Optimization of Cytogenetic and Physical mapping of Culicinae genomes

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Abstract

Understanding chromosome structure and genome organization of Culicine mosquitoes can potentially contribute to the development of novel approaches to vector control. However, because of highly repetitive nature of the *Aedes* and *Culex* genomes, the structure of their polytene chromosomes is damaged by ectopic contacts that make the analysis difficult. Mitotic chromosomes from imaginal discs of 4^{th} instar larvae of *Aedes aegypti* were tested as a source for the physical genome mapping for this mosquito. Chromosomes in imaginal discs are 10 times more abundant than chromosomes in nervous ganglia, and they do not accumulate chromosomal mutation as cell line chromosomes do. Prometaphase chromosomes in imaginal discs of *Ae. aegypti* are 4-5 times longer than metaphase chromosomes and can provide higher resolution for physical mapping. Cold temperature ($+16^{o}C$) was proven to increase the number of the chromosomes. Hypotonic solution treatment of live larvae was proven to elongate chromosomes and improve banding patterns. We differentially stained these mitotic chromosomes with Giemsa and YOYO-1 to revile the banding pattern. We applied fluorescent *in situ* hybridization (FISH) procedure developed for human chromosomes to *Ae. aegypti* chromosomes. A strain from *Culex pipiens, Cx. quinquefasciatus* and their hybrids from the natural population in Virginia was successfully colonized in the laboratory. This strain can be used as a reliable source for cytogenetic studies.

Dedication

To those who work on vector borne diseases, I hope my research could contribute a little bit to this research.

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Introduction

Mosquitoes from family Culicidae are efficient vectors of arthropod-borne viruses and parasitic nematodes including yellow fever virus, dengue virus, West Nile virus and lymphatic filariasis worm. These diseases kill and disable millions of humans every year (Tolle 2009). Knowledge of the chromosome structure and genome organization of mosquitoes can help to better understand two fundamental characteristics: biology and population structure (Sharakhov and Sharakhova 2008). These two characteristics contribute to the inhibition of the disease transmission and vector control. For these reasons the genomes of *Anopheles gambiae, Aedes aegypti,* and *Culex quinquefasciatus* have been studied and sequenced (Holt, Subramanian et al. 2002; Nene, Wortman et al. 2007; Arensburger, Megy et al. 2010). However, further assembly of the whole genome sequence is required to fully utilize this data. Much more can still be learned about cryptic complexes of species like *An. gambiae* complex and *Cx. pipiens* complex. Further study of the phylogenetic relationships within complexes as well as vector competence is necessary for the better understanding of these vectors. Physical mapping that hybridizes a probe of known sequence to chromosomes via *in situ* hybridization has proven to be a good tool to orient and order sequences along chromosomes. The genome of the malaria vector *An. gambiae* was assembled based on the results of *in situ* hybridization of 2000 bacterial artificial chromosomes (BAC) clones to polytene chromosomes (Sharakhova, Hammond et al. 2007). More than 80% of genome scaffolds were placed to precise chromosomal positions using this method. However, compared with Anopheline polytene chromosomes, Culicine and Aedine polytene chromosomes are significantly underpolytenized, unspreadable, and useless for the physical mapping of the genome. For this reason, mitotic chromosomes are the alternative material used for mapping in Culicinae mosquitoes. Previous physical maps based on mitotic

chromosomes from the cell line of *Ae. aegypti* achieves a great improvement in fluorescent *in situ* hybridization (FISH) (Brown, Menninger et al. 1995; Brown and Knudson 1997). However, chromosomes from the cell line usually accumulate chromosomal aberrations. This might lead to misplacement of scaffolds to the chromosomes. The objectives of this study are to develop a tool for a high-resolution, precise physical mapping on mitotic chromosomes from *Ae. aegypti*, and colonize a strain from Virginian population of *Cx. pipiens*, *Cx. quinquefasciatus*, and their hybrids in the laboratory.

Recently, Maria V. Sharakhova identified imaginal discs (ID) that will develop as legs, which may provide a reliable source of mitotic chromosomes for physical mapping. Compared to mitotic chromosomes from cell line, these chromosomes, prepared from live larvae do not have genome rearrangements, and show improved banding patterns. Using 10% sugar mixed with rabbit blood, discovered by Sally L. Paulson, is an efficient source for the first blood feeding for *Culex* mosquitoes.

Hypothesis one: We can use prometaphase chromosomes with banding pattern from ID as a material to optimize cytogenetic and physical mapping of *Ae. aegypti*.

Hypothesis two: We can establish and maintain laboratory strains from *Cx. pipiens*, *Cx. quinquefasciatus*, and their hybrids in the laboratory.

The specific aims of this study are:

1) To test if ID in *Ae. aegypti* are a better source of mitotic chromosomes for physical mapping than other tissues used before;
    (i) To optimize conditions for *Ae. aegypti* colony maintenance that will help obtain the best quality and highest number of chromosomes;

(ii) To optimize slide preparation producing the best structure of the *Ae. aegypti* chromosomes;

(iii) To develop *in situ* hybridization procedure for physical mapping of the *Ae. aegypti* genome;

2) To establish a laboratory strain and to find an efficient blood feeding method for *Cx. pipiens, Cx. quinquefasciatus,* and their hybrids in insectary.

Chapter One: Literature review:

1. Important vector borne diseases are transmitted by Culicinae mosquitoes

Mosquito-borne diseases are a part of arthropod-borne diseases causing severe problems for humans (Marquardt, Kondratieff et al. 2005). With increases in global trading and global warming, some local diseases have become more pervasive, and some diseases, previously undetected in humans are becoming established in human populations. Vector, pathogen, vertebrate host and environment form a vector-borne cycle through blood feeding. In this cycle, pathogens are replicated in the vector, and then infect vertebrate animals by blood-sucking; other vectors are then reinfected by subsequent blood feeding on the infected animal.

Unlike Anopheline mosquitoes, which mainly transmit *Plasmodium*, Culicinae mosquitoes have a broader pathogen transmission capability. For example, *Ae. aegypti* and *Cx. pipiens* are responsible for yellow fever virus, dengue virus, West Nile virus, and Japanese encephalitis virus to name just a few. Every year diseases caused by these pathogens affect millions of people around the world (Tolle 2009). Without an effective vaccine, dengue virus causes 500,000 people to die every year. West Nile virus was first found in New York in 1999 and emerged as an epidemic disease across the U.S. *Culex spp* are the bridge-vectors that bite both birds and humans; as a result, West Nile virus can be easily transmitted to humans (Bernard and Kramer 2001). Yellow fever virus can still cause as many as 30,000 deaths annually. Mosquitoes from *Culex pipiens* complex are responsible for the transmission of lymphatic filarasis. It is estimated that more than 120 million people have been infected by lymphatic filarasis. In the absent of effective prophylaxis, vector control is the most effective way to reduce infections. Due to increased prevalence of drug and insecticide resistance, genetic controls such as the introduction

of sterile males or transgenic mosquitoes into populations may provide an alternative way to control transmission of these diseases (Severson, Brown et al. 2001).

2. Culicinae mosquitoes

Mosquitoes are two-winged, blood sucking insects. Based on fossil records, they evolved about 210 million years ago (MYA) (Rai, Black et al. 1999). All mosquitoes belong to family Culicidae in the order of Diptera. Culicidae is divided into three subfamilies: Anophelinae, Toxorhynchitinae, and Culicinae. With the development of molecular genetic data, phylogeneticists can analyze chromosomes and DNA sequences to make a detailed evolutionary tree. This tree could provide us with the answers as to where mosquitoes are derived, when they acquired the blood sucking habit and epidemiologically important questions. Based on chromosome length and genome size, Karamjit, S. Rao concluded that Anophelinae is the ancestral species followed by Toxorhynchitinae, and Culicinae (RAO and Rai 1990). Furthermore, ribosomal DNA and white gene supported this conclusion (Kumar and RAI 1990; Besansky and Fahey 1997; Miller, Crabtree et al. 1997). As the largest subfamily, the Culicinae includes 10 tribes, 33 genera, 117 subgenera and almost 2925 species out of 3436 species in Culicidae. Because Culinicae includes the majority of Culicidae species, and some of important vector are widely distributed, Culicinae attracts much research interest.

The Culicnae mosquitoes experience metamorphosis from the pupae to adult. Female adults from the tribe Culicini mosquitoes prefer to deposit more than 100 eggs holding together on the surface of water as an egg raft, while the tribe Aedine mosquitoes lay eggs individually on a moist surface. Compared to Anopheline larvae, Culicine and Aedine larvae have an elongated air tube used for breathing (Marquardt, Kondratieff et al. 2005).

Only female mosquitoes need blood to develop their ovaries for laying eggs. While feeding on the blood, the female injects saliva into the wound desensitizing the host and preventing coagulation. Saliva comes from salivary glands, located in the thorax of the mosquito. Because many parasites reside in salivary glands, saliva infects the host with diseases.

Furthermore, a change in host preference by the mosquito can also increase the incidence of infection of diseases in animals (Fonseca, Keyghobadi et al. 2004; Home and Table 2009). For example, *Cx. pipiens* prefers to bite birds, but also bites humans in USA. In Europe, this mosquito bites birds exclusively. This character is regarded as the reason for increased transmission of West Nile virus to humans. Due to the increase of trade and travel, Culicinae mosquitoes, like Aedine and Culicini, have been distributed around the world. Since this dissemination, mosquitoes have exerted different genetic factors to adapt to new local environments. For example, *Ae. aegypti*, also called the yellow fever mosquito, has resided in Asia; however, yellow fever in Asia is not a severe problem as in Africa (Marquardt, Kondratieff et al. 2005). The possible reasons are because mosquitoes in Asia are less susceptible to yellow fever virus than American and African strain and antibodies of Dengue and Japanese encephalitis can possibly reduce the infection of yellow fever (Gubler 2004).

Ae. aegypti belongs to Aedini in Culicinae with three subspecies: *Ae. aegypti aegypti*, the type form, *Ae. aegypti formosus*, a sylvan form, and *Ae. aegypti queenslandensis*, a pale type (Severson 2008). Although they originated from Africa, *Ae. aegypti* have been found in nearly all subtropical and tropical areas (Reiter 2010). *Ae. aegypti aegypti* are city dwellers and prefer human blood during daylight hours, these habits which amplify it's vector capability.

Since it has been documented that better control of mosquitoes can help control the yellow fever and dengue, global elimination of *Ae. aegypti* has been attempted (Morrison, Zielinski-Gutierrez et al. 2008). However, they failed because insecticide resistance genes emerged, and no continued efforts to control mosquitoes were made. Genetic control has been shown to be an effective way to control vector population. For example, mating behavior in mosquitoes is monogamous: once the male and the female have mated, females remained inseminated for rest of life. After a blood feeding, females have enough nutrients to develop their ovaries, and then lay eggs. This mating behavior can be exploited by releasing sterile male mosquitoes, controlling the female that make them fertile. Additionally, researchers are focusing on genetic differences to transmit the dengue among *Ae. aegypti* (Lozano-Fuentes, Fernandez-Salas et al. 2009). *Ae. aegypti* from different areas have shown tremendous variety among alleles in genome sequence (Marquardt, Kondratieff et al. 2005). If mutations influencing vector capacity can be found, it will be a chance to create transgenic mosquitoes, and inhibit the transmission of dengue.

The *Cx. pipiens* complex has emerged as an interesting complex of cryptic species (Fonseca, Keyghobadi et al. 2004). There is little consensus about how many the members are included in this complex. Currently, scientists in the U.S. have placed 5 species in this complex: *Cx. pipiens pipiens, Cx. p. quinquefasciatus*, biotype "molestus" and "pallens" and in Australia two sibling species *Cx. atstralicus* and *Cx. globocoxite*. In Europe however, different members are included in this complex (Fonseca, Keyghobadi et al. 2004; Vinogradova, Shaikevich et al. 2007; Atkinson 2009).

As the most widely distributed species in the world, member of the *Cx. pipiens* complex demonstrate a variety of genetic traits in different areas. For example, *Cx. pipiens* in London are autogenous, laying its first batch of eggs without blood feeding. Another difference can be seen

in the proportion of hybrids. It is estimated that 40% of *Cx. pipiens* in USA are hybrids; in Europe this number is only 10% (Couzin 2004). Because of their importance in medical entomology, many researchers have focused on *Cx. pipiens* and *Cx. quinquefasciatus*. The distribution of these two species is different, with *Cx. pipiens* found mainly in the north, and *Cx. quinquefasciatus* predominantly in the south. *Cx. pipiens* has been found in all temperate regions, except Australia. *Cx. quefasciatus* on the other hand, disseminates in tropical and subtropical climates around the world (Cornel, Mcabee et al. 2003). In North America, their hybrids are distributed between 36^{o}N and 39^{o}N. This area includes Virginia where these hybrids can be fertile (Rozeroom 1951). Hybrids may represent a bridge vector, which allows transmission of West Nile virus between humans and birds (Cornel, Mcabee et al. 2003; Couzin 2004).

Identification of hybrids can be accomplished by comparison of morphological differences and molecular markers. The window area of first instar larvae, siphonal index of 4th instar larvae and the male genitalia ratio are three morphological markers to identify two species and their hybrids (Sundararaman 1949; Brogdon 1984). In addition, molecular markers, like acetylcholinesterase-2 (ACE-2) gene and ribosomal DNA (r-DNA) on chromosome 1 and cytochrome oxidase I (COI) gene from mitochondrial DNA have been used to differentiate species and their hybrids.

3. Culicinae genomes

Most mosquitoes contains three pairs of chromosomes, with the exception of *Chagasia bathana* in Anophelinae which has four pairs of chromosomes (Marquardt, Kondratieff et al. 2005). Generally Culicinae mosquitoes have one pair of large, one pair of small, and one pair of averaged sized chromosomes. The centromere is located in the middle, or near the middle of

chromosome. Unlike anophelinae mosquitoes, which display sex chromosome dimorphism, sex in Culicinae mosquitoes is determined by a single locus in chromosome 1 (Kumar and RAI 1990; Severson, Brown et al. 2001).

As for genome structure, eighteen to eighty-four percent of genomes in mosquitoes are repetitive sequence. Anophelinae mosquitoes have 18% repetitive sequence in their genome while in Culicinae mosquitoes, the proportion of repetitive sequence percent increases linearly with the phylogenetic tree. DNA reassociation kinetics can be used to measure repetitive sequences in genomes. This method firstly denatures double stranded DNA into single stranded DNA, and then cools them into a low temperature and measures by spectrophotometer for single and double stranded DNA. Usually, the high repetitive sequence reassociates faster than the single repetitive sequence. To better measure the repetitive sequence, the $C_o t$ value has been used to describe the high repetitive sequence, moderately repetitive sequence, and single repetitive sequence. C_o is the concentration of DNA, and t is the time in seconds. The highly repetitive sequence anneals in a short time, and a longer time is required for reannealing of the unique sequences. In Culicinae mosquitoes, they have a short period interspersion, a pattern that include single copy sequences, 1000-2000 bp in length with short (200-600 bp) or moderately long (1000-4000 bp) repetitive sequence. Anophelinae mosquitoes have a long period interspersion, a pattern that includes long repeats (more than 5600bp) with long unique sequence (more than 13,000 bp). Polytene chromosomes are an interpahse giant chromosome forming when multiple rounds of replication produce many sister chromatids that remain synapsised together (Zhimulev 1996). While many tissues in Anopheline mosquitoes provide good polytene chromosomes, the Culicinae mosquitoes do not have those readable chromosomes. This is because a high number

of small repetitive sequences form ectopic contacts. That links different parts of chromosomes together, inhibiting the production of the readable chromosomes. (Campos, Andrade et al. 2003).

4. The *Ae. aegypti* genome mapping

Ae. aegypti has a typical karyotype chromosome in Culicinae. Among the three chromosomes, sex determination is a locus located in the shortest chromosome 1. Chromosome 2 is the longest and submetacentric chromosome. The chromosome is divided by the centromere into a long arm "q" and a short arm "p" (Rai 1963). A recently released genome sequence of *Ae. aegypti* of Liverpool strain (LVP) provided a genomic platform for mosquito research and incited the requirement of cytogenetic and high-resolution physical mapping for this sequence (Nene, Wortman et al. 2007).

Currently, 1376 million base pairs of *Ae. aegypti* are available in Vectorbase.org. There are 36206 contigs that represent 4758 supercontigs in these genome sequences. Almost 180 markers have been hybridized and 30% of supercontigs have been assigned on chromosomes, but without order and orientation (Nene, Wortman et al. 2007). Forty seven percent of the *Ae. aegypti* genome consist of transposable element. Those repetitive sequences contribute to the larger genome size in Culicidae. The physical map for *Ae. aegypti* has a low-resolution compared to the physical map of *A. gambiae*, ~ 2000 makers have been hybridized to the ovarian nurse cell polytene chromosomes which cover 88% of genome (Sharakhova, Hammond et al. 2007).

The emergence of insecticide resistance and the need for a better understanding of the associated mechanisms and population dynamics led to the development of a classical genetic map (Severson 2008). The first genetic map was constructed for the DDT resistant gene by three different resistant strains (Coker 1958). Later more than 70 loci including morphological mutants,

insecticide resistances, and isozyme markers were to generate the linkage map in *Ae. aegypti* (MUNSTERMANN and CRAIG 1979). With the development of molecular technology, a DNA based genetic map allows the creation of a higher-resolution genetic map. The first DNA molecular based map was the restriction fragment length polymorphism (RFLP) by David W. Severson (Severson, Mori et al. 1993). This map was combined with four morphological marker loci, called as a composite map which used 50 DNA molecular markers that identified 53 loci covering 134 centiMorgans (cM) across the three linkage groups. The second map, random amplified polymorphic DNA (RAPD) and the third map, single strand conformation polymorphism (SSCP) or single nucleotide polymorphism (SNP), used 96 RAPD loci and 57 loci and covered 168 cM and 134 cM (Antolin, Bosio et al. 1996; Fulton, Salasek et al. 2001). A composite map for the RFLP, SSCP, and SNP markers consisted of 146 loci and covered 205 cM (Severson 2008). An additional map using 148 amplified fragment length polymorphism (AFLP) loci and 6 SSCP loci were constructed to cover 180.9 cM (Zhong, Menge et al. 2006). Those saturated linkage maps provide a mechanism for beginning to resolve the complex phenotypes into their quantitative trait loci (QTL) component, like dengue, filarioid nematode, *Brugia malayi* (Severson, Mori et al. 1994; Bosio, Fulton et al. 2000; Gomez Machorro, Bennett et al. 2004).

The physical mapping is a more difficult task for *Ae. aegypti* than for the Anophelinae mosquitoes. In *Ae. aegypti* genome, 47% of genome are transposable elements (TEs) and most eukaryotic TE families have been found in here. Usually in addition to the probe with the repetitive DNA, it is necessary to use an unlabeled competitor DNA to block those labeled repetitive sequences before hybridization (Trifonov 2009). The third problem for the physical map is absence of good quality spreadable polytene chromosomes in Aedini mosquitoes. In 1995,

fluorescent *in situ* hybridization (FISH) on metaphase chromosomes from ATC-10 cell line was developed by Dennis L. Knudson in Colorado State University to achieve 1 Mb resolution (Brown, Menninger et al. 1995). Chromosomes and chromosomal arms were identified by measuring the length. The position of a probe was located on the chromosomes by fractional length from *p*-terminus (FLpter). This FLpter measures the probe position relative to the end of the short arm of the chromosome. Finally C_ot I DNA was utilized to block unspecific hybridization and cosmid probes were successfully hybridized to the metaphase chromosomes by *in situ* hybridization ,(Brown 2006). Because chromosome 1 and 3 do not have the distinctive difference in length in metaphase stage, and those two chromosomes have almost equal length of arms, the measurement method for identifying chromosomes and arms was not accurat. In 1996 a FISH landmarks method was developed to identify chromosome and chromosomal arms (Ferguson, Brown et al. 1996). . At the next step, the genetic and physical map were integrated together by hybridizing 21cDNA markers and 8 cosmid markers containing RFLP markers on metaphase chromosomes (Brown, Severson et al. 2001). This integration of the genetic map and physical map provides a platform for positional cloning to isolate genes of potential interest for the vector control, such as insecticide resistant genes, mosquito-pathogen interaction genes and adaption genes. High-resolution physical mapping in genome sequence will improve the assembly of current genome sequence and correct genome annotation. Precise assembled genome sequence facilitates the study of evolution (Waterhouse, Wyder et al. 2008; Lewin, Larkin et al. 2009).

5. Imaginal discs (ID) in insect and mitotic chromosome

ID will develop as wings, legs, antennae or halters during the pupal transformation (Held 2002). From 4^{th} instar larva to pupa and adult, IDs grow faster than in earlier instar stages, due to

rapid cell division taking place which facilitates their development. The cell division, also called mitosis, is a part of the cell cycle in eukaryotes (Snedden 2007). Mitosis includes four phases: prophase, prometaphase, metaphase and anaphase. In prophase, chromatin condenses and chromosome shape and banding pattern becomes visible. A centromere connects two identical sister chromatids, which were synthesized in the interphase state proceeding mitosis. In prometaphase, chromosomes continue to condense. The nuclear envelope dissolves, microtubules grow out from the centrosomes, and begin attaching to the chromosomes. In metaphase, condensed chromosomes align in the center of the cell, and microtubules attach to kinetochore proteins in centromeres and in anaphase, chromosomes are pulled apart by microtubules to opposite pole, forming z daughter cells.

The number of dividing chromosomes in ID tissue is greater than in the other tissues and the tissue is soft enough for squashing. These chromosomes keep their morphology and three chromosomes from same nucleus localize together on the slide, making it easy to recognize them by length and banding pattern.

6. The *Cx. quinquefasciatus* genome sequence

The genome size of *Cx. quinquefaciatus* is about 540 Mb. Although the newly released genome sequence of *Cx. quinquefasciatus* from Johannesburg strain demonstrates that although *Cx. quinquefaciatus* has middle genome size between *Ae. aegypti* and *An. gambiae*, its repertoire of protein-coding gene is larger than these two. There is a larger number of genes related to olfaction in *Cx. quinquefasciatus*, which contribute to its variety of host preference (Arensburger, Megy et al. 2010).

Chapter Two: Material and methods

1. Mosquito strains

a. *Ae. aegypti*

Ae. aegypti Liverpool (LVP) strain was used in this experiment. Eggs were hatched out at 28^{o}C, and then some of the larvae were transferred to 16^{o}C for two weeks. The 4th instar larvae were dissected to obtained chromosomes.

b. *Cx. pipiens, Cx. quinquefasciatus* and their hybrids

Culex mosquitoes were collected from pig center and horse center and pine tree areas around Virginia tech in Blacksburg VA, Montgomery County in 2009. Hay infusion (114 g straws, 1 g lactalbumen, 1.32 g brewer's yeast, and 26.5 liters of warm tap water) was used as an oviposition attraction for *Culex* mosquitoes (JACKSON, PAULSON et al. 2005). The egg rafts were hatched out in distilled water at 28^{o}C (Madder, MacDonald et al. 1980; Reiter 1986). The first instar larvae differentiated from *Cx. restuans* screened by windows area on the top of head (Darsie, Ward et al. 2005).

2. Chromosomes in *Ae. aegypti*

a. Chromosome preparation

The 4th instar larvae were put in container with distilled water on ice for 20-30 mins, and then were transferred on glass slide. Mosquitoes were placed in cold PBS for dissection. Head and midgut were removed by opening the thorax with a scissor. After that, the cold PBS was wiped off. And hypotonic solution (0.075 M KCl) was dropped on ID for 10 min. Then the hypotonic solution was removed. And a drop of freshly made Carnoy's fixative solution (3:1

ethanol:acetic acid) were placed on ID for 30 sec. The Carnoy's fixative solution was removed. A drop of 50% freshly propionic acid was added to ID for 1 min. Then ID were covered by coverslips and squashed by pencil with rubber side. Slides were frozen in liquid Nitrogen for 2 min, and then dipped in cold 50% ethanol (-20^{o}C) for more than 1 h, in cold 70% ethanol (4^{o}C) 5 mins, 90% ethanol (4^{o}C) 5mins, room temperature 100% ethanol and dried at room temperature. Chromosomes were analyzed using microscope (Olympus SZ).

b. Chromosome counting

To determine size effect in ID, the single, middle ID from 4th instar larvae was dissected out from thorax. After measurement using a micrometer, 10 of IDs were selected with average size between 0.3-0.45 mm, and 10 between 0.1-0.25mm were selected. Single ID was squashed to release mitotic chromosomes for chromosomal counting. To determine the temperature effect in ID, the single, middle ID from 4th instar larvae was dissected out from thorax. Ten IDs from larvae grown at 28 ^{o}C and ten that grew at 16 ^{o}C from 4th instar larvae were squashed to release mitotic chromosomes for chromosomal counting. For the ganglia and ID comparison experiment, 10 ganglia and 10 ID (average size between 0.1-0.45 mm) from 4th instar larvae were selected. The tissue was squashed to release mitotic chromosomes for chromosomal counting. Different stages of chromosomes were counted under microscope (Olympus BX). The result was compared by one way analysis of variance (ANOVA), a statistical model used to compare variance.

c. Cytogenetic and physical mapping

i. Giemsa-trypsin banding method

The slides were aged in a dry oven at 55-60^{o}C for 1 hour. The aged slides were put into trypsin solution (2.5%) for 5 seconds, and then in fetal bovine serum solution for 5 min, Gurr's buffer, Giemsa solution for 5 min, and finally in distill water for 1 min. The slides were air dried and examined with oil objective under 40X the microscope (Olympus BX) (Czepulkowski 2001).

ii. DNA probe

Two kinds of probes were used in this experiment. Primers of ribosomal DNA of *Ae. aegypti* were designed by Primer 3.0 (http://frodo.wi.mit.edu/primer3/) and amplified by PCR. Bacterial artificial chromosomes (BACs), obtained from David W. Severson from Norte Dame University were isolated by Qiagen Large Construction Kit (Qiagen).

iii. DNA probe labeling

1. Random primer labeling

A reaction mixture with 1 μl DNA (at least 10 ng) was mixed with 10 μl 2.5 × Random Primer Solution (Invitrogen Corporation, Carlsbad, CA, USA), and 2.5 μl sterile water in a PCR tube. Then the mixture was held at 95^{o}C for 5 min to denature the double stranded DNA. And then placed on ice immediately. To continue the reaction, 10 μl dNTP mixture (dNTP mixture: T=1 mM; A=3.5 mM; G=3.5 mM; C=3.5 mM from Fermentas Corporation) and 1 μl Klenow Fragment (Invitrogen Corporation, Carlsbad, CA, USA) were added into mixture on ice. 0.5 μl of Cy3 or Cy5 were added (GE Healthcare UK Ltd, Buckinghamshire, England). This was followed by a 1.5h incubated at 37 ^{o}C.

2. Nick-translation labeling

A reaction mixture was prepared in a PCR tube by mixing 2.5 μl of 10 × reaction buffer from Fermentas, 1.25 μl of 1.0 mM 3 dNTP Mix (without a labeled dNTP), 0.5 μl of Cy3 or Cy5, 1 μl of DNase I (Fermentas), 1 μl of Polymerase from Fermentas, and 1 μg of template DNA. Water was added to the reaction mixture to a volume of 25 μl. Finally, the mixture was incubated at 15 ^{o}C for 3 hour.

iv. DNA labeled probe precipitation

Labeled probes were precipitated by following steps: 2.5 μl (1/10 volume) of 3M sodium acetate and 75 μl (3 volumes) of 100% Ethanol were added into the labeled probes, and then mixed by inverting the tubes, stored at -20^{o}C overnight, or -80^{o}C for at least 3 hours or overnight. Probes were collected by centrifugeing at 14,000 rpm at 4 ^{o}C for 10-20 min.

v. C_ot I DNA

C_ot I DNA was made from Genomic DNA. 100 μl of Genomic DNA (150 ng/μl) that dissolved in water and 11 μl of 10 × DNase I Buffer, 0.02U DNase I were put in a tube at 37^{o}C to digest for 20-30 min. The DNase I activity was stopped at -80^{o}C. Three volumes of 100% ethanol and 1/10 volume of 3M sodium acetate were added to the mix by inverting several times. After that the samples were placed in -80^{o}C for at least 3 hours or overnight. The digested DNA was collected by centrifuging at 14,000rpm at 4^{o}C. The ethanol was poured off. 100 μl of EDTA was added. The collected DNA was incubated in 95^{o}C for 10 min, at 37^{o}C 1 hour for annealing highly repetitive sequence. Preheated S1 nuclease Buffer 10 × Buffer (37^{o}C) and 50 U S1 nuclease (Invitrogen Corporation, Carlsbad, CA, USA) were added to each 100 μg of DNA at

37°C for digestion the single strain DNA. Phenol-chloroform was added to extract the protein from DNA. DNA was precipitated again by previous method and stored at -20°C.

vi. *In situ* hybridization

a. Prehybridization

The slides were incubated in 2 × SSC for 5-10 min at room temperature (RT). An ethanol of 70%, 80% and 100% were used to wash them for 5 min in each at RT. Slides were dried at 37°C. 100 µl of working RNase solution were put on slides for 30 min at 37°C. The slides were put in 2 × SSC 5 min at 37°C. 0.01% pepsin solution was applied for 5 min at RT. Slide were washed in 1 × PBS for 5 min at RT. The paraformadehyde was used to fix the slides for 10 min at RT. The series of ethanol 70%, 80% and 100% were used to wash for 5 min in each at RT. Slides were dried at 37°C.

b. Hybridization

Slides were denatured in 70% formamide 2 min at 72°C. The series of cold (-20°C) ethanol (70%, 80% and 100%) were used for washing 5 min in each. Slides were dried at 37°C. The hybridization mix (5 µl of 100% formamide; 2.5 µl of 50% dextran sulfate; 5µg of C_0t I DNA (1 µg/µl); 100 ng of labeled DNA probes) was incubated at 97°C 10 min and then for 30 min at 37°C. Glue was used to seal the coverslip. Slides were held in a humid chamber at 37°C for overnight (at least 8 hour) for hybridization.

c. Washing and staining

Slides were put into solution 1 (20xSSC 2ml, NP-40 300 µl, water 100ml) for 2 min at 73°C, and then solution 2 (20xSSC 10ml, NP-40 100µl, water 100ml) for 5 min at RT. 1 × PBS

was used to rinse slides. 1: 5,000 YOY)-1 was used to stain (Invitrogen Corporation, Carlsbad, CA, USA) for 15 min. Then put slides into 1 × PBS. The DABCO was used to protect fluorescent on slides.

d. Detection

Zeiss LSM 510 Laser Scanning Microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) was used to acquire the pictures.

3. *Cx. pipiens, Cx. quinquefasciatus* and their hybrids

a. *Culex* colony maintaining in insectary

Bovine liver power was used to feed the *Culex* larvae. After larvae emerge out as adult, 10% sugar water were used to feed them first week. Rabbit blood containing 10% sugar (by Sally L. Paulson) was used to blood feed the adult in dark overnight. Before next blood feeding, the *Culex* mosquitoes were starved for one day, and then guinea pigs were used as a host.

b. Molecular identification

After morphology screening we also use the molecular marker like ACE-2 gene to confirm our first screening. These primers are ACEquin (5'-CCTTCTTGAATGGCTGTGGCA-3'), ACEpip (5'-GGAAACAACGACGTATGTACT-3'), B1246s (5'-TGGAGCCTCCTCTTCACGG3'). In the Gogreen amplification system (Promega), there are 100 ng genomic DNA as template, 0.2 μM ACEquin, 0.2 μM ACEpip, 0.4 μM B1246s.The PCR protocol is 95 ^{o}C, 1min, 55^{o}C, 30s, 72 ^{o}C 1 min, 40 cycles (Smith and Fonseca 2004).

Chapter Three: Results and Discussions

A. Results

1. *Ae. aegypti* genome mapping

a. ID of 4th instar larvae of *Ae. aegypti*

From the pictures of ID in Figure 3.1, we can see IDs at various stages of development. Larvae are depicted after treatment with Carnoy's fixative solution (3:1 ethanol:acetic acid) to fix them overnight. From the front side, we can see that there are three IDs on each side under the skin in the thorax. Images A and B show IDs at an intermediate stage of development, which is the best time to dissect them. In image B, the two middle imaginal discs are dissected from the larva in image A.

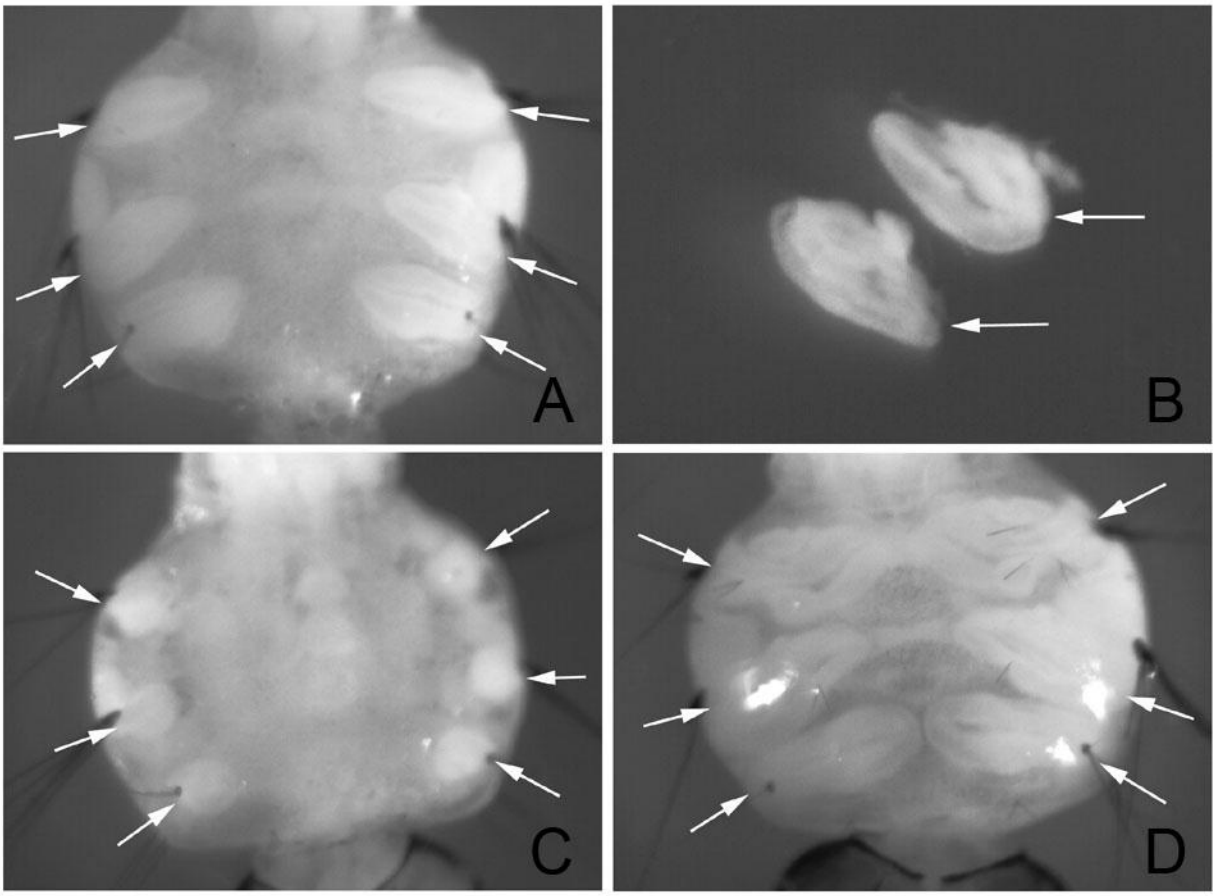

Figure 3.1 Different stage of imaginal discs from 4th instar larva of *Ae. aegypti*, Liverpool strain

In image C, we can see the IDs are starting to grow. When the larva grows to 4th instar, about 0.8 cm in length, the imaginal discs begin to develop. Now they are about 0.23 to 0.38 mm.

In image D, we have late stage of ID. At this point, 4th instar larvae are going to pupae stage. The ID reached to the longest in length.

b. Comparison of mitotic chromosomes in number from different sized IDs

In Figure 3.2, one way ANOVA was used to compare the statistical difference of individual stage of mitosis between different tissues. The large size (0.3-0.45 mm) of IDs yields more of prophase and metaphase chromosomes than small size of ID. Although small size (0.1-0.25 mm)

of IDs yield greater numbers of anaphase chromosomes, our experiment requires prophase and metaphase stage chromosomes. For this reason, larger sizes of IDs are the better tissues than small ones for our purposes. The mean number of chromosomes from small size of IDs is 17.5, while the mean number of chromosomes from large size of ID is 108.7. The mean number of prophase chromosome from small size is 16.4. The mean number of prophase chromosome from large size is 35.7. The mean number of metaphase chromosome from small size is 21.0. The mean number of metaphase chromosome from large size is 129.1. After compare individual stage of chromosomes from different tissues by one way ANOVA, we can say there is highly significant statistical evidence that the mean numbers of chromosomes from ID of different size are not all equal ($P<0.0001$). These data suggest that the sizes of 0.3-0.45 mm IDs are optimal for our experiment because of greater number of prophase and metaphase chromosomes.

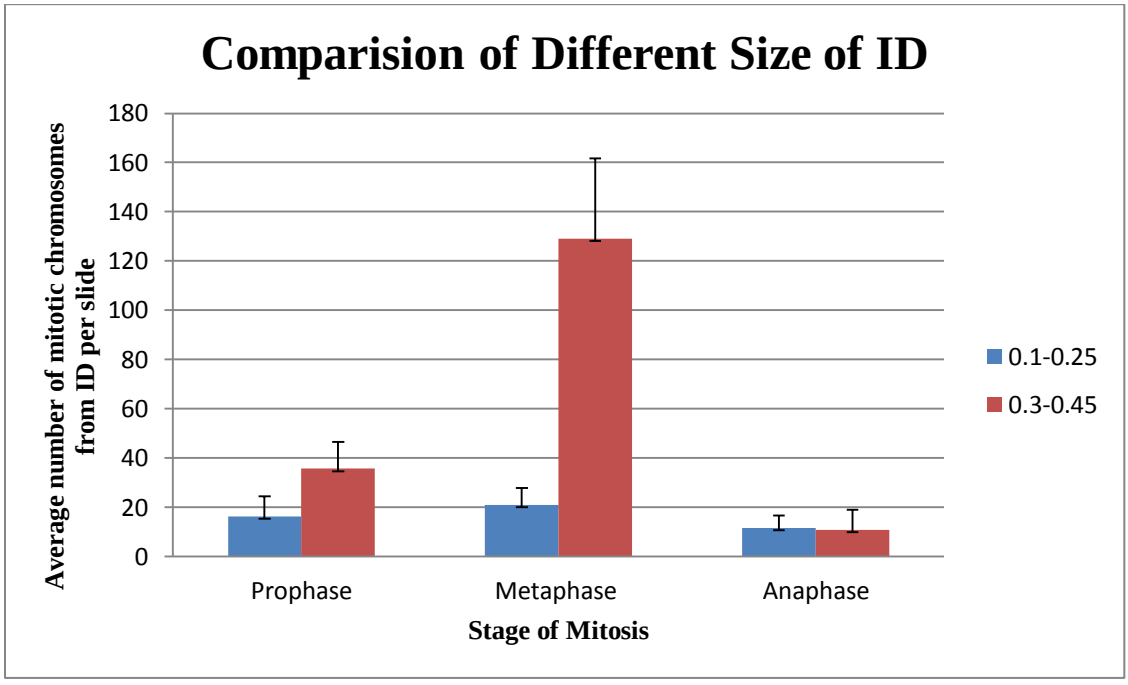

Figure 3.2 Comparison the number of chromosomes at different stage from ID of different size

c. Comparison of mitotic chromosomes from ganglia and ID

Figure 3.3 showed single ID released more mitotic chromosomes than single ganglia. The mean number of chromosomes from Ganglia is 11.9. The mean number of chromosomes from imaginal discs is 175.7. The one way ANOVA result showd to calculate the P-value of total number of chromosome between ganglia and ID was less than 0.0001. The mean number of prophase chromosomes in ganglia is 4.3. The mean number of prophase chromosomes in ID is 58.3. The P-value is less than 0.0001. The mean number of metaphase chromosomes in ganglia is 5.1. The mean number of metaphase chromosomes in ID is 62.9. The P-value is less than 0.0001. There is highly significant statistical evidence that the mean numbers of total chromosomes, prophase chromosome, metaphase chromosome from different tissue (ganglia and ID) are not all equal. From these, we conclude that ID is the better source to provide larger number of the chromosomes than ganglia.

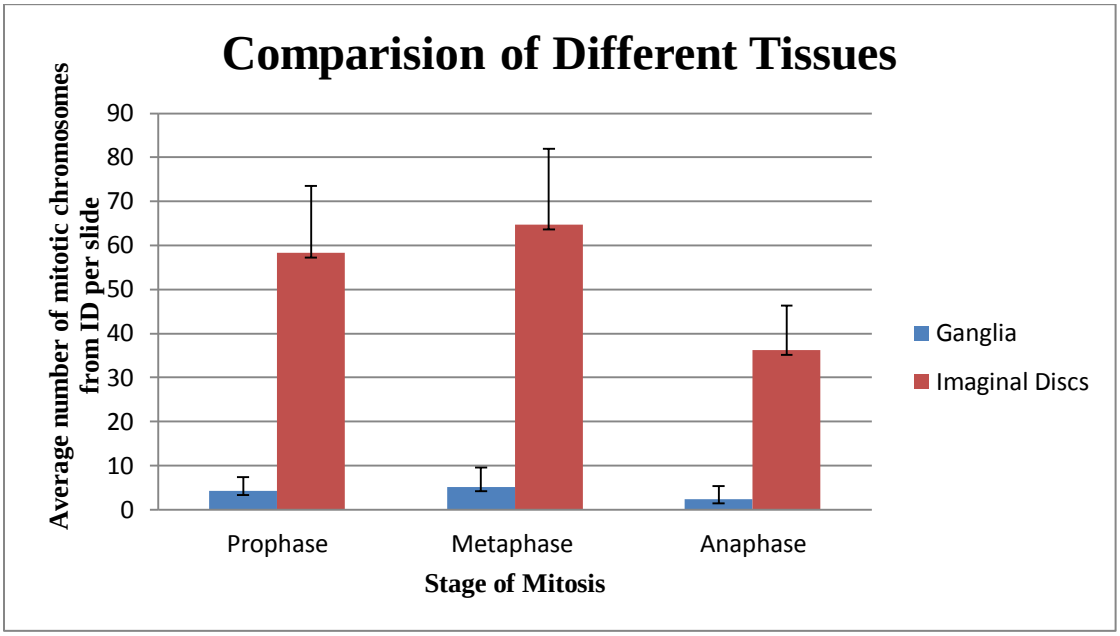

Figure 3.3 Comparison the number of different stage chromosomes from ganglia and imaginal discs

d. Development of ID chromosomes at different temperature

Data in Figure 3.4 showed ID grew in cold temperature (16^{o}C) yields higher amount of mitotic chromosomes than high temperature (28^{o}C). Larva in cold temperature (16^{o}C) can generate more in number than high temperature. Because in cold temperature cell division grows slowly, at same time, a more number of cells undergo mitosis stage. Consequently, there are more number of mitosis chromosomes. The mean number of total mitotic chromosomes from ID at high temperature (28^{o}C) is 150.56. The mean number of total mitotic chromosomes from ID at cold temperature (16^{o}C) is 192.91. The P-value is 0.0075, less than 0.05. The mean number of prophase chromosomes from ID in high temperature (28^{o}C) is 58.3. The mean number of prophase chromosomes from ID in cold temperature (16^{o}C) is 96. The P-value is less than 0.0001. The mean number of metaphase from ID in high temperature is 64.7. The mean number of metaphase chromosome from ID in cold temperature is 112.1. The P-value is less than 0.0001. By one way ANOVA, we can say that there is highly significant statistical evidence that the mean numbers of total chromosomes from ID under different temperature are not all equal. From those, we conclude that cold temperature can yield more mitotic chromosomes than high temperature.

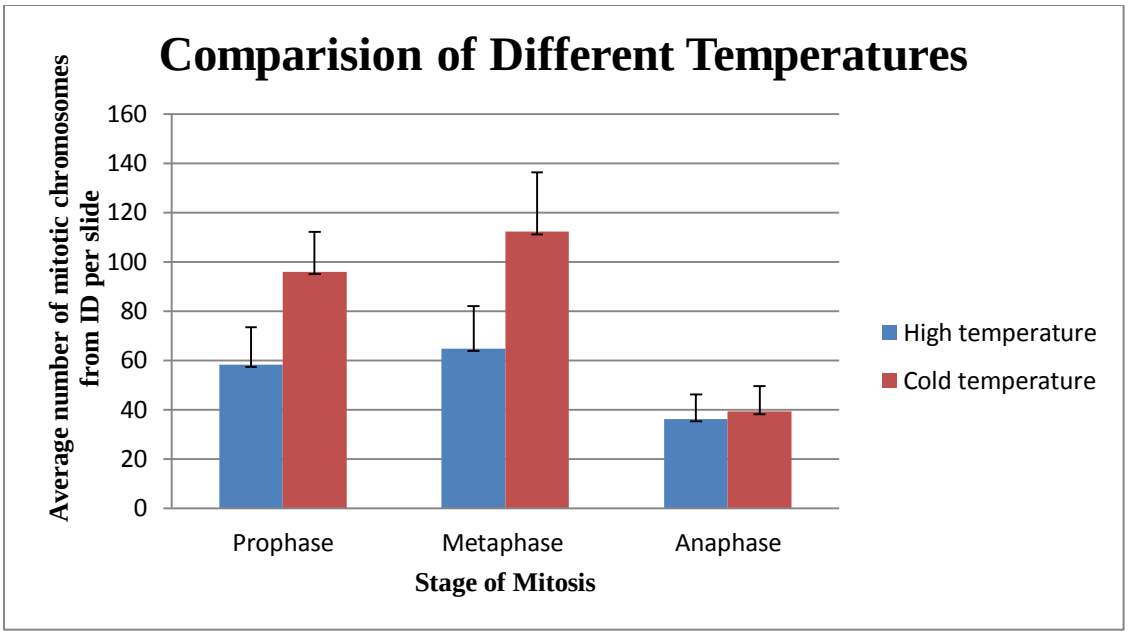

Figure 3.4 Comparison the number of different stage chromosomes from ID of different temperatures

e. Differential staining of ID chromosomes

In Figure 3.5, Giemsa staining was used to visualize the banding pattern of mitotic chromosomes from *Ae. aegypti*. From the length of chromosomes, chromosome 2 was identified. And banding patterns in chromosome 2 were visible under microscopy.

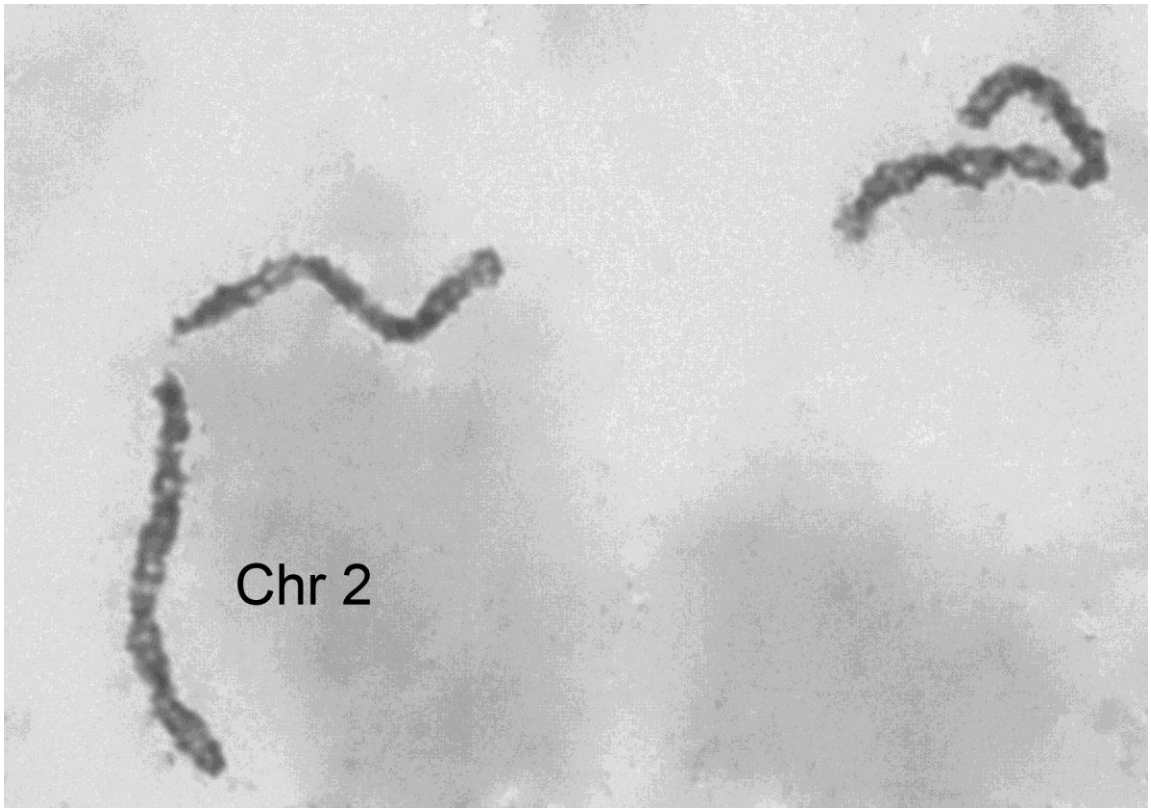

Figure 3.5 Giemsa staining of prometaphase chromosomes from *Ae. aegypti*

In Figure 3.6, YOYO-1 staining was used to stain different stage of mitotic chromosomes from *Ae. aegypti*. In image A, prophase chromosomes were presented with some banding patterns. In image B, prometaphase chromosomes were stained with YOYO-1 showing banding pattern. Based on the length of chromosome, chromosome 1, 2 and 3 was identified. In image C, the length of mitotic chromosomes was less than prometaphase chromosomes. Banding pattern was not clear. In image D, anaphase chromosomes did not produce any morphology and banding pattern characteristics for identification.

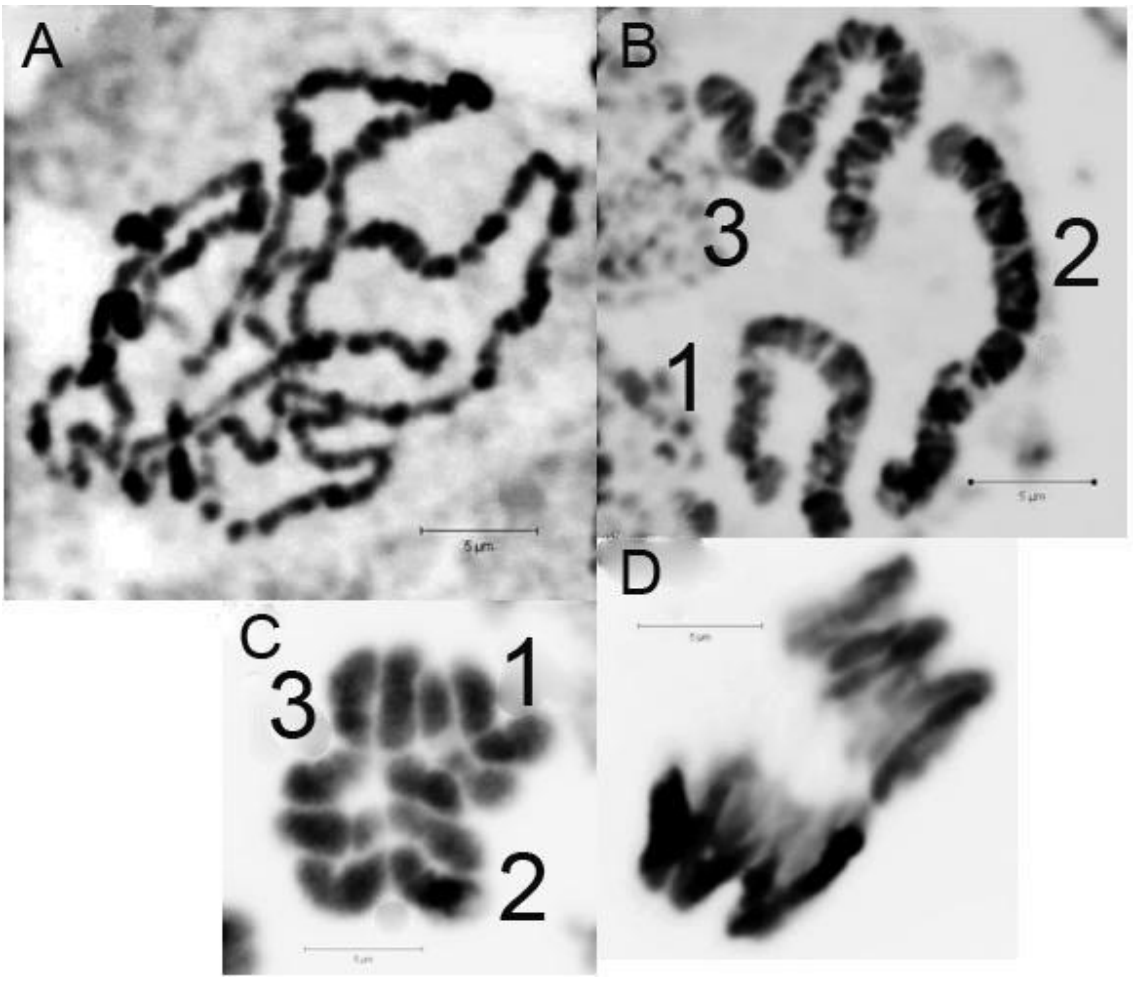

Figure 3.6 YOYO-1 staining of different stage of chromosomes

f. Chromosome preparation with hypotonic solution treatment

The hypotonic solution is a treatment to induce the animal cell to swell. This treatment has been used to help uncoil and elongate prometaphase and metaphase chromosomes (Rønne 1989). In Figure 3.7, YOYO-1 staining was used to stain mitotic chromosomes from *Ae. aegypti*. In image A, mitotic chromosomes were dissected from fixed larva. In image B, mitotic chromosomes was dissected from live mosquito larva. In addition, ID was also treated by hypotonic solution. It turned out that mitotic chromosomes uncoil with banding pattern.

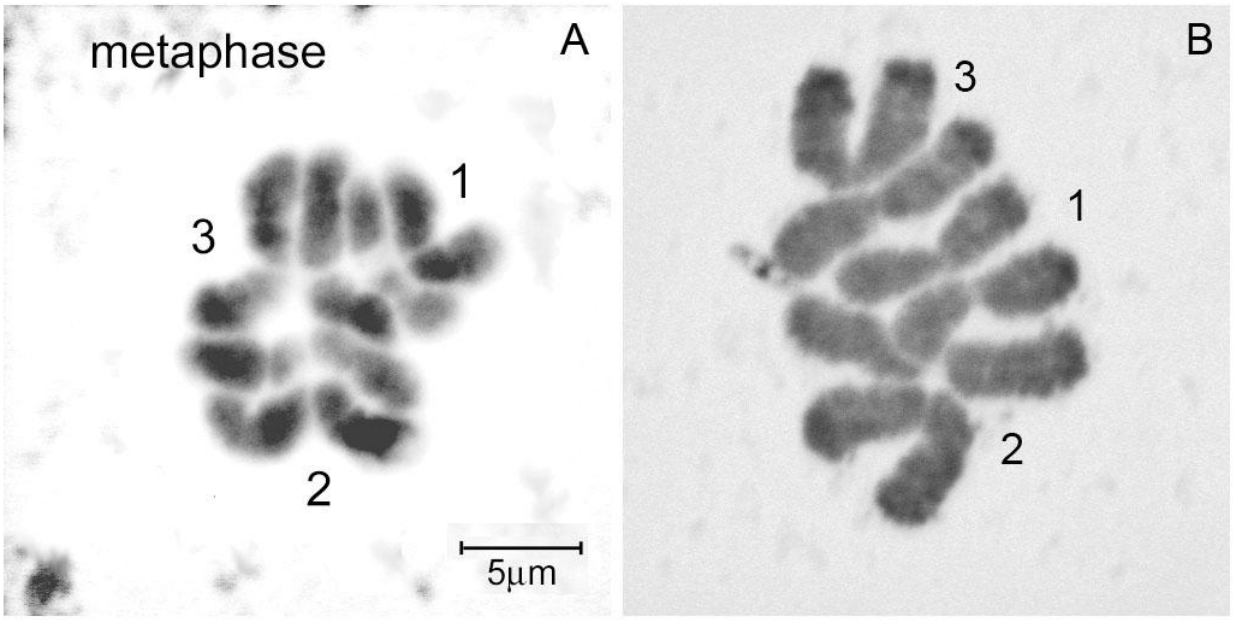

Figure 3.7 Metaphase chromosomes preparation without hypotonic solution (Left) and with hypotonic solution (Right)

g. Optimization of *In Situ* hybridization

i. Optimization of Labeling

In Figure 3.8 A, a single clear band was amplified from genomic DNA. The size was about 500-650 bp.

In Figure 3.8 B, r-DNA was labeled by random primer labeling method with Cy3 and Cy5. The size of probe was below 200 bp.

In Figure 3.8 C, BACs (AEW) was labeled by nick-translation method with Cy3. The size of probe was below 100 bp.

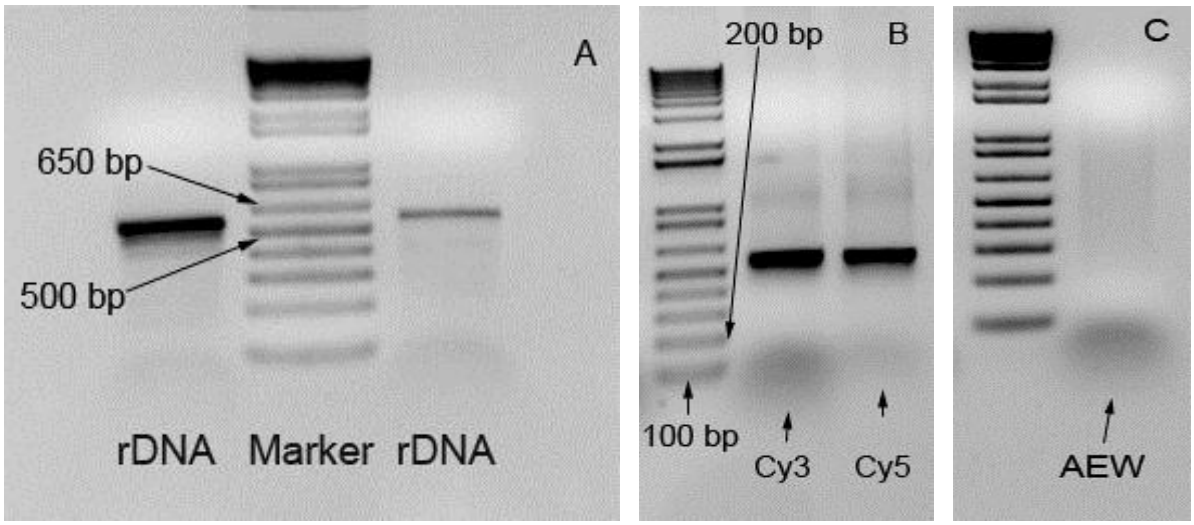

Figure 3.8 rDNA amplification, labeling rDNA by Cy3 and Cy5 and labeling of AEW

ii. Optimization of C_0t I DNA

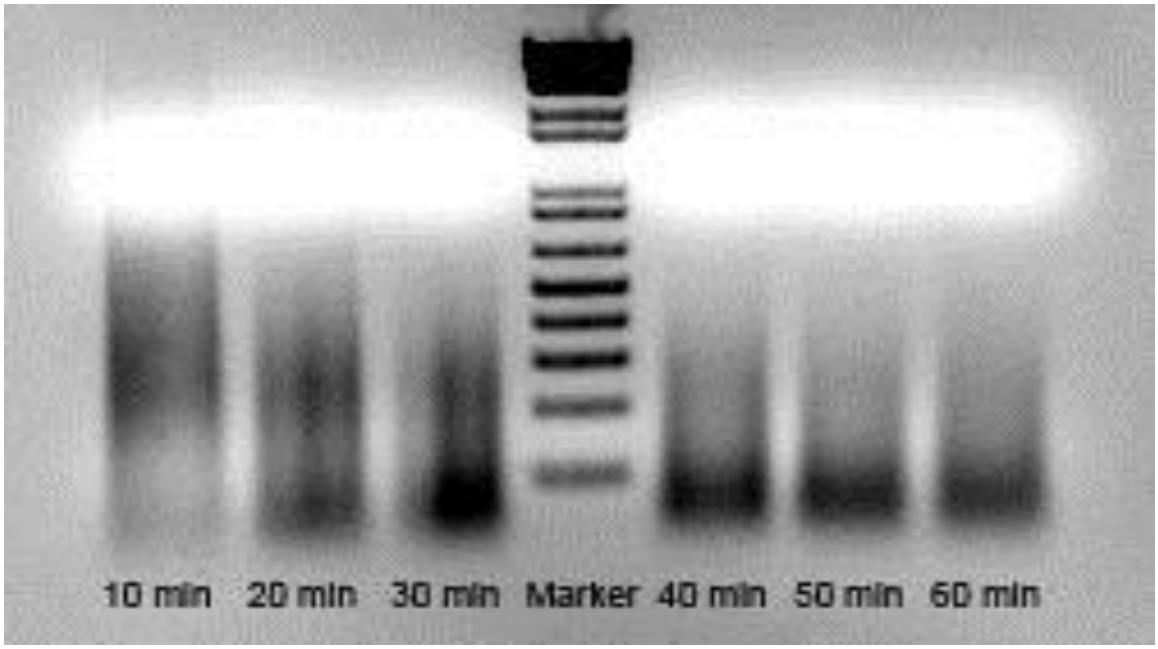

Figure 3.9 The digestion of Genomic DNA by DNase I by different temperature

In Figure 3.9, genomic DNA was digested by DNAse I at 37^{o}C for different time. The efficient C_0t I DNA was required to match with the size of probe to block inside repetitive

sequence for clear signal. In that case, the size of C_0t I DNA was required below than 100-200 bp. From the gel images, 30-40 min turned out to be an efficient time for DNase I digestion to generate correct small size of genomic DNA.

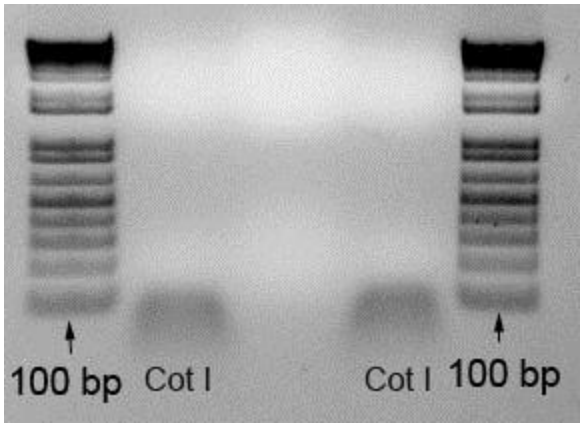

Figure 3.10 C_0t I DNA

In Figure 3.10, the size of C_0t I DNA was shown below than 100 bp, which matched with the size of BACs probe.

iii. Optimization of Hybridization

In Figure 3.11, we can see rDNA hybridizes on both pair of chromosome 1 (Chr 1) from *Ae. aegypti*. The intensity of signal is different in different pair Chr 1. This proved that FISH procedure from human was successfully applied on *Ae. aegypti*.

In Figure 3.12, we can see two BACs (AEW and nAcBP) from *Ae. aegypti* hybridized on Chr 1 proved that BACs labeling, C_0t I DNA and hybridization procedure work successfully.

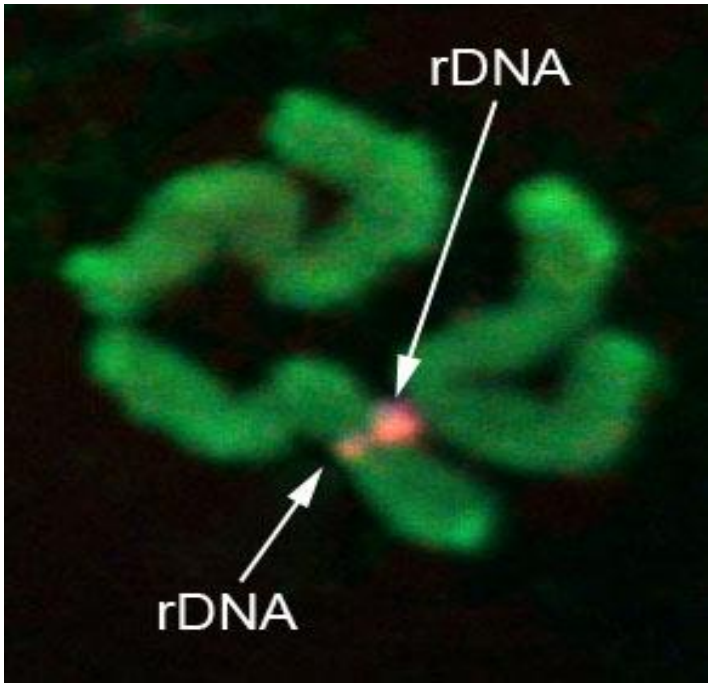

Figure 3.11 rDNA labeled with Cy3 and Cy5 hybridized on prometaphase chromosome Chr 1 of *Ae. aegypti* Liverpool strain

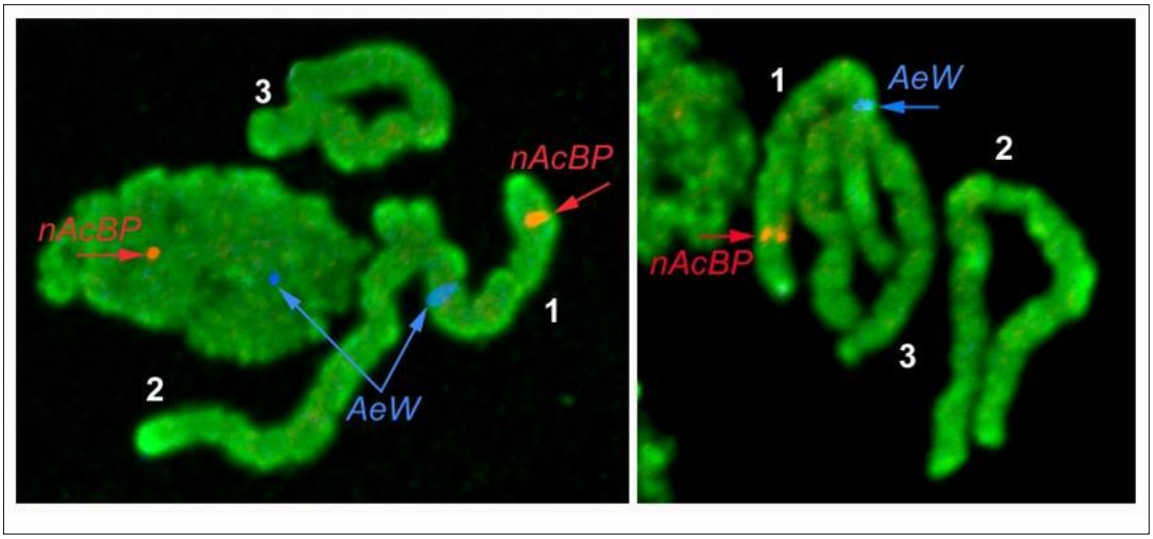

Figure 3.12 BACs (AEW and nAcBP) hybridized on Chr 1 of *Ae. aegypti*, Liverpool strain

2. *Cx. pipiens* complex project

a. *Culex* mosquito collection and morphology screening

To collect the *Culex* mosquito, we set up three traps around pig center and horse center by hay infusion. From 7/8/09 – 8/20/09, 775 egg rafts had been colleceted, and 206 (without window area) had been identified as *Culex pipiens*, *Culex quinquefasciatus* and their hybrids by window area on the top of head of the first instar larva as the primiary screen result.

b. Identification of *Cx. pipiens* complex by the molecular marker

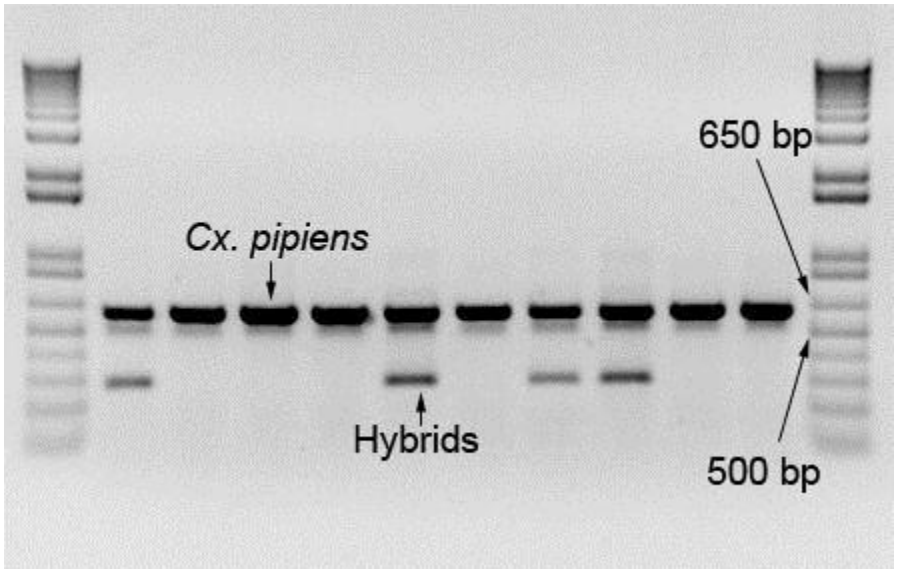

Figure 3.13 Identification of *Cx. pipiens* complex by ACE-2; First and Last lane are marker; The second, sixth, eighth and ninth lane are hybrids; The rest are *Cx. pipiens*

In Figure 3.13, ACE-2 gene was used as molecular marker to identify *Cx. pipiens*. If one higher band (610 bp) was amplified, then this species is *Cx. pipiens*. If one lower band (274 bp) was amplified, then this species is *Cx. quinquefasciatus*. If two bands were amplified together, then this species is hybrids.

80 individual genomic DNA were isolated from *Cx. pipiens* complex mosquitoes. 13 (16%) turned out to be hybrids. The rest are *Cx. pipiens*. No *Cx. quinquefasciatus* had been identified. It was probably because my collecting area was really near to each other. In this area, *Cx. quinquefasciatus* did not exist in there.

c. Colonization of *Cx. pipiens* complex

From Aug, 2009 to Sep 2010, *Culex* colony was maintained in lab for more than a year. Artificially blood feeding with parafilm and midgut had been tried to feed them with the help from John Machen in insectary in Fralin. But they did not work. However, 10% sugar mixed with rabbit blood can be served as the first blood meal overnight in dark place. After that, *Cx. pipiens* mosquitoes can take blood out from guinea pig by themselves in dark place. This blood feeding behavior guarantees female mosquitoes to acquire fresh blood for ovary development to lay eggs.

Cx. pipiens complex maintaining in lab can provide a reliable resource for further studies of vector competence and chromosome arrangements. For example in Figure 3.14, this is the polytene chromosome from *Cx. pipiens*. From this picture, we can see the banding pattern can be recognized. There is also an inversion on the chromosome. Further study can compare more slides with good quality of polytene chromosomes from this strain with cytogenetic map from *Cx. quinquefasciatus* and maybe also cytogenetic map from *Cx. pipiens*. We could get material from laboratory directly.

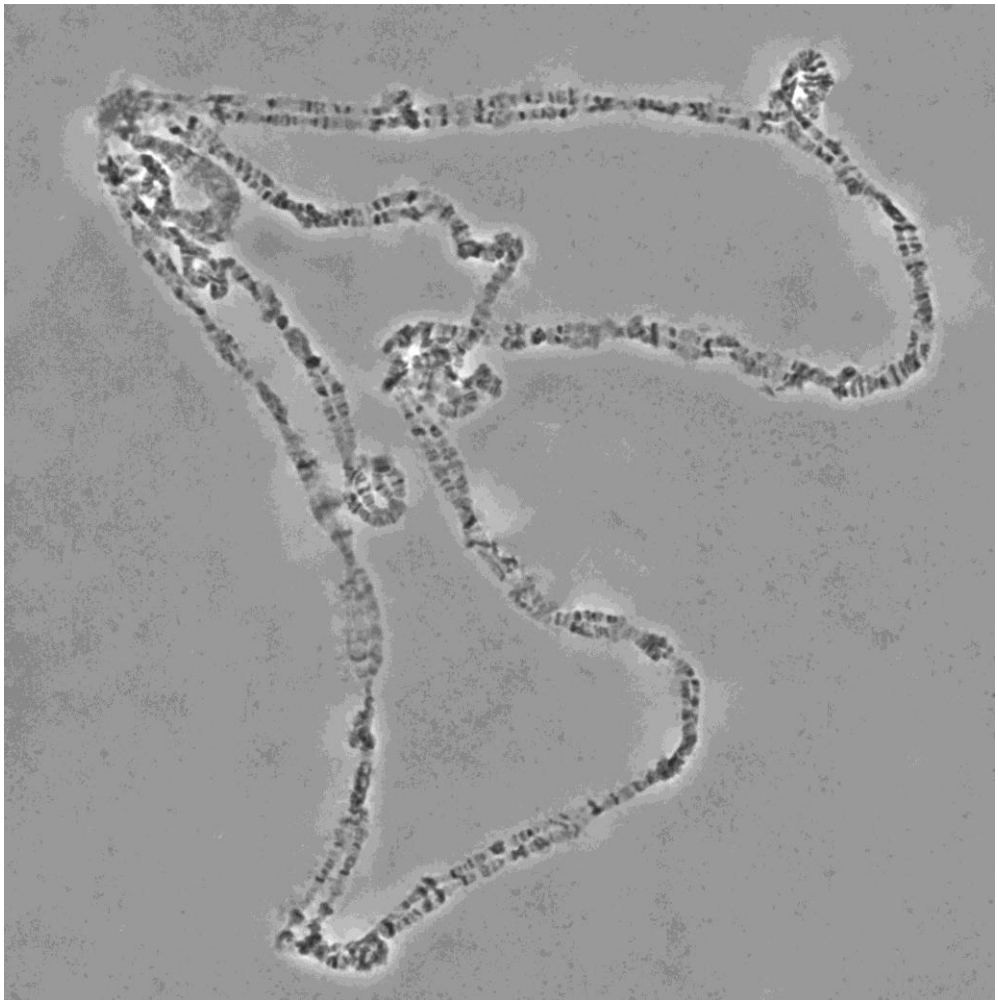

Figure 3.14 Polytene chromosomes from *Cx. pipiens*

B. Discussions

1. *Ae. aegypti* genome mapping project

We found ID were the better tissue providing a great number of mitotic chromosomes. Special treatments such as low temperature (16^{o}C) and hypotonic treatment, and dissection from live larvae can improve the quality and quantity of chromosomes. Prometaphase chromosomes are longer with banding pattern. Hybridization of BACs labeled with Cy3 and Cy5 on

prometaphase chromosomes proved to increase the precision and resolution in cytogenetic map and physical mapping. Dennis L. Knudson, who hybridized probes on metaphase chromosomes from the cell line of *Ae. aegypti*, demonstrated that a variety of probes can be localized on specific chromosomal positions. However, due to the possibility of genome rearrangements present in chromosomes from the cell line, precise mapping is not possible. The FLpter measurement methods and molecular landmarks can not accurately locate the positions of fluorescent signals. In future, based on mitotic chromosomes, high resolution cytogenetic map could be produced, which will give a clear position of banding pattern on chromosomes. Integration of genetic marker on physical map could be more precise. Well assembled *Ae. aegypti* genome sequence could facilitate the study such as mosquito taxonomy, population genetics and comparative genomics.

2. *Cx. pipiens* complex strain maintenance in laboratory project

Cx. pipiens complex strain was maintained in insectary for more than a year. . After taking 10% sugar mixed with rabbit blood in first week, *Cx. pipiens* mosquitoes can take blood from guinea pig by themselves. In that case, *Culex* mosquitoes can take fresh blood every time. This colony from Virginia contained *Cx. pipiens* and hybrids. Further study such as taxonomy, vector competence and comparative genomics can be done on a strain colonized in laboratory in this method, instead of collecting from field and identifying them.

Conclusions

1) Imaginal discs of *Ae. aegypti* have proven to be a better source of mitotic chromosomes for physical genome mapping.

   (i) Larvae of *Ae. aegypti* reared at cold temperatures (16^{o}C) can provide more numerous and higher quality chromosomes;

   (ii) Live mosquitoes of *Ae. aegypti*, after treatment with hypotonic solution, can provide well-spread, banded chromosomes;

   (iii) A procedure for physical mapping of the *Ae. aegypti* genome was developed;

2) *Cx. pipiens* and hybrids can be maintained in the laboratory for further cytogenetic mapping study.

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