1.4.1 Cytogenetic studies in *Culex***

When WNV was introduced to the United States in 1999, the spread of it to Central and South America motivated cytogenetic research for *C. pipiens* complex species (Unger, Sharakhova et al. 2015). *C. quinquefasciatus* polytene chromosomes have unique features, and this includes telomere fusions, ectopic contacts, and extended chromosomal arms, which result in a low number of available slides (2015, Unger, Sharakhova et al. 2015). Chromosomal rearrangement might provide information about genetic linkage, and the genomic difference between *Culex* species will help us understand species distribution and improve vector/disease control methods (Dickson, Sharakhova et al. 2016).

The measurements of *Cx. quinquefasciatus* and *Ae. aegypti* mitotic chromosomes were previously studied. Finding the locations of genes helps to improve the understanding of genome organization, contributes to the deciphering of how the genome functions, and provides landmarks, such as inversions that may be associated (Unger, Sharakhova et al. 2015). In *Cx. pipiens*, two genes, 18S and 28S ribosomal DNA, have been physically on to the smallest mitotic

chromosome (Kumar and Rai 1990). Mitotic chromosomes from *Cx. pipiens* was briefly described as three pairs of metacentric chromosomes and numbered in the order of increasing size as chromosomes I, II, III (Rai 1963). A new nomenclature was established for *Cx. quinquefasciatus*, the chromosomes were renumbered as 1 being the smallest, 2 being the largest, and 3 being the intermediate chromosome (Naumenko, Timoshevskiy et al. 2015). The chromosomes of *Cx. pipiens* are smaller than those in *Ae. aegypti* (Naumenko, Timoshevskiy et al. 2015).

However, very little is known about the genomic and chromosomal makeup of *Cx. tarsalis* mosquitoes. For decades there has been extensive field and laboratory research on *Cx. tarsalis*. This research resulted in a better understanding of variation in its physiology, vector competence, and vectorial capacity (Venkatesan, Broman et al. 2009). Polytene chromosome physical maps for most culicines have been difficult to construct (David W. Severson, Susan E. Brown et al. 2001, Unger, Sharakhova et al. 2015). A population genetic analysis of the entire western United States identified three genetically distinct populations by using 12 microsatellite markers; the Pacific, Sonoran, and Midwest clusters (Venkatesan and Rasgon 2010). The microsatellite variation was consistent with the possibility that genetic variation may be connected to phenotypic variations in populations (Venkatesan, Hauer et al. 2007).

Translocation heterozygotes can be massed produced and released to introduce sterility into fertile populations (McDonald, Asman et al. 1978). Translocation heterozygotes are important for the development and application of genetic control mechanisms such as sex-sorting systems, translocation homozygotes, and compound chromosomes (McDonald, Asman et al. 1978).

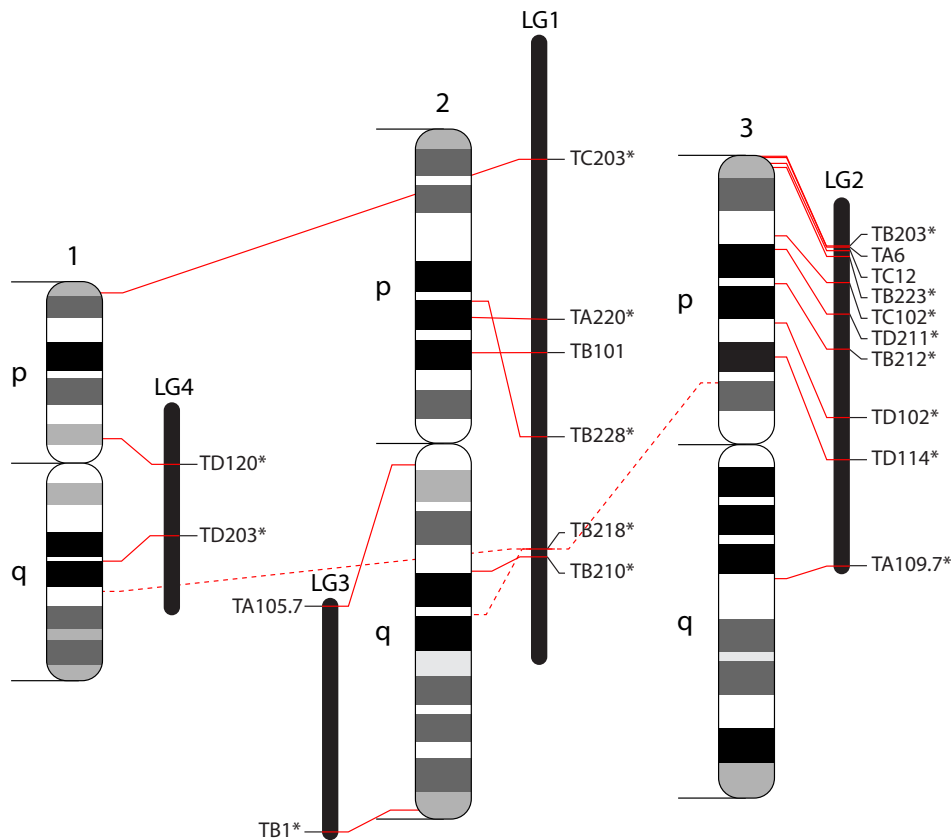
There is an evident lack of understanding of the genetic makeup of the different *Culex* mosquito genomes. Identification of the structural variations in the chromosome

of *Culex tarsalis* will bring together a gap in knowledge in this poorly studied mosquito (Venkatesan, Broman et al. 2009). Physical mapping of *Ae. aegypti* helped to clarify genetic regions related to pathogen transmission, and this suggests that various pathogens can be controlled by the same genomic loci (Timoshevskiy, Kinney et al. 2014). Physical mapping is the mapping of genes and DNA markers on to chromosomes. All three pairs of chromosomes are metacentric in genus *Culex* but are much shorter in length than *Aedes* chromosomes (Rai 1963).

#### **1.4.2 Genetic mapping in *Culex tarsalis***

M. Venkatesan *et al.* constructed the first genetic linkage map for the arboviral vector *Cx. tarsalis* (Venkatesan, Broman et al. 2009). In an earlier study of sex linkage in *Cx. tarsalis*, the sex locus is shown to be located on the longest chromosome (McDonald, Asman et al. 1978). In contrast to *Cx. quinquefasciatus* and most *culicines* mosquitoes, where the sex locus is located on the shortest chromosome (Sharakhova, Timoshevskiy et al. 2011). The cytology of sex-linked translocation heterozygotes in *Cx. tarsalis* indicated that the shortest and longest chromosomes were involved in an interchange (McDonald, Asman et al. 1978). Five of the translocations were linked the sex locus to linkage groups I and II (McDonald, Asman et al. 1978). Cytology of an autosomal translocation that involved linkage groups II and III confirmed that the sex locus is located on the longest chromosome (McDonald, Asman et al. 1978).

However, it is currently unknown if this difference is associated with chromosomal rearrangements (Venkatesan, Broman et al. 2009). In our preliminary study, the genetic linkage map for *Cx. tarsalis* was integrated with the *Cx. quinquefasciatus* idiogram. This is used as a foundation for quantitative trait loci mapping of phenotypic traits such as virus susceptibility, autogeny, and diapause in *Cx. tarsalis* (Venkatesan, Broman et al. 2009).



**Figure 1.1** Microsatellite containing sequences from *Cx. tarsalis* was compared to the *Cx. quinquefasciatus* genome. *Cx. tarsalis* linkage groups schematic representation (black) is next to the idiogram representing *Cx. quinquefasciatus*. The microsatellites were highly similar to regions of the *Cx. quinquefasciatus* genome. For the three chromosomes, four linkage groups were established for *Cx. tarsalis* (Naumenko, Timoshevskiy et al. 2015). The sex locus in *Cx. tarsalis* is located between microsatellite markers TB210 and TB218 on the longest chromosome (Naumenko, Timoshevskiy et al. 2015).

## **Chapter 2: Materials and Methods**

### **2.1 Mosquitoes strains**

#### **2.1.1 *Culex tarsalis***

The following reagent was obtained through BEI Resources, NIAID, NIH:

*Culex tarsalis* YOLO, NR-43026. *Cx. tarsalis* YOLO strain was used in these experiments. Eggs were hatched at 28 degrees Celsius. Then 3rd instar larvae were transferred to 16 degrees Celsius.

#### **2.1.2 *Culex pipiens***

A laboratory strain of *Cx. pipiens* Chicago was used. This colony was established by mosquitoes collected in areas throughout Chicago, IL.

#### **2.1.3 *Culex quinquefasciatus***

Johannesburg (JHB) strain was used for *Cx. quinquefasciatus*. This strain was previously sequenced (Arensburger, Megy et al. 2010) and also used for the cytogenetic studies (Naumenko, Timoshevskiy et al. 2015, Unger, Sharakhova et al. 2015).

### **2.2 Chromosome preparation**

4th instar larvae were put on ice for several minutes for immobilization and then transferred onto a glass slide. Larvae were then covered in a drop of cold hypotonic solution (0.5% sodium citrate) for dissection. The larvae were then decapitated, and the cuticle was cut from the ventral side of the thorax using scissors. Next, the guts were dissected from the fat bodies. Then the hypotonic solution was wiped off, and a new drop was added. Once the hypotonic solution was removed, Carnoy's solution (ethanol/acetic acid in 3:1 ratio) was applied.

The imaginal discs immediately turned white and became easily visible. The imaginal discs were then removed from the larva using dissection needles, and any other guts and fat bodies were removed. The imaginal discs were then put in 50% propionic acid. The imaginal discs were covered with a coverslip for 10 minutes, and then the slide was covered with a filter paper and squashed by the tapping with the eraser of a pencil. The slide was then observed under a microscope and slides with greater than 50 chromosome spreads were considered suitable for measurements and fluorescent *in situ* Hybridization (FISH).

These slides were then dipped in liquid nitrogen until there were no bubbles left, and the coverslip was then removed using a razor. The slide was then put into 50% ethanol and chilled at -20 degrees Celsius. The slides were then dehydrated in a series of 70%, 80%, 100% ethanol at 4 degrees Celsius for 5 minutes each, and then air-dried at room temperature.

## **2.3 Fluorescent *in situ* Hybridization**

### **2.3.1 DNA probe preparation**

Genomic DNA was extracted from the YOLO county using the Qiagen Blood & Cell Culture DNA Maxi Kit (Qiagen Science, Germantown, MD, USA). An 18S rDNA probe was amplified with forward primer CCTATATGGTGGCGCTTGAT and reverse primer AACTAAGAACGGCCATGCAC. It was then labeled with Cy3- and Cy5-deoxyuridine 5-triphosphate (dUTPs) (GE Healthcare UK Ltd., Buckingham-shire, UK) in a PCR reaction using PCR IMMOMIX (Bioline USA, Taunton, MA).

### **2.3.2 Prehybridization**

Slides were put into a humid chamber with 100 ul of 2 x SSC with RNAase solution and then covered with parafilm as a coverslip, at 37 degrees Celsius for 30 minutes. Slides were then put into a Caplin jar containing pepsin and HCL solution and incubated at 37 degrees Celsius for 4-5 minutes. Slides were washed in 1 x PBS for 5 minutes at room temperature. Slides were dehydrated in a series of ethanol of 70%, 80%, and 100% for 5 minutes each at room temperature.

### **2.3.3 Hybridization**

Slides were denatured in 70% formamide for 5 minutes at 72 degrees Celsius and then were washed in a series of cold ethanol 70%, 80%, and 100% that was at -20 degrees Celsius at 5 minutes each. The hybridization mix (50% formamide, 10% dextran sulfate, and 0.1%) was incubated at 97 degrees Celsius and then for 30 minutes at 37 degrees Celsius. 5 ul of the probe in hybridization buffer were placed on each slide and then covered with a coverslip and sealed with rubber cement. Slides were incubated in a humid chamber at 37 degrees Celsius for at least 8 hours overnight for Hybridization.

### **2.3.4 Washing/staining**

Coverslip and glue were removed from the slides. Slides were put into solution 1 (49 ml distilled water, 1 ml 20xSSC, 150 ul NP4O) for 2 minutes at 73 degrees Celsius, and then solution 2 (50 ml 2xSSC and 50 ul NP4O) for 5 minutes. Slides were rinsed with 1 x PBS. Oxazole Yellow (YOYO-1) iodide (Invitrogen Corporation, Carlsbad, CA, USA) was used to stain for 15 minutes. Then rinsed in 1xPBS, antifade Prolong Gold (Invitrogen Corporation, USA) is covered with a coverslip.

### **2.3.5 Image processing**

Pictures were acquired using the Zeiss LSM 510 Laser Scanning Microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) at 1000x magnification.

### **2.3.6 Chromosome measurements**

Early metaphase and mid-metaphase chromosomes were utilized for chromosome measurements and chromosome idiogram development. The lengths of the chromosomes were measured using Zen 2009 Light Edition software. Proportions of chromosomes were calculated using the standard curve measurements in Zen 2009 Light Edition software.

### **2.3.7 Statistical analysis**

Statistical analysis was performed using JMP Pro 14 software program (Fit X by Y, ANOVA). The relative length and centromeric index were calculated in order to develop the nomenclature for *Cx. tarsalis*. Chromosomes were identified based on the ratio of their arms and lengths. The long chromosome arm is  $q$  and  $p$  the short arm. The centromeric index was calculated by dividing the shortest arm by the length of the chromosome arm. The percentage relative chromosome length was calculated by the length of the chromosome divided by the total length of all chromosomes multiplied by 100.

## **2.4 Idiogram development**

The best images of chromosomes from imaginal discs were stained with Oxazole Yellow (YOYO-1) iodide (Invitrogen Corporation, Carlsbad, CA, USA) were selected for the development of idiograms. The images were converted to grayscale images and straightened and

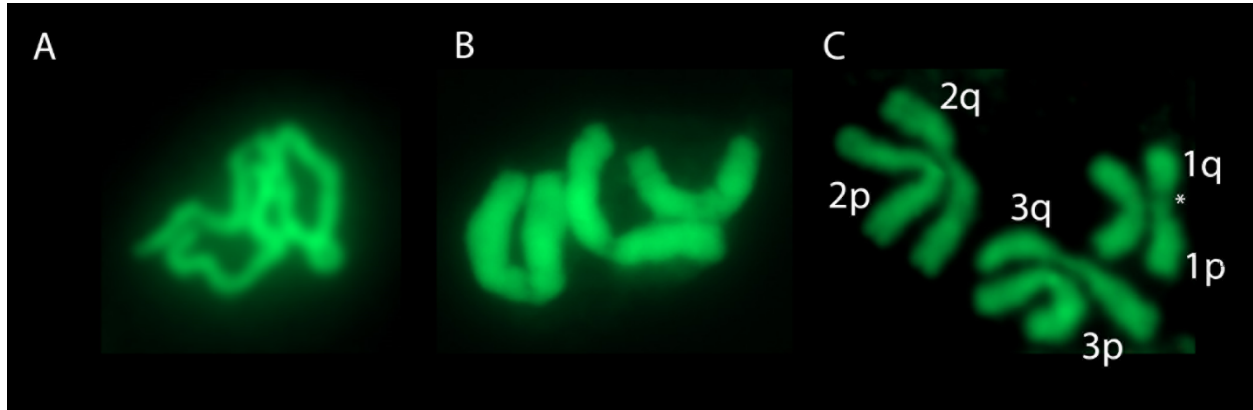
aligned for comparison using ImageJ program. 120 chromosome measurements were analyzed for *Culex tarsalis* and *Culex pipiens*, and *Culex quinquefasciatus*.

### **3 Results and discussion**

#### **3.1 Chromosome map for the genome mapping in *Culex tarsalis***

For the development of the chromosome map suitable for the physical mapping of the *Cx. tarsalis* genome the metaphase mitotic chromosomes from fourth instar larvae imaginal discs were utilized. Mosquito mitotic chromosomes are diploid sets. The chromosome nomenclature for *Cx. quinquefasciatus* was revised from the original nomenclature which were in order of increasing size (Rai 1963), to 1 being the smallest, 2 being the largest, and 3 being the intermediate (Naumenko, Timoshevskiy et al. 2015). The chromosomes were identified according to their length.

*Cx. tarsalis* mitotic chromosomes were stained with YOYO-1 iodide Fig. 1 shows a range of different stages of mitosis (prophase, prometaphase, and metaphase). Homologous chromosomes are paired at prophase and prometaphase (Fig. 4A, B). Chromosomes in prometaphase and prophase are not used due to the homologous chromosomes being tightly paired similar to other *Culicinae* mosquitoes (Sharakhova, Timoshevskiy et al. 2011). At prometaphase, the chromosomes start segregating from each other and become completely segregated at metaphase (Fig. 3.1C). During metaphase, six chromosomes are visible.



**Figure 3.1 Stages of Mitosis (A-C) chromosomes in early metaphase (C) were used for idiogram development.** Prophase (Fig. 3.1A) and prometaphase (Fig. 3.1B) cannot be used for the measurements or idiogram development. During these two phases, three chromosomes are visible. During metaphase (Fig. 3.1C) six chromosomes are visible and labeled.

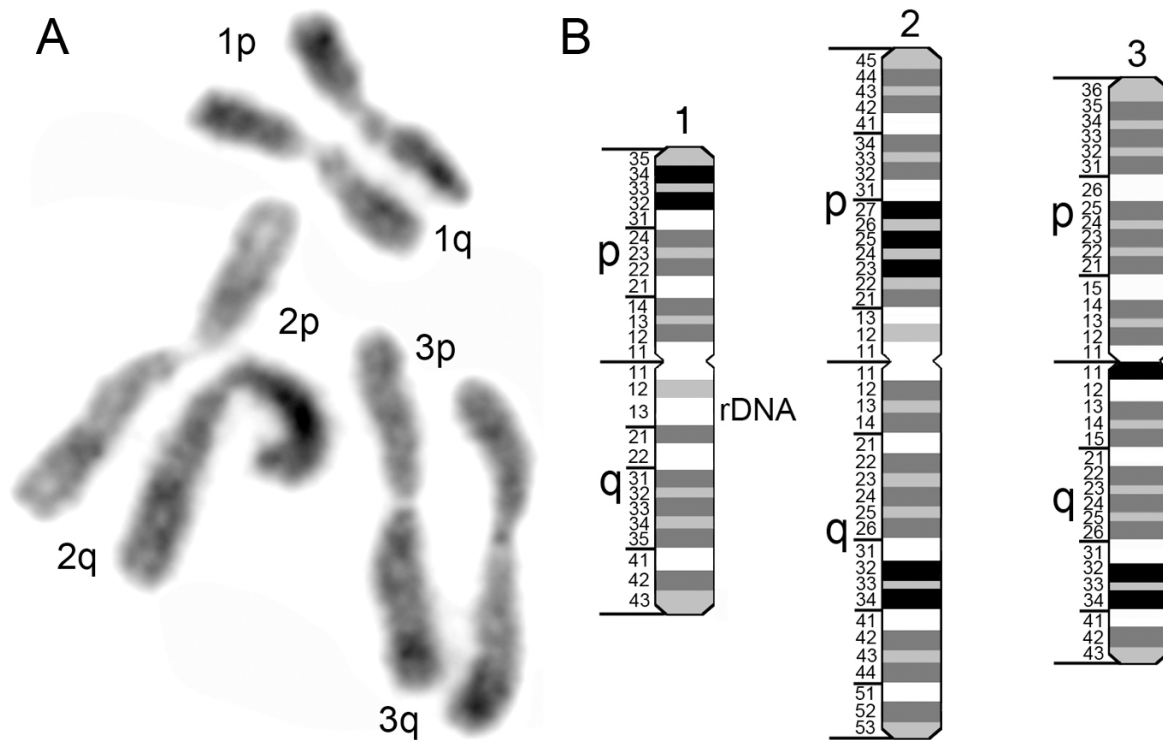
The lengths of chromosomes, arms, relative lengths, and centromeric index were computed using the standard curve measurements in Zen 2009 Light Edition software. The mean value of all measurements for each lengths of metaphase chromosomes were calculated (Table 1). About 120 chromosomes were measured. The average chromosome lengths of *Cx. tarsalis* at metaphase are 6.17  $\mu\text{m}$  for chromosome 1, 9.04  $\mu\text{m}$  for chromosome 2 and, 7.92  $\mu\text{m}$  for chromosome 3 (Table 1). The relative lengths of *Cx. tarsalis* chromosomes were 26.68%, 39.08%, and 34.32% for each chromosome, respectively. All three chromosome pairs of the species studied are metacentric based on the calculated relative lengths and centromeric index (Levan, Fredga et al. 1964). Based on these calculations the chromosomes meet the characteristics of metacentric chromosomes. The centromeric indexes, the relative length of the p-arm, were 46.05%, 45.20%, and 47.56% for three chromosomes, respectively.

**Table 3.1 Measurements of *Cx. tarsalis* mitotic chromosomes in comparison to *Cx. pipiens* and *Cx. quinquefasciatus*. Chromosome measurements of *Cx. quinquefasciatus* were published earlier (Naumenko, Timoshevskiy et al. 2015).**

<b>Mosquito species</b>	<b><i>Cx. tarsalis</i></b>	<b><i>Cx. pipiens pipiens</i></b>	<b><i>Cx. quinquefasciatus</i></b>
<b>Chromosome</b>	6.17	5.82	4.04
<b>1 Average length, <math>\mu\text{m}</math></b>			
Relative length, %	26.68%	26.71%	23.30%
Centromeric index, %	46.05%	44.94%	43.1% or 48.1%
<b>Chromosome</b>	9.04	8.40	6.37
<b>2 Average length, <math>\mu\text{m}</math></b>			
Relative length, %	39.08%	38.60%	39.80%
Centromeric index, %	45.20%	47.43%	47.4%
<b>Chromosome</b>	7.92	7.55	5.59
<b>3 Average length, <math>\mu\text{m}</math></b>			
Relative length, %	34.32%	34.69%	34.9%
Centromeric index, %	47.56%	46.18%	46.9%

The chromosome idiograms are diagrammatic representations of the chromosome banding patterns. The several best images of mitotic chromosomes from imaginal discs were stained with Oxazole Yellow (YOYO-1) iodide were selected for the development of idiograms. YOYO-1 iodide stains euchromatin and provides a detailed banding pattern (Timoshevskiy, Sharma et al. 2012). The chromosome images were converted to grayscale images and straightened and aligned for comparison. Idiograms for the *Cx. tarsalis* chromosomes were

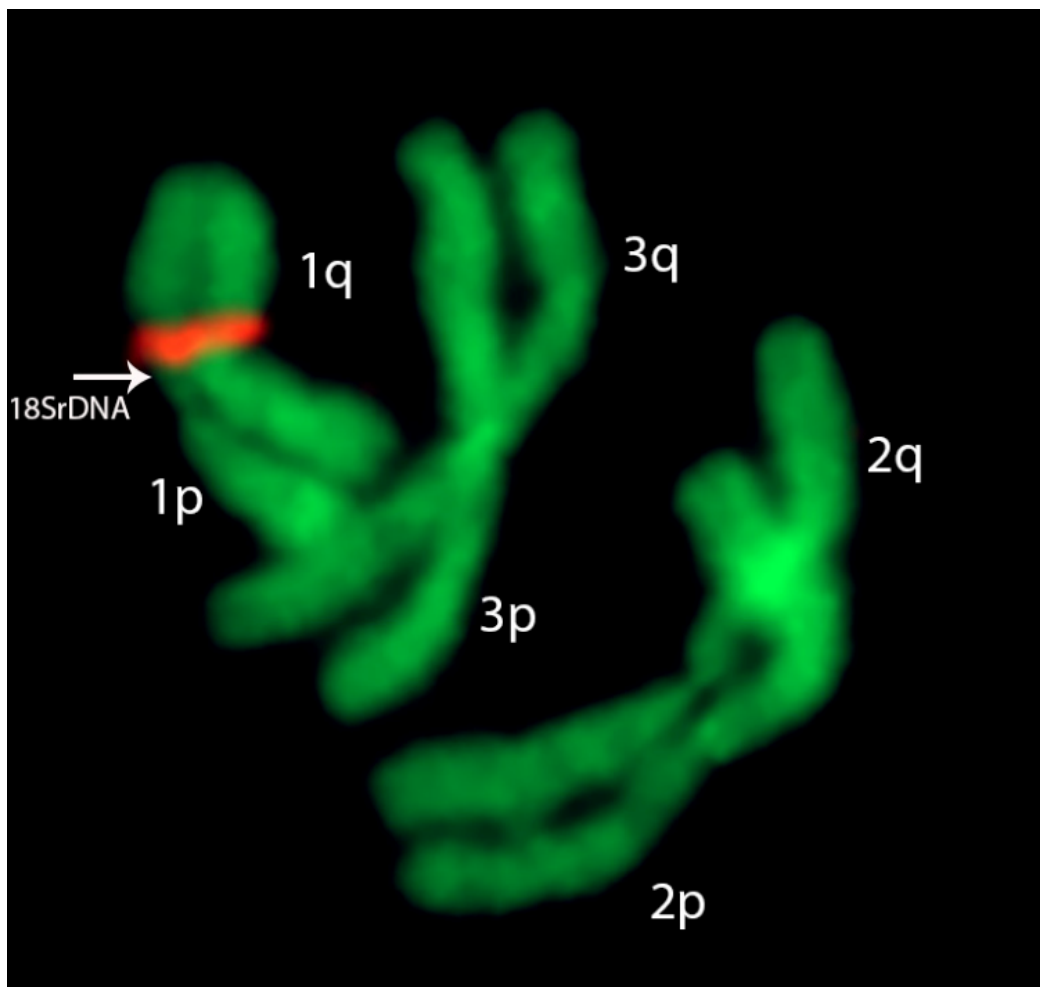
developed using standard human nomenclature (Shaffer, Slovak et al. 2009). Centromeric regions of the chromosomes were aligned and chromosome divisions and subdivisions were numbered from the centromeres to the telomeres. A total of 101 bands were identified: 26, 40 and 35 for the chromosomes 1, 2, and 3 respectively. Chromosome bands of 4 different intensities were determined: black, dark gray, light gray, and negative. Large negative bands on the chromosomes served as boundaries between divisions. This map would be suitable for the physical mapping of *Cx. tarsalis* genome. However, the physical mapping will require further validation of these idiograms on a bigger set of the chromosomes.



**Figure 3.2.** the development of chromosome idiograms for *Cx. tarsalis*. Gray-scale image with banding pattern is shown on panel A. Idiograms are shown on panel B. p and q indicate

short and long chromosome arms, respectively. Chromosomes indicated by numbers, Chromosome 1, 2, and 3. The ribosomal DNA is labeled in chromosome 1 on the q arm.

Chromosome divisions and subdivisions are shown on the left side of the chromosome. The location of rDNA is indicated on the right side of the chromosome 1. To prove the suitability of the map for the physical mapping we mapped 18S ribosomal gene to the chromosomes of *Cx. tarsalis* (Fig. 3.3). The rDNA locus was placed in division 1q13. This region forms a secondary constriction on the chromosome 1 that is indicated as a large negative band in *Cx. tarsalis* idiogram.

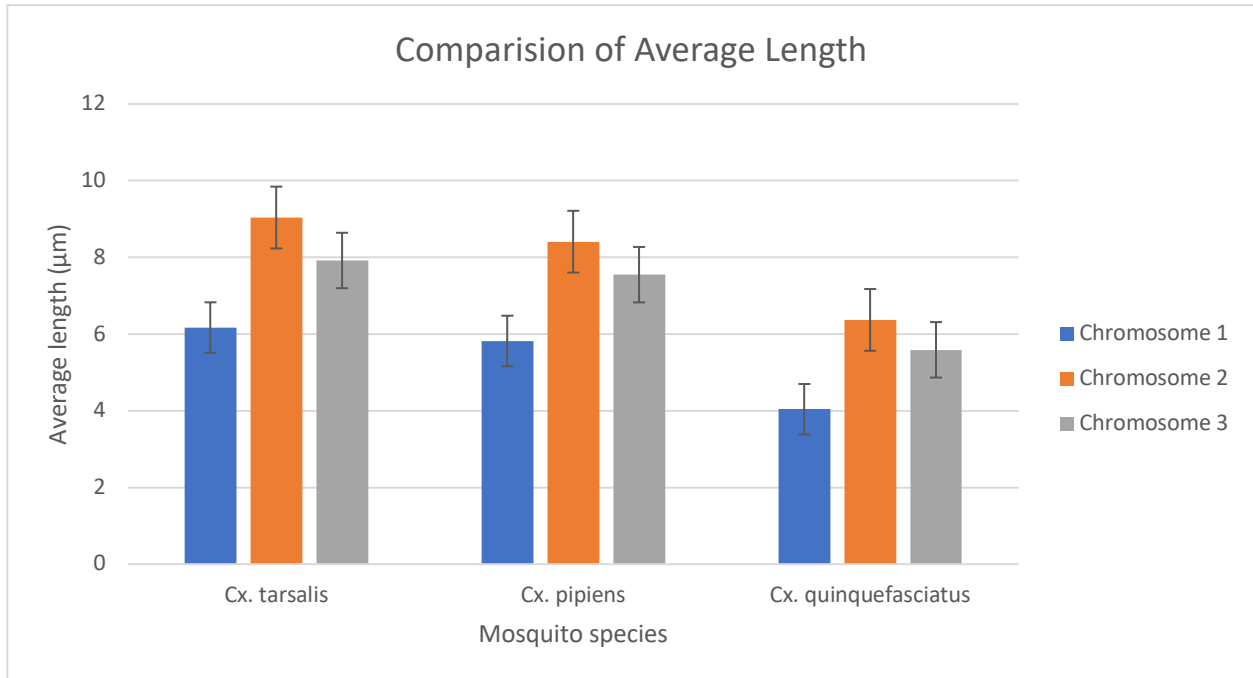


**Figure 3.3 Fluorescent *in situ* hybridization of 18S ribosomal DNA in chromosomes of *Cx. tarsalis*.** The location of 18S rDNA on chromosome 1 is indicated by the arrow.

The preliminary idiogram developed for *Cx. tarsalis* are comparable to previously developed *Ae. aegypti* and *Cx. quinquefasciatus* idiograms (Naumenko, Timoshevskiy et al. 2015). The number of bands is higher in *Cx. tarsalis* than in *Cx. quinquefasciatus*. Overall this comparison suggests that the idiograms developed in this study for *Cx. tarsalis* are suitable for further genome mapping in this species.

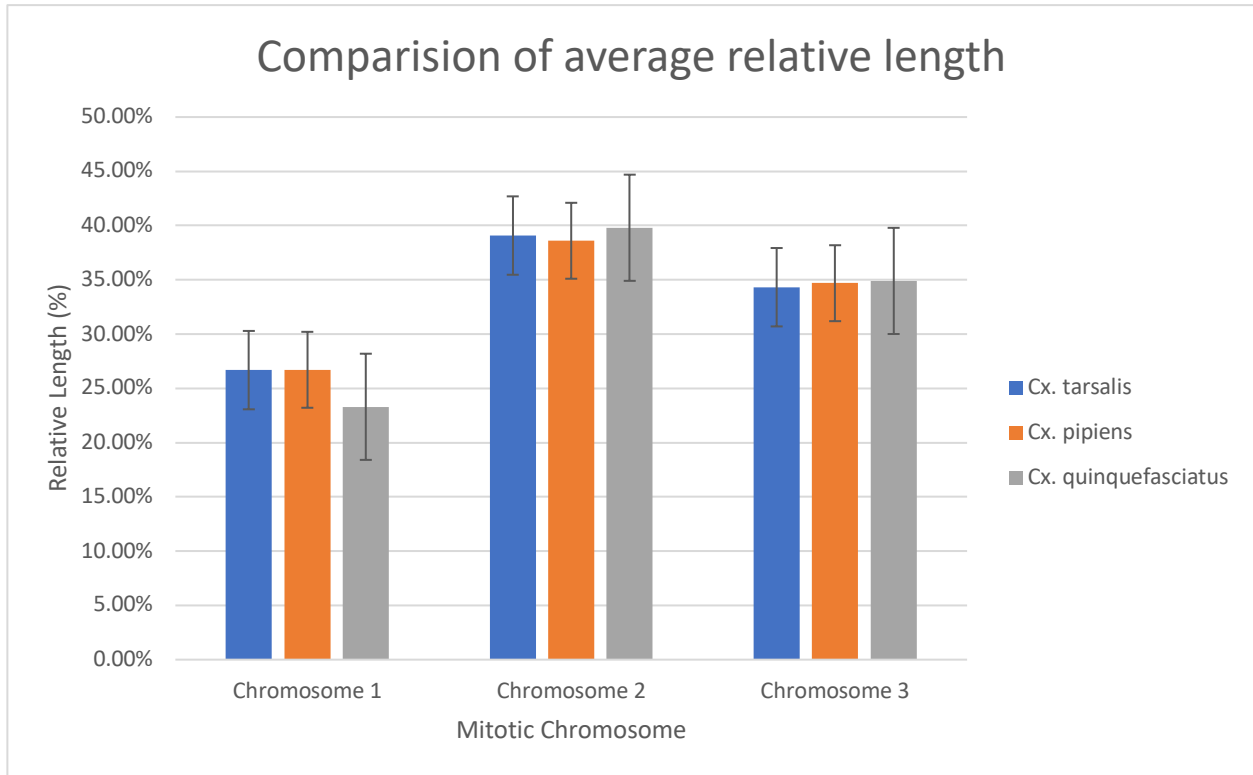
### **3.2 Comparative analysis of *Cx. tarsalis*, *Cx. pipiens*, and *Cx. quinquefasciatus* chromosomes**

We analyzed mitotic chromosomes in three mosquito species *Cx. tarsalis*, *Cx. pipiens*, and *Cx. quinquefasciatus*. According to our measurements the total chromosome length in *Cx. tarsalis* is longer than *Cx. quinquefasciatus* and *Cx. pipiens* (Table 3.1, Fig. 3.4). This difference is reflected in the genome sizes of *Cx. quinquefasciatus* and *Cx. tarsalis*. Indeed, *Cx. quinquefasciatus* genome is 579 Mb (Arensburger, Megy et al. 2010) but *Cx. tarsalis* genome is 800 Mb (Main, Brown et al. 2020)



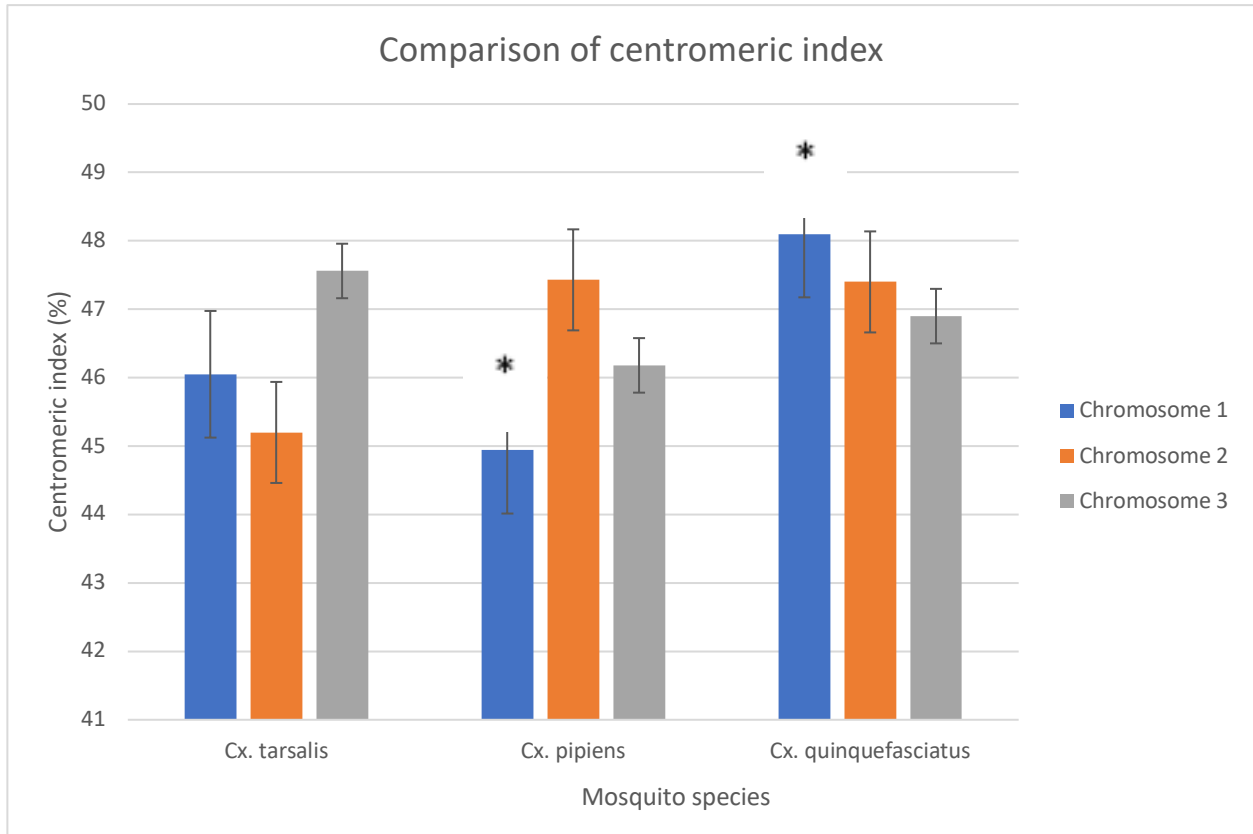
**Figure 3.4 Comparison of Average length of *Cx. tarsalis*, *Cx. pipiens*, and *Cx. quinquefasciatus* chromosomes.** Chromosome measurements of *Cx. quinquefasciatus* were published earlier (Naumenko, Timoshevskiy et al. 2015).

The comparison of the relative length of the chromosomes detected absence of significant differences between the species suggesting no large chromosomal rearrangements between the chromosomes in three species. However, small rearrangements that are not detectable by cytogenetic analysis cannot be excluded. Thus, future genome mapping will shade light to this question.



**Figure 3.5 Comparison of the average relative lengths of *Cx. tarsalis*, *Cx. pipiens*, and *Cx. quinquefasciatus* chromosomes.** Chromosome measurements of *Cx. quinquefasciatus* were published earlier (Naumenko, Timoshevskiy et al. 2015).

Intriguingly, we found significant differences in the centromeric indexes between *Cx. pipiens* and *Cx. quinquefasciatus* in chromosome 1 (Fig. 3.6) that may suggest presence of pericentric inversion in this chromosome between the species or amplification of ribosomal genes in *Cx. pipiens*.



**Figure 3.6 Comparison of centromeric index of *Cx. tarsalis*, *Cx. pipiens*, and *Cx. quinquefasciatus* chromosomes.** Chromosome measurements of *Cx. quinquefasciatus* were published earlier (Naumenko, Timoshevskiy et al. 2015). There is a significant difference in the centromeric index between *Cx. pipiens* and *Cx. quinquefasciatus*. This is labeled on the graph by asterisks.

Chromosomal rearrangements play important role in evolution of different organisms. For, example, the comparative analysis of five species of Anopheles mosquitoes: *An. atroparvus*, *An. albimanus*, *An. funestus* and *An. stephensi* and *An. gambiae* revealed highly nonuniform rates of gene order reshuffling among the chromosomes (Neafsey, Waterhouse et al. 2015). The highest rate of the chromosome evolution was determined for the sex chromosome X. It was shown that the ratio of the rates of evolution of sex chromosome to all chromosomes in malaria

mosquitoes is significantly higher in *Anopheles* than in *Drosophila*,  $t(10)=7.299$ ,  $p=0.000026$ , with means of 2.726 and 1.200, respectively.

The comparison of the X chromosome evolution in malaria mosquitoes and sex-determining autosome 1 in *Ae. aegypti*, which is homologous to the X and 2R arm in *An. gambiae*, demonstrated significantly more breaks per mega base (Mb) than the rest of the autosomes (Timoshevskiy, Kinney et al. 2014). This study determined a high abundance of simple tandem repeats in both the X chromosome and sex-determining chromosome 1 suggesting their role in genomic plasticity. In addition, a large translocation between chromosome 2 and chromosome 1 was determined between *Ae. aegypti* and *An. gambiae*. A number of chromosome rearrangements occurred within and between chromosome arms in *Ae. aegypti*.

In previous study, the linkage groups of *Cx. tarsalis* were identified (Venkatesan, Broman et al. 2009). Four linkage groups were established for the three chromosomes. Based on previous studies done in 1978, sex-linked translocation was evident in *Cx. tarsalis* resulting in the sex locus being located on the longest chromosome (McDonald, Asman et al. 1978). Unlike other culicine mosquitoes where the sex determining locus is located on chromosome 1, the shortest chromosome (McDonald, Asman et al. 1978). The ribosomal locus was determined by FISH of 18s rDNA probe. This probe hybridized on the q arm of chromosome 1 (Figure 3.3).

#### **4 Conclusion**

1. This project developed a cytogenetic tool for the further development of the genome map for the major vector of West Nile virus in the USA *Culex tarsalis*. Mapping

18S rDNA to the chromosome 1 confirmed suitability of this map for the physical genome mapping.

2. Chromosome measurements of *Cx. tarsalis*, *Cx. pipiens*, and *Cx. quinquefasciatus* suggest absence of a large chromosomal rearrangement between the species. This result suggests de novo formation of the sex-determining locus on chromosome 2 in *Cx. tarsalis*. However, small translocation cannot be excluded.

## Summary

This project advances our understanding of cytogenetics in *Culex* mosquitoes and will help to develop a physical genome map for this mosquito. The idiograms for *Cx. tarsalis* was developed and the 18S ribosomal gene was mapped to chromosome 1. Comparing the karyotypes of three species of *Culex* mosquitoes is putting us in steps closer to the better understand chromosomal evolution in *Culicinae* mosquitoes. The comparative analysis of *Cx. tarsalis* to *Cx. pipiens* and *Cx. quinquefasciatus* chromosomes showed that the total chromosome length in *Cx. tarsalis* is longer than the other two species suggesting a bigger genome size in this species. Comparison of the relative chromosome length between the species indicated no significant differences suggesting that no large chromosomal translocation occurred between the species. Based on the comparisons of the centrometric indexes there is a significant difference in chromosome 1 of *Cx. pipiens* and *Cx. quinquefasciatus* in chromosome 1 that suggests a presence of pericentric inversion between the species or an amplification of ribosomal genes in *Cx. pipiens*. Chromosomal rearrangement plays an important role in the evolution of different organisms. The next steps in this study could be to look at different genes that can play a role in making *Culex* species mosquitoes different from other mosquitoes. Then map the genetic

markers to their precise position on the chromosomes to make a more detailed physical map for *Cx. tarsalis*. Availability of high-quality genome for *Cx. tarsalis* anchored to the chromosomes will improve our understanding of the genetic makeup of this mosquito and how it is able to transmit viruses. That knowledge may help with targeting specific *Culex* genes that are involved with the transmission of West Nile Virus and other arthropod-borne viruses.

### **List of abbreviations**

FISH - fluorescent in situ hybridization

Mb - mega base pairs

Kb- kilo base pairs

PCR - polymerase Chain Reaction

DNA - deoxyribonucleic acid

dUTP - deoxyuridine 5-triphosphate

YOYO - Oxazole Yellow

rDNA - ribosomal DNA

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Supplementary Table: Measurements for *Culex tarsalis* and *Culex pipiens*

Table 2: *Cx. tarsalis* measurements

<b>Cx. tarsalis</b>	<b>1p</b>	<b>1q</b>	<b>arm total</b>	<b>2p</b>	<b>2q</b>	<b>arm total</b>	<b>3p</b>	<b>3q</b>	<b>arm total</b>	<b>total</b>
	2.72	3.217	5.937	4.317	4.894	9.211	3.23	3.918	7.148	22.296
	2.063	2.392	4.455	3.132	4.173	7.305	2.691	3.352	6.043	17.803
	2.494	3.16	5.654	3.787	4.685	8.472	3.202	3.407	6.609	20.735
	3.138	3.754	6.892	4.794	5.96	10.754	4.547	5.281	9.828	27.474
	3.018	4.017	7.035	3.957	4.937	8.894	4.021	4.349	8.37	24.299
	2.699	3.019	5.718	4.809	5.434	10.243	4.073	4.612	8.685	24.646
	3.169	3.747	6.916	3.971	4.782	8.753	3.973	4.213	8.186	23.855
	2.458	2.514	4.972	3.791	4.773	8.564	3.067	3.231	6.298	19.834
	3.31	4.04	7.35	4.698	5.471	10.169	4.309	5.072	9.381	26.9
	2.926	3.391	6.317	4.604	5.104	9.708	3.716	4.398	8.114	24.139
	1.994	2.134	4.128	3.696	4.417	8.113	2.962	3.051	6.013	18.254
	2.822	2.903	5.725	4.609	5.264	9.873	4.634	4.792	9.426	25.024
	2.276	2.671	4.947	3.981	4.732	8.713	3.597	3.649	7.246	20.906
	2.726	3.769	6.495	4.51	5.467	9.977	3.943	4.394	8.337	24.809
	3.116	3.286	6.402	4.023	5.266	9.289	3.699	4.094	7.793	23.484
	2.911	3.084	5.995	3.715	4.293	8.008	3.54	3.87	7.41	21.413
	3.228	3.88	7.108	3.801	4.388	8.189	4.188	4.328	8.516	23.813
	1.887	2.27	4.157	3.495	4.231	7.726	2.698	3.173	5.871	17.754

	2.72	3.217	5.937	3.617	4.694	8.311	3.23	3.918	7.148	21.396
	3.063	3.392	6.455	4.032	4.873	8.905	4	4.352	8.043	23.403
	3.494	4.16	7.654	3.587	4.285	7.872	3.202	3.407	6.609	22.135
	3.138	3.754	6.892	3.894	4.96	8.854	3.547	4.281	7.828	23.574
	3.368	3.597	6.965	3.959	4.904	8.863	4.139	4.615	8.754	24.582
	3.018	4.017	7.035	4.057	5.237	9.294	4.021	4.349	8.37	24.699
	2.748	3.317	6.065	4.045	4.962	9.007	3.281	3.516	6.797	21.869
	2.819	3.226	6.045	4.092	5.151	9.243	3.816	4.142	7.958	23.246
	3.359	3.621	6.98	4.054	5.329	9.383	4.073	4.314	8.387	24.75
	2.299	3.019	5.318	3.819	4.734	8.553	3.073	3.612	6.685	20.556
	3.169	3.747	6.916	3.971	4.782	8.753	3.973	4.213	8.186	23.855
	2.055	2.433	4.488	3.436	4.369	7.805	3.054	3.623	6.677	18.97
	3.458	3.514	6.972	4.991	5.973	10.964	5.067	5.231	10.298	28.234
	2.51	3.04	5.55	4.309	5.072	9.381	3.798	4.071	7.869	22.8
	3.711	4.279	7.99	4.501	5.398	9.899	4.208	4.646	8.854	26.743
	2.926	3.391	6.317	4.804	5.104	9.908	3.716	4.398	8.114	24.339
	3.394	3.934	7.328	4.696	5.317	10.013	3.962	4.051	8.013	25.354
	2.822	2.903	5.725	3.709	4.664	8.373	3.634	3.792	7.426	21.524
	2.076	2.671	4.747	3.781	4.982	8.763	3.597	3.649	7.246	20.756
	2.81	3.19	6	3.918	4.581	8.499	3.895	4.228	8.123	22.622
	2.726	3.769	6.495	4.51	5.267	9.777	3.943	4.394	8.337	24.609
	3.116	3.986	7.102	4.023	4.966	8.989	4.699	5.094	9.793	25.884
	2.917	3.152	6.069	3.707	4.556	8.263	3.507	3.654	7.161	21.493

	2.911	3.384	6.295	4.215	5.093	9.308	4.44	4.87	9.31	24.913
	2.62	3.217	5.837	4.317	5.494	9.811	4.23	4.918	9.148	24.796
Total										
Average	2.84190	3.32972	6.17162	4.08683	4.95390	9.04074	3.7647	4.15167	7.91646	23.1288
	698	093	791	721	698	419	907	442	512	372

**Table 3: *Cx. pipiens* measurements**

Cx. Pipiens	1p	1q	arm total	2p	2q	arm total	3p	3q	arm total	total
	2.469	2.769	5.238	3.717	4.153	7.87	2.791	3.575	6.366	19.474
	2.095	2.076	4.171	4.441	4.538	8.979	3.436	3.55	6.986	20.136
	2.188	2.939	5.127	3.639	3.661	7.3	3.042	3.083	6.125	18.552
	1.87	2.514	4.384	3.656	4.141	7.797	2.999	4.091	7.09	19.271
	2.389	2.913	5.302	3.575	3.623	7.198	3.36	3.667	7.027	19.527
	3.233	2.753	5.986	4.043	4.077	8.12	3.665	3.87	7.535	21.641
	2.843	3.08	5.923	3.774	4.361	8.135	3.287	3.945	7.232	21.29
	2.923	2.779	5.702	3.825	4.154	7.979	3.2	4.639	7.839	21.52
	3.654	4.5	8.154	5.361	5.428	10.789	4.664	5.576	10.24	29.183
	2.436	3.179	5.615	3.829	4.21	8.039	2.956	3.854	6.81	20.464
	2.567	3.826	6.393	4.4	4.855	9.255	3.878	4.34	8.218	23.866
	2.128	2.53	4.658	2.974	3.791	6.765	2.932	3.267	6.199	17.622
	2.427	2.856	5.283	3.768	4.085	7.853	2.866	3.841	6.707	19.843
	2.487	3.519	6.006	3.826	3.735	7.561	3.045	3.321	6.366	19.933

	2.458	2.874	5.332	3.818	4.22	8.038	3.129	4.684	7.813	21.183
	2.935	3.53	6.465	4.102	4.52	8.622	3.716	4.281	7.997	23.084
	3.048	3.653	6.701	4.9	5.5	10.4	4.19	4.364	8.554	25.655
	2.879	3.516	6.395	3.973	5.225	9.198	4.366	4.621	8.987	24.58
	4.323	4.341	8.664	5	6.252	11.252	5.34	5.355	10.695	30.611
	2.619	4.859	7.478	5.067	5.284	10.351	4.542	5.441	9.983	27.812
	2.38	2.955	5.335	3.457	4.674	8.131	3.195	3.97	7.165	20.631
	2.46	3.6	6.06	4.15	4.26	8.41	3.331	3.72	7.051	21.521
	2.06	3.1	5.16	3.13	3.991	7.121	3.151	3.52	6.671	18.952
	2.369	2.669	5.038	3.617	4.053	7.67	2.691	3.475	6.166	18.874
	2.195	2.176	4.371	4.541	4.638	9.179	3.536	3.651	7.187	20.737
	2.088	2.839	4.927	3.539	3.561	7.1	2.942	2.983	5.925	17.952
	1.77	2.414	4.184	3.556	4.041	7.597	2.899	3.991	6.89	18.671
	2.289	2.813	5.102	3.475	3.523	6.998	3.26	3.567	6.827	18.927
	3.133	2.653	5.786	3.943	3.977	7.92	3.565	3.773	7.338	21.044
	2.743	2.982	5.725	3.674	4.261	7.935	3.187	3.845	7.032	20.692
	2.723	2.579	5.302	3.625	3.954	7.579	3.01	4.439	7.449	20.33
	3.554	4.451	8.005	5.261	5.328	10.589	4.564	5.476	10.04	28.634
	2.336	3.079	5.415	3.729	4.113	7.842	2.856	3.754	6.61	19.867
	2.767	3.26	6.027	4.1	4.55	8.65	3.878	4.34	8.218	22.895
	2.028	2.43	4.458	2.874	3.691	6.565	2.832	3.167	5.999	17.022
	2.227	2.856	5.083	3.668	3.885	7.553	2.666	3.141	5.807	18.443
	2.482	3.51	5.992	3.821	3.731	7.552	3.04	3.32	6.36	19.904

	2.358	2.674	5.032	3.718	4.124	7.842	2.929	4.384	7.313	20.187
	2.835	3.43	6.265	4.002	4.32	8.322	3.516	4.281	7.797	22.384
	2.948	3.453	6.401	4.73	5.31	10.04	4.09	4.264	8.354	24.795
	2.679	3.516	6.195	3.973	5.225	9.198	4.366	4.621	8.987	24.38
	4.32	4.34	8.66	5.33	6.052	11.382	5.34	5.355	10.695	30.737
	2.619	4.859	7.478	5.067	5.284	10.351	4.54	5.41	9.95	27.779
	2.38	2.955	5.335	3.457	4.674	8.131	3.195	3.97	7.165	20.631
	2.46	3.6	6.06	4.15	4.26	8.41	3.331	3.72	7.051	21.521
	2.06	3.1	5.16	3.13	3.991	7.121	3.151	3.52	6.671	18.952
Total										
average	2.6137	3.2021	5.8159	3.9870	4.4192	8.4062	3.4883	4.0656	7.5540	21.776
e	8261	5217	3478	6522	1739	8261	6957	9565	6522	2826