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In Situ Co-Hybridization of Retroposon Sine-CTRT1 and Hemogoblin Family Genes in the Polytene Chromosomes of Chironomus Riparius (Diptera)

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In situ co-hybridization of retroposon SINE-CTRT1 and hemoglobin family genes in the polytene chromosomes of *Chiromomus riparius* (Diptera)

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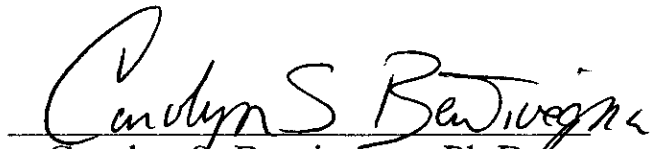
Approved by



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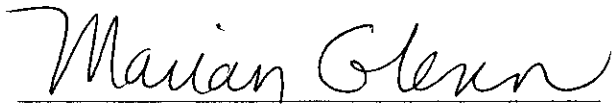

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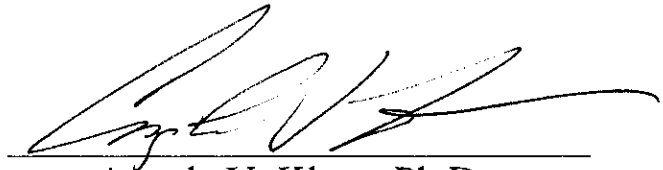

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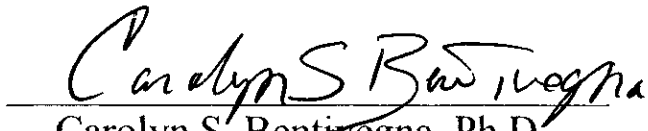
Committee member



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Abstract

The hemoglobin family genes in the dipteran *Chironomus riparius* are environmentally relevant for the species' oxygen tolerant lifestyle. The number of gene copies far exceeds what is required to encode the 28 monomeric and homodimeric hemoglobin (Hb) polypeptides. Transposons such as SINE (short interspersed repetitive element) have been found to relocate genetic information and may account for amplification and dispersion of Hb genes in the chironomid genome. In this study, different Hb genes (Hb-13, Hb-7 and Hb-4) and SINE-CTR1 were hybridized *in situ* to salivary gland polytene chromosomes in order to investigate the extent to which they might co-localize. The relationship between the positions of transposons and the different hemoglobin genes on chromosomes should provide insights into the microevolution of multi-gene families. Results showed that SINE-CTR1 was inserted into Hb-13 and co-localized with two homodimeric Hb genes, Hb-13 and Hb-7, at one locus but not with a monomeric hemoglobin gene, Hb-4, which was located on a different chromosome. This indicated that SINE activity can cause mutation in multi-gene families; however, it may not account for the expansion of the hemoglobin gene family. Adaptive selection appeared to favor mechanisms of gene amplification but not necessarily gene transposition by SINEs.

Introduction

Chironomidae (Family, Diperta) are a worldwide-distributed and abundant group of species in freshwater (Armitage et al, 1995). They play a key role in many lake and river systems because of their detritus consumption, and they represent an important food resource for many bird and fish species. Chironomidae larvae are commonly called chironomids. The larval stage is the most critical, in that it is the most metabolically active stage of their life cycle and responsive to environmental stress.

Chironomids have been extensively studied for their ecological response to pollutants (Govinda et al, 2000; Groenendijk et al, 2002), and are EPA-approved test organisms in the U.S.A (USEPA, 1996). Chironomids are often the only larval insects found in lake sediments of the profundal zone where hypoxic (oxygen concentrations less than 3 mg l^{-1}) and even anoxic conditions sometimes occur (Pinder et al, 1995). Chironomids have been uniquely successful in exploiting these environments as a result of behavioral and physiological adaptations. Perhaps the best understood is the ability to tolerate reduced levels of dissolved oxygen.

Among the aquatic insects, only certain chironomid species and a few motonectid bugs possess hemoglobin (Pinder et al, 1995). Chironomids can saturate their hemoglobin by undulating their bodies within their silken tubes or substrate burrows. This movement causes oxygenated water to flow over their cuticles. They contain at least 16 different hemoglobin (Hb) proteins and 12 globin polypeptides (Gruhl et al, 1997), which constitute about 80% of their total hemolymph proteins (Laufer et al, 1982). In a number of chironomid species, larvae can have a number of different Hb genes. The number and

types of Hb genes varies among genetically different strains, and their expression can vary during larval development.

The multiple copies of Hb genes are believed to have arisen by duplication and variation of a single ancestral gene (Kleinschmidt et al., 1981). During duplication, the ancestral gene is replicated, so that each of the resulting daughter cells carries two identical copies of the ancestral hemoglobin gene. Gradually, during successive cell divisions, small variations or mutations in the sequence of nucleotides start to appear. In this way, the two genes that started out identical acquired sequence differences and later, functional differences (Hardison et al, 1999).

Hemoglobins in chironomids exist as monomers and dimers: this has been shown for two different genera, *Chironomus ramosus* (CR) and *Chironomus thummi thumrni* (CTT) (Das et al, 1996). The different quaternary structures include three that are monomers (I, IV and VI), eleven that are dimers (III, V, VII, VIII, IX, X, XI, XII, XIII, XVI, XVII) and two (II and XIV) that exists in both monomeric and dimeric states (Das et al, 1996). The numerous hemoglobin genes in the *Chironomus* genome are expressed in a stage and tissue-specific way. They are essential genes in that they enable the aquatic larvae of chironomids to survive in organic laden sediments where dissolved oxygen levels are low. This makes them of value as potential indicators of environmental stress and for studies of the genetic mechanisms that permit such survival.

Chironomids also are potential subjects for cytogenetic monitoring because their polytene chromosomes have a large size and a very good banding pattern (Michailova et al, 1989). Polytene chromosomes were originally observed in the larval salivary glands of *Chironomus* larave by Balbiani in 1881. They begin as normal chromosomes, but through

repeated rounds of DNA replication without any cell division, they become large, banded chromosomes. The standard karyological characteristics of the polytene chromosomes can be used as a basis for mapping genes and for revealing chromosomal aberrations and changes in functional activity in response to environmental mutagens (Hagele, 1970).

It is well known in eukaryotes that transposable genetic elements are one of nature's tools for genome mutation. Transposable elements (TEs) are ubiquitous components of all living organisms, and in the course of their coexistence with their respective host genomes, these parasitic DNAs have played important roles in the evolution of complex genetic networks (Miller, 2004). The interaction between TEs and the host genomes are diverse, ranging from modifications of gene structure and regulation to alterations in general genome architecture. Thus, these elements are indicated as natural molecular tools for evolution of species over time and are essential for studying more about genome evolution and gene function.

There are two classes of transposable elements that have been found in eukaryotes. Class I is retrotransposon that works by copying itself and pasting the copies back into the genome in multiple places. To do this, retrotransposon first transcribes itself into RNA, next the RNA is copied into DNA by a reverse transcriptase and then the DNA is inserted back into the genome. Class II is DNA transposon, which copies itself as above, but does not require an RNA intermediate. They usually move by a mechanism analogous to cut and paste, rather than copy and paste, using the transposase enzyme.

In chironomids, there are different transposable elements that have been found such as LINEs (long interspersed transposable elements), SINEs (short interspersed transposabl elements), MEC (mechanosensory-defective) and NLRCth1 (non long

terminal repeat –*Chironomus thummi* 1) (Blinov *et al.*, 1991). Recently, sequencing of Hb-13 in *C. thummi* revealed that an exon of this gene is interrupted by a SINE (short interspersed repetitive element, Bergtrom *et al.*, 2002). SINEs are retrotransposons: this means that their movement around the genome is dependent on the presence of an RNA intermediary. In this system, SINEs produce RNA transcripts, which are then converted back into DNA by an enzyme called reverse transcriptase. The new DNA copies then insert themselves into other spots in the genome. In chiromomids, it was called SINE-CTRT1 (*Chiromomus thummi* reveal interrupted 1) because it was first found in *Chiromomus thummi* (The species name was later revised to *Chironomus riparius*.). Whenever a transposon inserts itself into a functional gene, it will probably damage it. Insertion into exons, introns, and even into DNA flanking the genes, which may contain promoters and enhancers, can destroy or alter the gene's activity.

Because there was evidence that SINEs were involved in the mutation of a hemoglobin gene, SINE was chosen to investigate the relationship between tranposable elements and the distribution of hemoglobin genes on polytene chromosomes from salivary glands of *C. riparius* using fluorescent *in situ* hybridization (FISH). FISH is a technique in which a labeled nucleic acid probe (usually DNA, but RNA may also be used) is permitted to interact so that it complexes, or hybridizes, to molecules with sufficiently similar, complementary sequences. Through nucleic acid hybridization, specific DNA sequences can be detected and located on a given chromosome. The large size of the polytene chromosomes facilitates the co-localization of different probes at relatively low magnifications (200x).

It was hypothesized that SINE elements contributed to the spread of hemoglobin genes in the genome, creating a multi-gene family, which may ultimately have increased the fitness of chironomid species in low oxygen environments. Hb-4 and Hb-7 were selected in addition to Hb-13 because they have been fully sequenced, and they belong to two different types of hemoglobin. Hb-4 is a monomeric hemoglobin (Schmidt et al, 1988) while Hb-7 and Hb-13 are dimeric hemoglobin (Bergtrom et al, 1995), therefore, they were expected to be located on different chromosomes (Schmidt et al, 1988). A single copy gene known to be induced by ecdysteroid hormone, called Ecdysteroid I-18C, was used as a negative control. Chromosome region I-18C of *Chironomus tentans* is the first region in which experimentally a puff could be induced by ecdysone, i.e., injection of the purified hormone caused larvae to molt (Clever and Karlson 1960). It was used as a control for three reasons: 1) its location on chromosomes of C. *tentans* has been published, 2) its sequence has been published and 3) its response to ecdysone has only ever been recorded at one locus, chromosome I, and band 18C. According to the hypothesis, this single-copy gene should not co-localize with SINEs as compared to members of the Hb gene family.

Materials and methods

Animals

The laboratory population of *Chironmomus riparius* were maintained in 20 gallon aquarium containing acid-washed play sand (American Stone-Mix, Inc., Towson, Maryland) as substrate and carbon filtered water (CDPRM1206 and CDFC01204: Millipore Corporation, Billerica, MA) that had a hardness of approximately 170 mg/L. Chironomids were fed ground fish food (TetraCichlid, Tetra GMBH, Germany) weekly and held at a photoperiod of 12 light/ 12 dark. Temperature was 21 ± 0.5 °C.

DNA extractions, PCR, cloning and sequencing

Genomic DNA was extracted from whole body tissue by homogenizing each larva in DNAzol reagent (Invitrogen, 10503-027) according to the manufacturer's protocol. Briefly, after homogenation in DNAzol, the homogenate was centrifuged at 10,000 x *g* for 10 min at 4 °C. Following centrifugation, the resulting viscous supernatant was transferred to a fresh tube. This step removed insoluble tissue fragments, RNA, and excess polysaccharides from the lysate/homogenate. The genomic DNA was then precipitated from the lysate by adding 100% chilled ethanol and centrifuging at 4,000 x *g* for 1-2 min at 4 °C. The pelleted DNA was washed with 70 % ethanol and then solubilized in 8 mM NaOH and stored at -80 °C. DNA concentration was determined by using a spectrophotometer at an absorbance of 260 nm.

PCR amplification was carried out using sense and antisense primers specific for C. *ripaius* SINE-CTRT1, Hb-13, Hb-7, Hb-4 and Ecdysteroid (EDS) (Synthesized by IDT, Skokie, Illinois) (Table 1). PCR reactions were performed using standard conditions

in accordance with the manufacturer's protocol (Invitrogen, 10966-018). The PCR reaction was as follows: 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 53 °C to 56 °C depending on the primer set for 30 sec, extension at 72 °C for 30 sec, and then a final 7 min extension at 72 °C before storing at 4 °C. PCR products were separated on a 1.5 % agarose gel using horizontal electrophoresis and visualized with ethidium bromide.

Table 1. Information on PCR primers for Hb-13, Hb-7, Hb-4, SINE-CTRT1 and the ecdysteroid responsive gene.

PRIMER	SEQUENCE	TM (°C)	GENEBANK ACCESSORY NUMBER	EXPECTED SIZE OF PCR PRODUCTS (nucleotides)
Hb-13	F: 5'CTTCTCTCATCCTTGCTG 3'. R: 5' GAGGACATGCATCCATTC 3'	53.65 54.04	AF001292 (Bergtrom, 2000)	692
Hb-7	F 5'AAATCCTTGCTGCTGTCTTC3' R 5'CGAGTTGTCCGACTAAGGTT3'	54.02 53.92	AF001292 (Bergtrom, 2000)	371
Hb-4	F:5'ACCAATCAAAATGAAACTCCTCA3' R:5'ACATCTCCATCAATGTTTGGAAG3'	49.03 49.86	(X00920) Genbank	286
SINE-CTRT1	F: 5'CTTCTCTCATCCTTGCTG 3'. R: 5' GAGGACATGCATCCATTC 3'	55.6 56.0	AF248853 (Bergtrom, 2000)	367
EDC	F 5'GTCAGTCACGTACAAACACG3' R 5'TCTTCCTAGTCCTATGCTC3'	55.03 54.95	X06212 (Markus, 1989)	459

PCR products were cloned using TOPO cloning kit (Invitrogen, Carlsbad, CA). The TOPO cloning reaction was set up by mixing together PCR products and TOPO vectors and incubating the mixture for 5 min at room temperature. The PCR-vector recombinant was then mixed with One- Shot® Competent *E. coli* cells and incubated on ice for 5 to 30 min in order to allow transformation to take place. The cells were heat-shocked for 30 sec at 42 °C without shaking and immediately placed on ice. S.O.C. medium (250 µl) was added to the reaction. The tube was caped tightly and shaken horizontally (200 rpm) at 37 °C for 1 hour. At the same time, LB plates containing 50 µg/ml kanamycin and 40 mg/ml X-gal in dimethylformamide (DMF) were prepared and incubated at 37 °C until ready for use. About 10-50 µl of each transformation was spread on to a pre-warmed selective (antibiotic-containing) plate and incubated overnight at 37 °C. An efficient TOPO cloning reaction should produce several hundred colonies. White colonies should contain the PCR-vector recombinants. From each plate, 10 white or light blue colonies were picked for analysis. PCRs were performed at standard conditions with the white colonies containing Hb-13 gene fragments or SINE. The PCR products and adequate primers were sent out for sequencing by McLab (South San Francisco, CA). The products cloned and sequenced included SINE and Hb-13 from the laboratory population of *C. riparius*, Hb-13 from *C. tentans* and Hb-13 from a wild population of *Chironomus* species collected from Kearny Marsh, NJ.

Fluorescent In situ hybridization (FISH)

Like Northern and Southern Blots, FISH is a technique that can indicate the presence of a particular RNA or DNA sequence, but FISH differs from blots in that the

labeled probe reveals the actual location of the sequence on chromosomes in the cell. FISH is the only procedure that allows the location of the sequence of interest to be studied (Polak et al, 1990). The method is comprised of three basic steps: fixation of a specimen on a microscope slide, hybridization of fluorescent labeled probe to homologous fragments of genomic DNA and detection of the tagged target hybrids.

To conduct FISH, the salivary glands of 4th instar larvae were dissected into 45% acetic acid and transferred to a drop of fixative (ratio of 1/2/3 mixture of lactic acid/water/glacial acetic acid) on a plastic coverslip and incubated for 5 min. The slides were frozen and the coverslips popped off using a razor blade. Prior to performing fluorescent *in situ* hybridization, the chromosomal DNA was denatured in order to allow the labeled probe to find the target sequence. This involved treating them with 2X SSC at 65 °C for 45 min, dehydrating using a 50%, 70%, 90%, 100% ethanol series for 2 min for each solution and treating with 0.1 N sodium hydroxide for 3 min. Finally, the slides were thoroughly washed with 1x PBS before applying the probe.

A variety of probes can be developed for FISH, depending upon the desired binding sensitivity. For DNA, FISH probes include double-stranded DNA, single-stranded DNA and synthetic oligodeoxyribonucleotides. The probes can be labeled directly, where the reporter molecule is directly attached to the probe, or indirectly, where a specific antibody or labeled binding protein is used to detect another molecule that is attached to the probe sequence (James Kling, 1996).

In this study, double-stranded DNA probes were labeled directly. SINE-CTRT1, Hb-13, Hb-7A, Hb-4 and Ecdysteroid I-C18 probes were generated by the PCR-labeling method. This was done by using a thermostable polymerase to incorporate 5-(3-

aminoallyl)-dUTP (Ambion, AM8438), which is an amine-modified nucleotide. When using aa- *dUTP*, the dTTP in the reactions had to be reduced in order to make the total amount of aa-dUTP and dTTP equal to the other three nucleotides. The incorporated aa-dUTP functioned as a reactive site for subsequent labeling of the probe. Activated fluorescent dyes such as fluorescein were covalently attached to the probes through the formation of a stable amide bond. In this study, Alexa 488 (Invitrogen, A20100) and Alexa 568 (Invitrogen, A20003) were conjugated with the aa-dUTP in the probes. To optimize the labeling intensity and visualization of PCR products, different dUTP:dTTP ratios were tested. It was important to achieve a high labeling density to ensure bright signal, it was also important to achieve a high yield of probes. In order to select the best ratio, both DNA synthesis yield and number of dyes attached to 100 nucleotides of probes were maximized.

PCRs for synthesizing probes were carried out using genomic DNA as a template. The PCR reaction contained 250 ng of genomic DNA in 2 μl, 2 μl of Hb-13 primers (20 pmol), 5 μl of 10X buffer, 1.5 μg of 50 mmol/L $MgCl_2$, 0.2 μl of Taq polymerase (25,000 U/L), and 1μl of 10μM dATP, dCTP, dGTP each in 50 μl total volume (the final concentration of each dNTPs in 50 ul is 200μM). The total concentration of dUTP and dTTP was kept equivalent with the other dNTPs. In order to optimize the aa-dUTP: dTTP ratio, different mixtures were used in the PCR reactions, including ratios of 0:10, 1: 9, 3: 7, 5:5, 7:3, 9:1, 10: 0, respectively.

Once PCR was completed, reactions were added to G-50 columns (GE Healthcare, 2753-3001) that removed the unincorporated aa-dUTP. The eluted PCR fragments were then incubated with Alexa dye in the presence of 0.05 M sodium bicarbonate, pH 9, at

room temperature for 1 hour. To stop the reaction, 4 µl of 4 M hydroxylamine was added, and the mixture was incubated at room temperature for 15 min. Finally, the unincorporated dye was removed using G-50 columns again. The end result was a double stranded DNA probe generated by binding the fluorescent dye to the amine-modified nucleotide that had been incorporated into the PCR product.

The relative efficiency of the labeling reaction can be evaluated by calculating the approximate ratio of bases to dye molecules. This ratio can be determined by measuring the absorbance of the nucleic acid at 260 nm and the absorbance of the dye at its absorbance maximum (λmax). λmax for Alexa 488 is 492 and λmax for Alexa 568 is 588. Alternatively, the ratio can be determined by using the Base:Dye Ratio Calculator on the Invitrogen website (probes.invitrogen.com) in the resources section. A good ratio for a labeled probe for FISH should be 8 to 20.

Before they could be hybridized to chromosomes, the probes were denatured by boiling for 10 min and then rapidly cooled on ice. The hybridization buffer was 50 % deionized formamide, 20 % Denhard solution, 4X sodium chloride sodium citrate (SSC) and 0.4 % sodium dodecyl sulfate (SDS). Each slide was treated with 100 pmol of probe and incubated overnight at 37 °C. After hybridization, the slides were washed twice in 2X SSC with 0.1% SDS for 10 min. The slides were then counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (1µg/ml) for 5 min and washed in phosphate buffered saline (PBS). Finally, the polytene chromosomes preparations were mounted in glycerol mounting medium (80% glycerol in PBS, 2% p-phenylenediamine) and visualized with a Leica inverted fluorescence microscope, using the Green- GFP/513852 and Red- N 2.1 - 513832 filters. Images were taken using a digital color camera, Leica DFC310 FX.

Cytogenesis analysis

Standard polytene chromosome maps of *C. riparius* (Ha¨gele, 1970) were used to localize hemoglobin, SINEs-CTRT1 and Ecdysteroid I-C18 genes. The diploid number for chromosomes in C. *riparius* was eight, 2n = 8. However, the homologous chromosomes were joined such that only 4 chromosomes were visualized in each cell, with chromosome arm combinations of AB, CD, EF and G (Figure 1). A well-defined centromere region was also characteristic of the chromosome set. (Michailova et al, 2005)

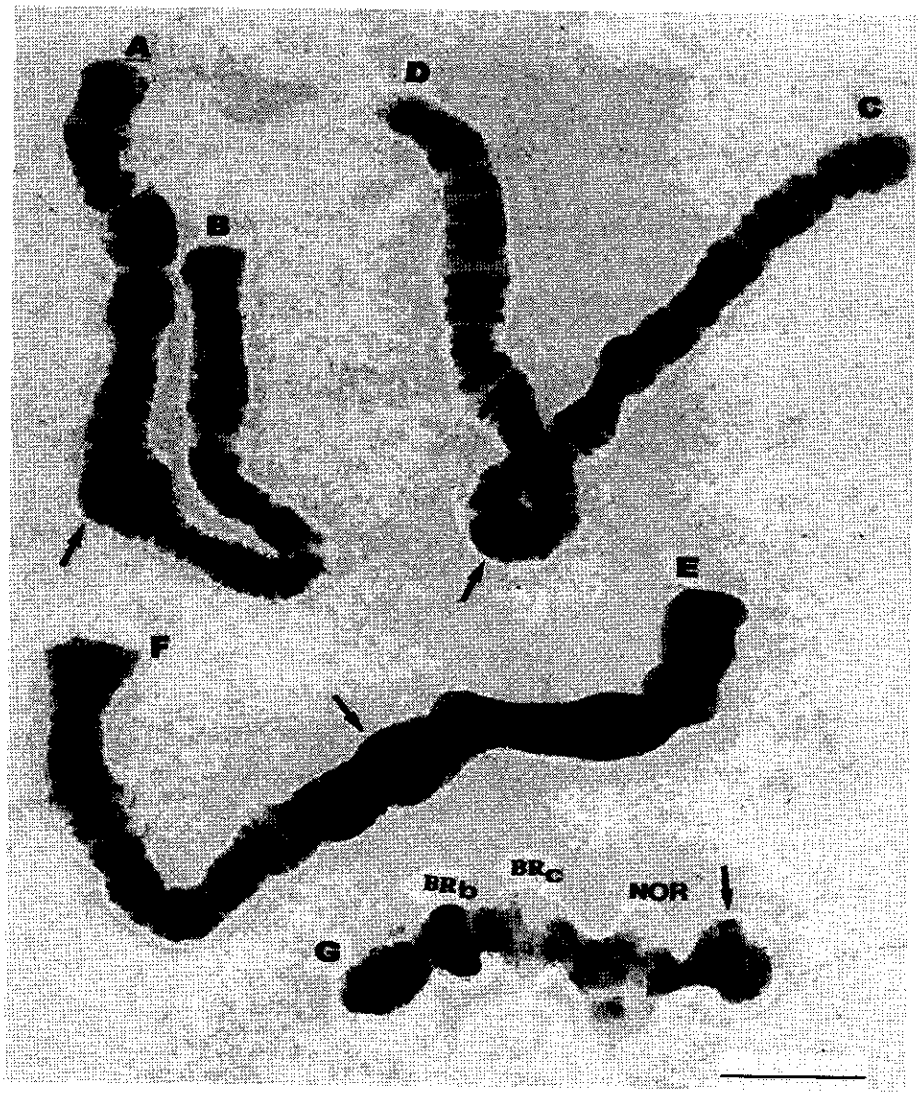

Figure 1: The karyotype of *Chironomus riparius* larvae. AB, CD, EF, G are chromosome arms; BRb and BRc represent Balbiani rings; NOR is the nucleolar organizer region. The arrow shows the centromere region. Scale bar: 100 mm. (Michailova et al, 2006)

Southern Blot

The Southern blot technique was performed to confirm probe specificity. Because Hb-7 was located adjacent to Hb-13 on chromosome II- arm D, it was necessary to demonstrate that the Hb-7 probe was not binding to the same sequence as Hb-13. The Southern blot was performed by first carrying out gel electrophoresis using the PCR products of Hb -13 and Hb-7 from C. *riparius*. The gel was then placed into 0.05 M sodium hydroxide to denature the double-stranded DNA. Denaturing with sodium hydroxide caused the following: it may have improved binding of the negatively charged DNA to the positively charged membrane, it separated the double-stranded DNA into single DNA strands for later hybridization to the probe and it destroyed any residual RNA that may still have been present in the DNA. After denaturation, a nitrocellulose membrane (Sigma-Aldrich, St. Louis, MO, N0639) was placed on top of the gel. The gel with membrane was place on top of a paper wick such that the gel was in between the membrane and wick. Both ends of the wick were in contact with the transfer buffer, 10x SSC. Pressure was applied evenly to the gel by placing a stack of paper towels and a weight on top of the membrane and gel. The paper towels drew the buffer up from the wick and through the gel by capillary action. The movement of the buffer drew the DNA in the gel up onto the membrane. The transfer was performed at room temperature overnight.

The membrane was then exposed for 7 min to a UV lamp DNA lamp transfer at 305 nm, 7000 μW/cm2 (Fotodyne, Hartland, WI, 2-1500) in order to permanently attach the transferred DNA to the membrane. The membrane was placed in a hybridization tube, DNA-side up, using forceps to prevent bubbles beneath the membrane. Then 20 ml of 2x

SSC was added, and the tube was rotated for 5 min in a Hybaid-Micro4 oven (Hybaid, Teddington, Middlesex, UK). The 2X SSC was replaced by 20 ml pre-hybridization buffer (2 % SDS, 50 % Formamide/ 5X SSC, 10X Denhardts, 0.05 % Na pyrophosphate) and rotated at 65 °C for 1 hour. The Hb-7 probe was denatured by boiling for 5 min and immediately thawed on ice until ready to use. The pre-hybridization buffer was removed and 20 ml of 1 μg probes/hybridization buffer mixture was added to the tube. The solution was incubated in the tube at 42 °C overnight. The membrane was then washed with 50 ml of 2X SSC three times in order to remove unspecific binding. Finally the membrane was scanned under fluorescent red laser diode (635 nm) and blue blue LED (450 nm) using a Storm 860 (GE, Healthcare)

Results

1. Optimized PCR labeling probes

Most fluorescent dyes absorb light at 260 nm as well as at their absorbance maximum (εmax). To obtain an accurate absorbance measurement for the nucleic acid, it is necessary to account for the dye absorbance using a correction factor (CF260). The CF260 value of Alexa 488 is 0.3.

$$Abase = A260 - (Adye \times CF260)$$

Calculate number of dyes per 100 bases:

$$\text{Number of dyes/100 bases} = 100/(Abase \times \varepsilon\text{-dye}) / (Adye \times \varepsilon\text{-base})$$

Where ε-dye is the extinction coefficient for the fluorescent dye, for Alexa 488, and is equal to 62,000 cm^{-1} M^{-1}, and ε-base is the average extinction coefficient for a base in double stranded DNA and is equal to 6600 cm^{-1} M^{-1}.

In order to select the ratio of aa-dUTP to dTTP, both the yield of DNA synthesized and number of dyes per 100 bases needed to be maximized for the PCR labeling reaction. The results revealed that the higher the amounts of aa-dUTP in the reaction, the lower the amounts of DNA synthesized (Figure 2). However, higher concentrations of aa-dUTP resulted in an increase number of dyes per 100 bases (Figure 3). Taken together, the ratio of 7:3 was selected for optimizing amplification of the genomic DNA and high labeling density to ensure a bright signal from the probe. For the

ratios of 0:10, 1:9, 3:7 and 5:5, the amount of DNA synthesized was higher than 7:3 ratio, but the results did not reveal enough labeling density for a good signal. On the other hand, the ratios of 9:1 and 0:10 resulted in higher number of dyes conjugated to the synthesized DNA but lower yields of PCR products overall. The optimal ratio of 7:3 was used to create all FISH probes in this study.

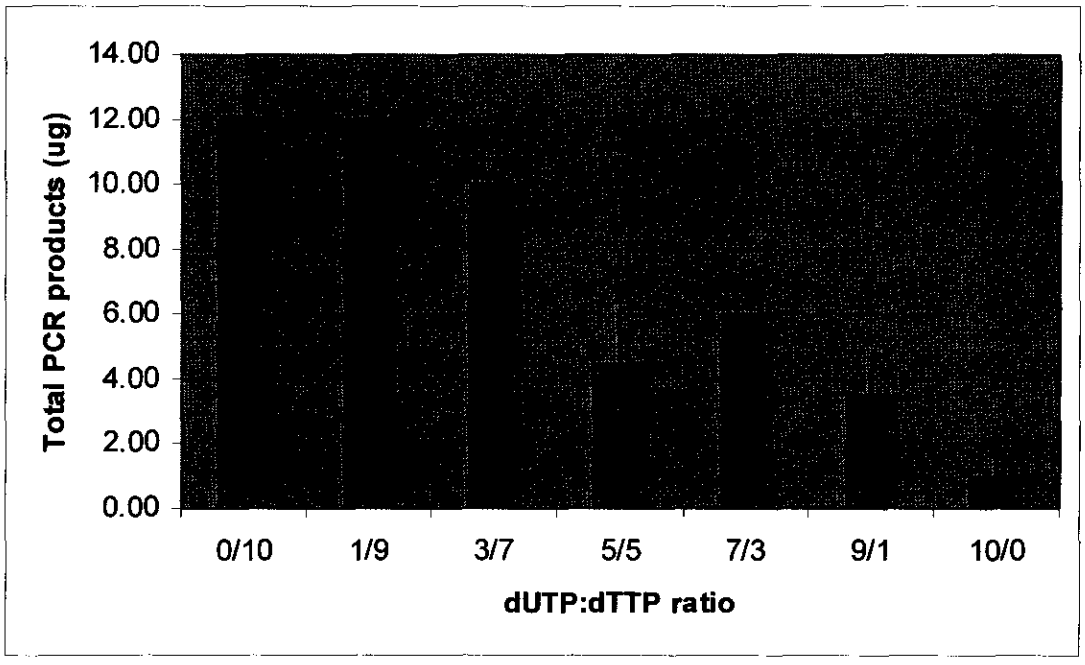

Figure 2: Average amounts of DNA synthesized from different ratios of aa-dUTP:dTTP. Increasing the amounts of aa-dUTP decreased the PCR yield. For each group n = 3, Error bar = 1 standard deviation.

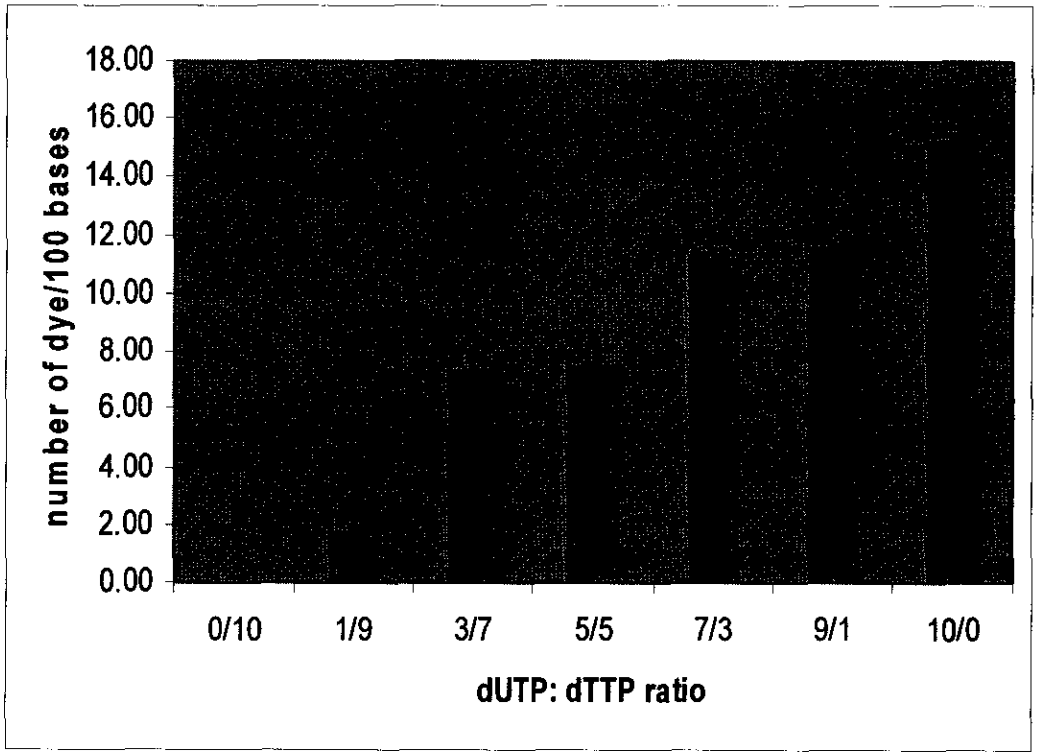

Figure 3: Higher concentrations of aa-dUTP resulted in an increased number of dyes conjugate into the DNA synthesis. Each group n = 3, error bar = 1 standard deviation

2. Alignment of Hb-13 coding sequences and SINE-CTRT 1 sequence

Published sequences for C. *riparius* Hb-13 and SINE-CTRT 1 were used to identify regions of similarity for the TOPO cloned sequences using NCBI *BLAST* program (blast.ncbi.nlm.nih.gov). The Hb-13 sequence from the C. *riparius* lab population was 630 bases long and found to be identical to Hb-13 sequence in the genomic clone l*gb2-1* (Gruhl et al, 1997) from C. *thummi*. The SINE-CTRT1 sequence was 360 bases long, rich in A T and 98 % similar to the SINE-CTRT 1 sequence of C. *thummi* (GenBank, accession No. AF001292). The alignment of Hb-13 sequence and SINE-CTRT-1 sequence showed the presence of SINE-CTRT1 inside of the Hb-13 sequence (Figure 4). This result confirmed that in our lab animal population, there is also an insertion of SINEs into the hemoglobin Hb-13. The insertion of SINE likely destroyed this gene's function.

To investigate the status of the Hb-13 gene in another species, PCRs with Hb-13 primers were performed with genomic DNA of C. *tentans* and a *Chironomus* species from Kearny Marsh. The larvae from the marsh were only identified to the genus level. The gel electrophoresis results showed bands of different sizes (Figure 5). A band of approximately of 650 nucleotides was shown for lab C. *riparius* PCR products, a band of approximately 280 nucleotides was shown on C. *tentans* PCR products and a band of approximately 270 nucleotides was shown for Kearny Marsh chironomids. It can be explained that the insertion of SINE increased the length of Hb-13 sequence of our lab C. *riparius* population, since the length of it was approximately equal to the sum length of Hb-13 of C. *tentans* and SINE-CTRT1 sequence.

PCRs with SINE-CTRT1 primers were performed with genomic DNA of C. *tentan* and *Chironomus species* from Kearny Marsh. Results showed bands of approximately 380 nucleotides for each species: however, no band was found when using the Hb-13 PCR products from these species as the template (Figure 6). It can be explained that there were SINE-CTRT 1 copies in the genomic DNA of C. *tentans* and *Chironomus* species from Kearny Marsh but no copies of this transposon inside their Hb-13 genes.

TTCCACGTGCTTTATGGCTGGCAGCAAGTTGTTCGGTAAGAGTGTAAAGTGCT
GGGACATTTGATTGATTTCCAATAAGTCCAACGATTTCAGAGAAGAAACCGAC
AATTCTACCTGTAAATTTGAAAACAGTTACAAATTATAAGAAGCTTCAACACTT
AGCTATTTATTTTACTCACCAGCATGTGTGGCGAATGCACCAGTATATATATA
ATATAATGTGTGAAGGGCCCAGCCTTTCAGCTGGAGGCACTGCGATTCAAATT
AGAACAGAATCTATTGTACCACACCCTTTAGACCTACAGTTTATAGTGGGATC
CGAACCACTTGCAAGCTCTGTACATTGCAAAGACACGAGTACGTCATAAGACC
TGATGTACCCTGCAATGTACCTAGTCACAATCCGTGTGCGCAAGATGTTGTAA
TGAAATCATCCAGATGCTAGTATATCCTTGTATACCTAGCAAAGGAGGCATAT
TCTATGCCAAAGACATCAATACTACACCCAACACACGAACCGAGCTTCGTAAC
TAGAACGGTGAAATGGAAAAAGAGCTTCGAAAATTAGACATGGCCTGGGATT
GAACCCAGGACCTTTGGCACGTAAGGCCAACGCTCTACCATTTAGGTCACTGA
TGCCATTTCACCGCAATGCACCAGTATCCTTGATTGAGTCGATGTCCTTGCCA
GCAAATTGAGGGAAACGAGCTTGGATGTCTGGATTGGCTGTGAAGATGGAAG
CAAGAATATCAACTTCACTATGTTTAACTTGAGCCCATGAAGATTTGACAAGT
GAAGCTTGATCAGCTGATAATGGAACAACAGCGGCTGAAGCTGCGGCAATGC
AAAGAGCCAAAATTAAGAATTTCAT

Figure 4. *Hb-13 and SINE-CTRT1* sequences cloned from PCR products of DNA extracted from the lab population of C. *riparius*. The Hb-13 sequence was identical to *Hb-13* in genomic clone l*gb2-1* (Gruhl et al., 1997). The location of SINE-CTRT1 inside of Hb-13 is underlined.

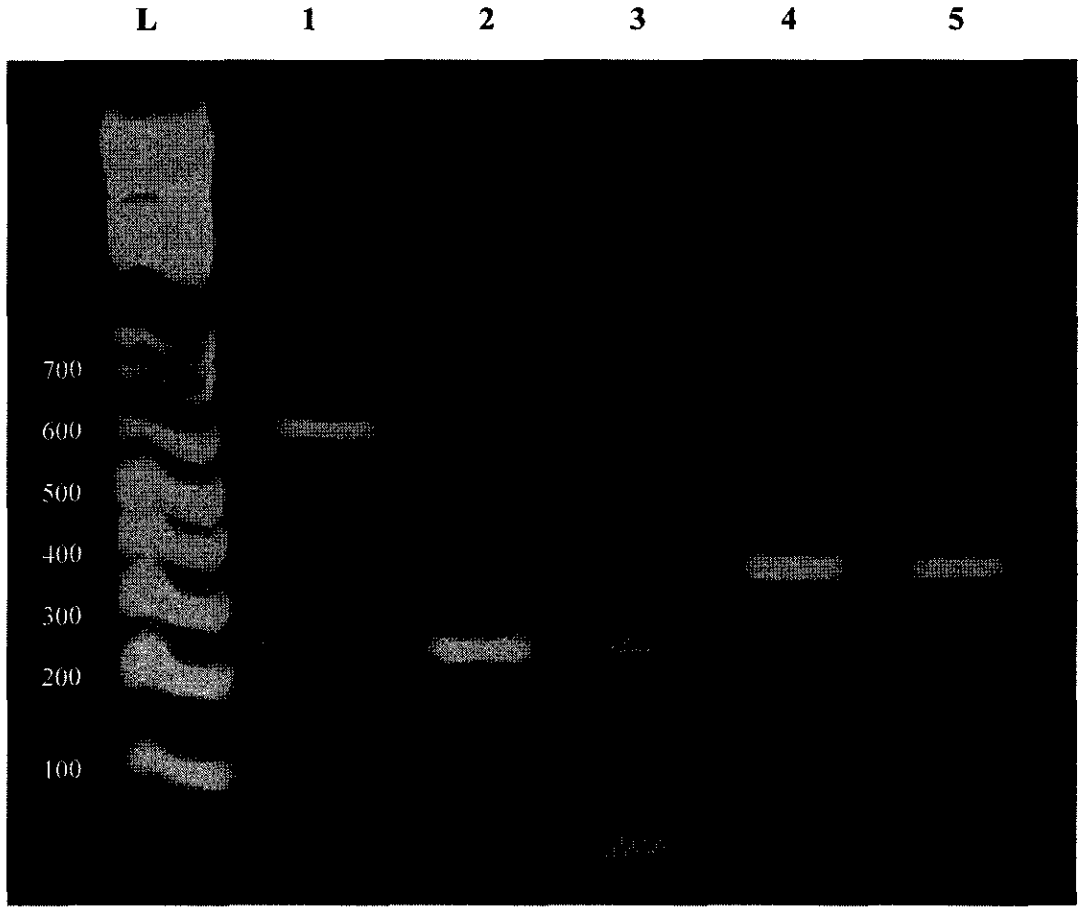

Figure 5: Agarose gel electrophoresis of PCR amplified products using Hb-13 and SINE-CTRT1 primers. L: 100 bp ladder, Lane 1: Hb-13 from our lab laboratory C. *riparius* population, lane 2: Hb-13 from C. *tentans*, lane 3: Hb-13 from chironomids of Kearny Marsh, NJ. The nucleotide lengths of DNA size markers in the left lane indicated that the Hb-13 sequence of C. *riparius* was longer than those of the other two species. Lane 4: SINE-CTRT1 from lab population of C.*riparius,* lane 5: SINE-CTRT1 from Hb-13 PCR products generated from C.*riparius* genomic DNA. This result confirmed the presence of SINE-CTRT1 inside the Hb-13 sequence.

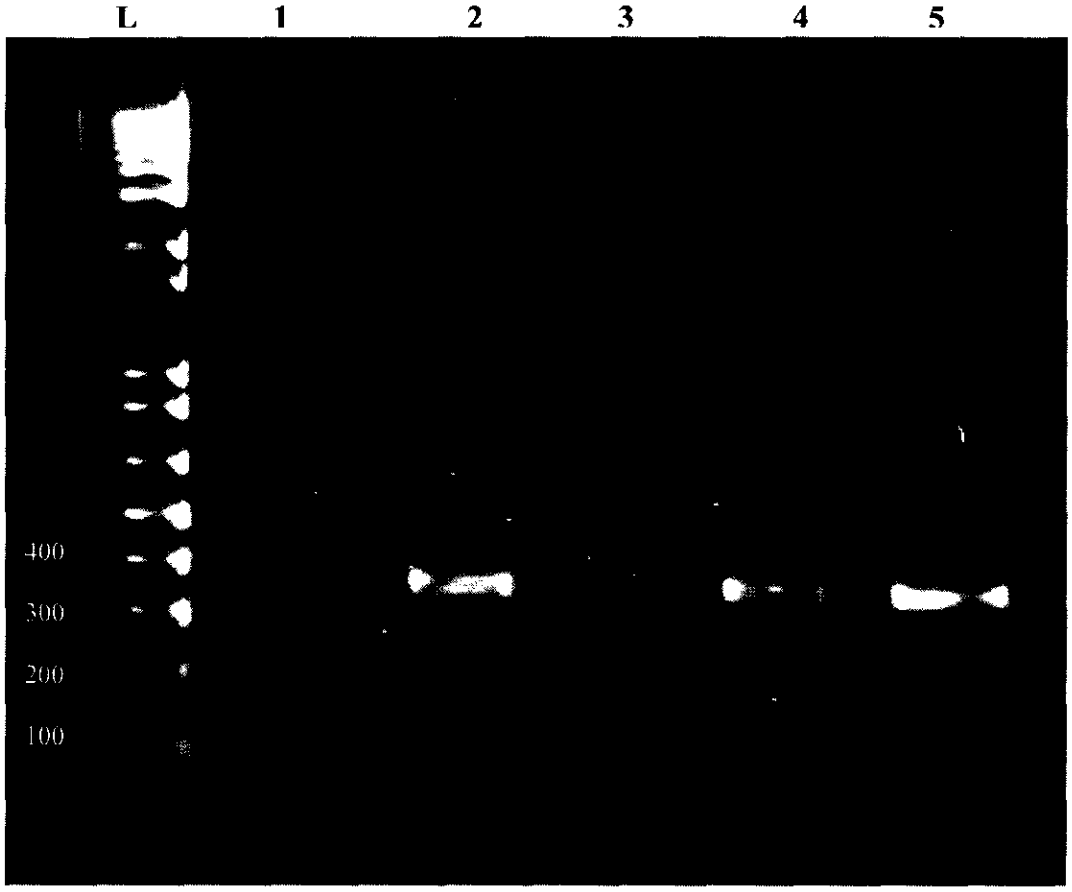

Figure 6: Agarose gel electrophoresis of PCR amplification products from different templates using SINE-CTRT1 primer sets. L: 100 bp ladder, Lane 1: Hb-13 PCR product of C. *tentans,* Lane 2: C.*tentans* genomic DNA, Lane 3: Hb-13 PCR product of *Chironomus* from Kearny Marsh, Lane 4: genomic DNA of Chironomus from Kearny Marsh, Lane 5: Hb-13 PCR product of C.*riparius* lab population. Results indicated that SINE-CTRT1 were distributed on genomic DNA of *C. tentans* and *Chironomus* from Kearny Marsh; however, there is no insertion of SINE into Hb-13 of these species as see in the C. *riparius* laboratory population.

3. Distribution of SINE-CTRT1

The gel electrophoresis results proved that SINE-CTRT1 was distributed in several chironomid genomes including our laboratory population. In order to determine the chromosomal distribution of the SINE-CTRT1, FISH analysis was carried out with Alexa 488 (green signal) SINE-CTRT1 labeled probes. The hybridization signals revealed that the SINE-CTRT1 sequence was interspersed throughout all 4 chromosomes (Figure 7). There were 68-70 distinct bands found: 23 on chromosome I, 22 on chromosomes II, 18 on chromosome III and 4 on chromosome IV.

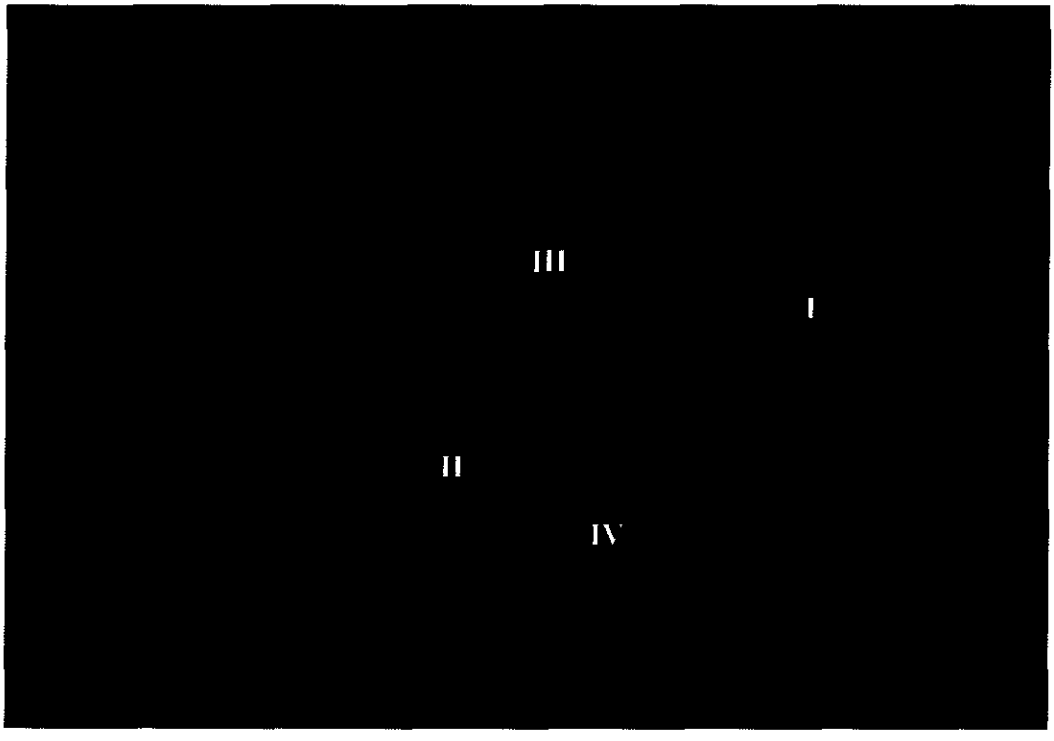

Figure 7: *In situ* hybridization of salivary polytene chromosomes of C. *riparius* hybridized with SINE-CTRT1 probe. SINE probes labeled with Alexa 488 were visualized in green under Green- GFP/513852 filter. There were 22 bands in chromosome I, 23 bands in chromosomes II, 18 bands in chromosomes III and 4 in chromosome III hybridized to the probe. Results showed multiple SINE elements throughout the genome.

4. *In situ* co-hybridization of SINE-CTRT1 and hemoglobin genes

In order to determine the involvement of the SINE-CTRT1 on chromosomal distribution of hemoglobin, FISH analysis was carried out with SINE-CTRT1 probes in combination with probes for Hb-4, Hb-7 or Hb-13 of the hemoglobin gene family. Co-hybridization of SINE-CTRT1 and Hb-13 probes to polytene chromosomes revealed an overlap on chromosome II, arm D (Figure 8). The overlap was visualized as a green band (SINE) laying along side of the red band (Hb-13) as well as the yellow tint of the red band. The yellow colored resulted from the combination of the green and red probes. This result was consistent with the early sequencing results: that there was an insertion of transposable element into Hb-13 gene in the laboratory population of *C. riparius*. The hybridization signals obtained with SINE-CTRT1 and monomeric Hb-4 probes revealed no overlap between their bands (Figure 9). Also, no co-localization was found from hybridization with SINE-CTRT1 and Ecdysteroid IC-18 (Figure 10).

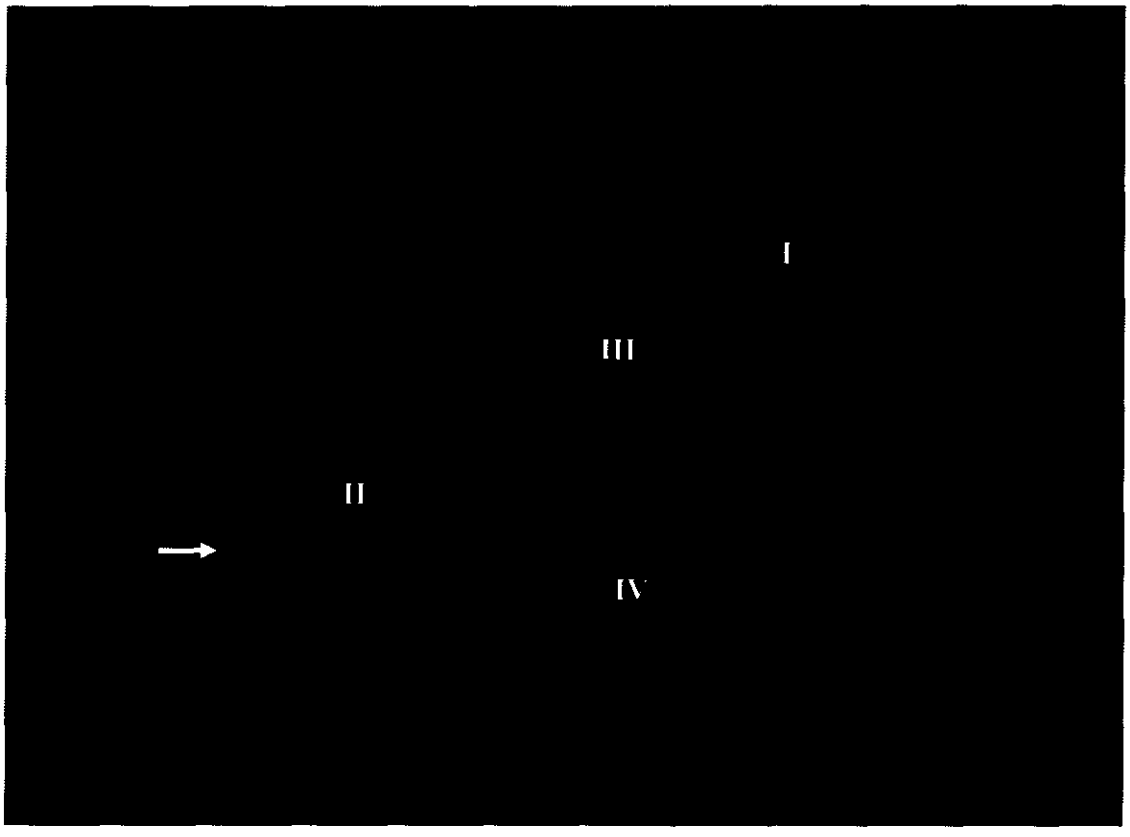

Figure 8: *In situ* hybridization of salivary polytene chromosomes of C. *riparius* with SINE-CTRT1 and Hb-13 probes. The SINE probe labeled with Alexa 488 was visualized in green under Green- GFP/513852 filter, and the HB-13 probe labeled with Alexa 568 was visualized in red under N 2.1 -513832 filter. Merging two channels showed the overlap in chromosome II- arm D. The arrow points to the locus containing Hb-13 and SINE-CTRT1. Note the green line next to the red one and that the red has a yellow color to it. The yellow color resulted from green and red dyes merging.

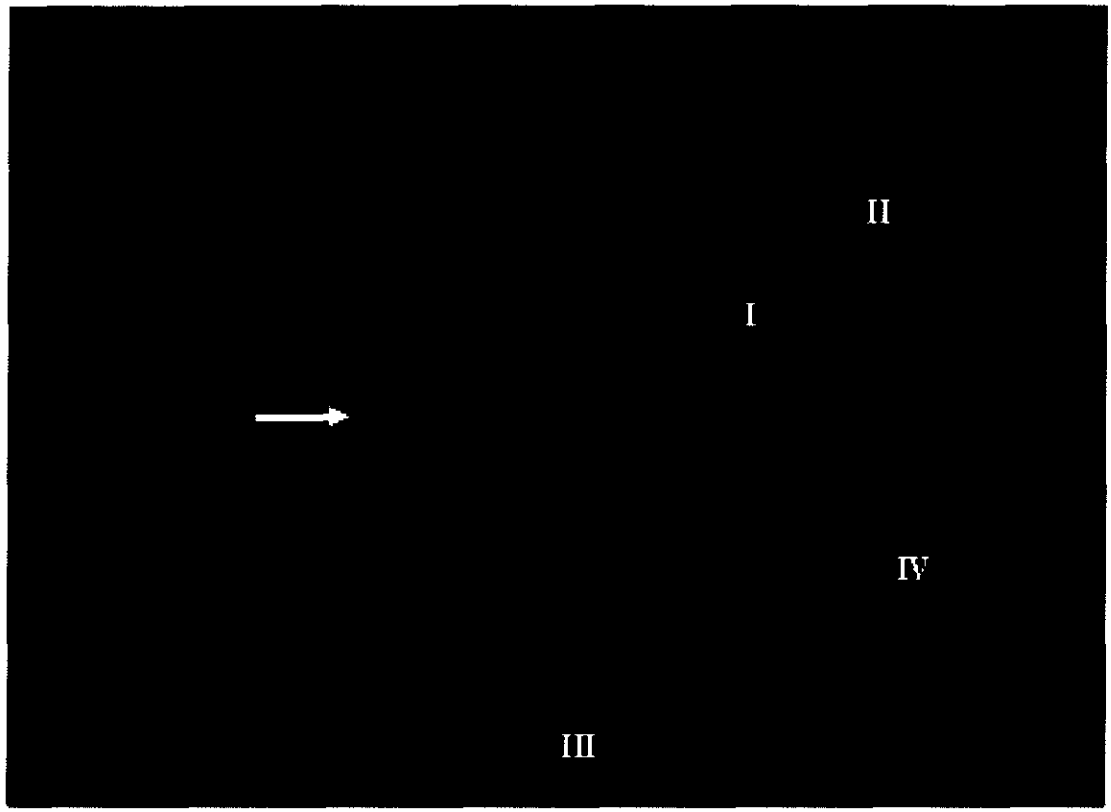

Figure 9: *In situ* hybridization of salivary polytene chromosomes of C. *riparius* hybridized with SINE-CTRT1 and Hb-4 probes. The SINE probe labeled with Alexa 488 was visualized in green under Green- GFP/513852 filter, and the Hb-4 probe labeled with Alexa 568 was visualized in red under N 2.1 -513832 filter. Hb-4 hybridized to the end of chromosome III-arm E. The arrow points to the locus containing Hb-4. The distinct red as opposed to yellow color at the Hb-4 locus indicated lack of co-hybridization.

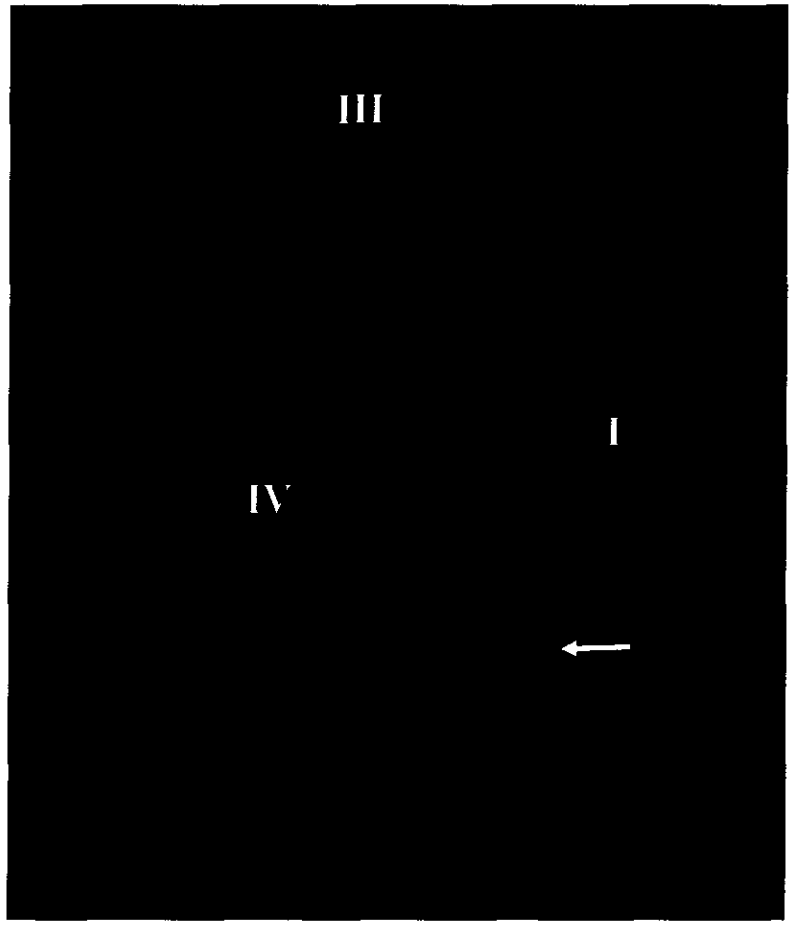

Figure 10: *In situ* hybridization of salivary polytene chromosomes of C. *riparius* hybridized with Ecdysteroid I-C 18 and SINE-CTRT1 probes. The SINE probe labeled with Alexa 488 was visualized in green under GFP/513852 and the Ecdysteroid I-C 18 probe labeled with Alexa 568 was visualized in red under N 2.1 -513832 filter. The arrow points to the locus containing Ecdysteroid I-C 18. The distinct red as opposed to yellow color at the ecdysteroid locus indicated lack of co-hybridization.

In order to further validate the loci of Hb-13 and Hb-4, the probes were co-hybridized (Figure 11). The signals obtained confirmed that the dimeric and monomeric hemoglobin genes were located on different chromosomes, chromosome II and III, respectively. Co-hybridization of probes for the two dimeric hemoglobin genes, Hb-13 and Hb-7, showed that they were both located in chromosomes II, arm D. Furthermore, they were neighbor genes, since the signals of Hb-13 and Hb-7 were visualized at the same site (Figure 12).

However, it was possible that the Hb-7 probe was not gene specific, that it could have been binding to the Hb-13 loci and *vice versa*. In order to verify that Hb-7 and Hb-13 were truly located next to each other and that their specific probes only hybridized with their genes, southern blot analysis was preformed. This was done using the Hb-7 probe and attempting to hybridize it to Hb-13 and Hb-7 PCR templates. The results confirmed that Hb-7 probe was specific for Hb-7 gene and would have only bound to the Hb-7 but not Hb-13 sequence during FISH (Figure 13).

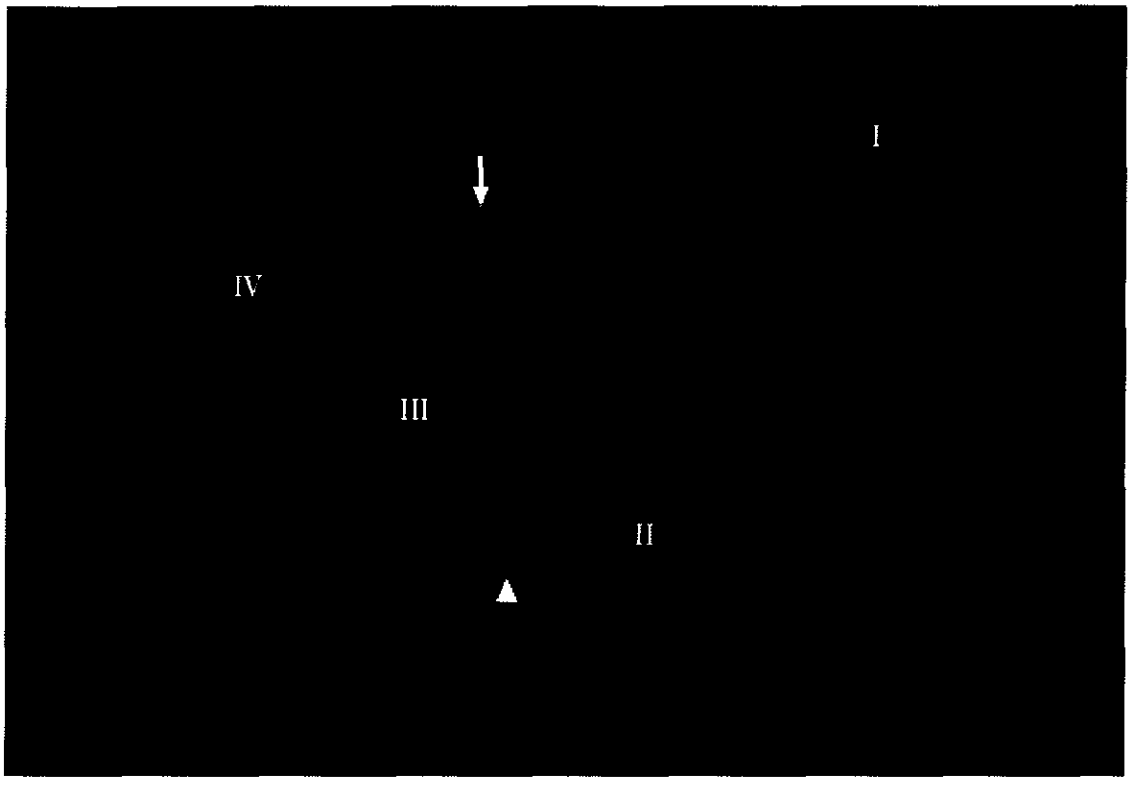

Figure 11: *In situ* hybridization of salivary polytene chromosomes of C. *riparius* hybridized with Hb-13 and Hb-4 probes. The Hb-13 probe labeled with Alexa 488 was visualized in green under GFP/513852 filter, and the Hb-4 probe labeled with Alexa 568 was visualized in red under N 2.1 -513832 filter. The arrow points to the locus containing Hb-4 and arrow head points to the locus containing Hb-13. Results indicated that the genes for the monomeric (Hb-4) and the homodimeric hemoglobin (Hb-13) were located at two separate loci.

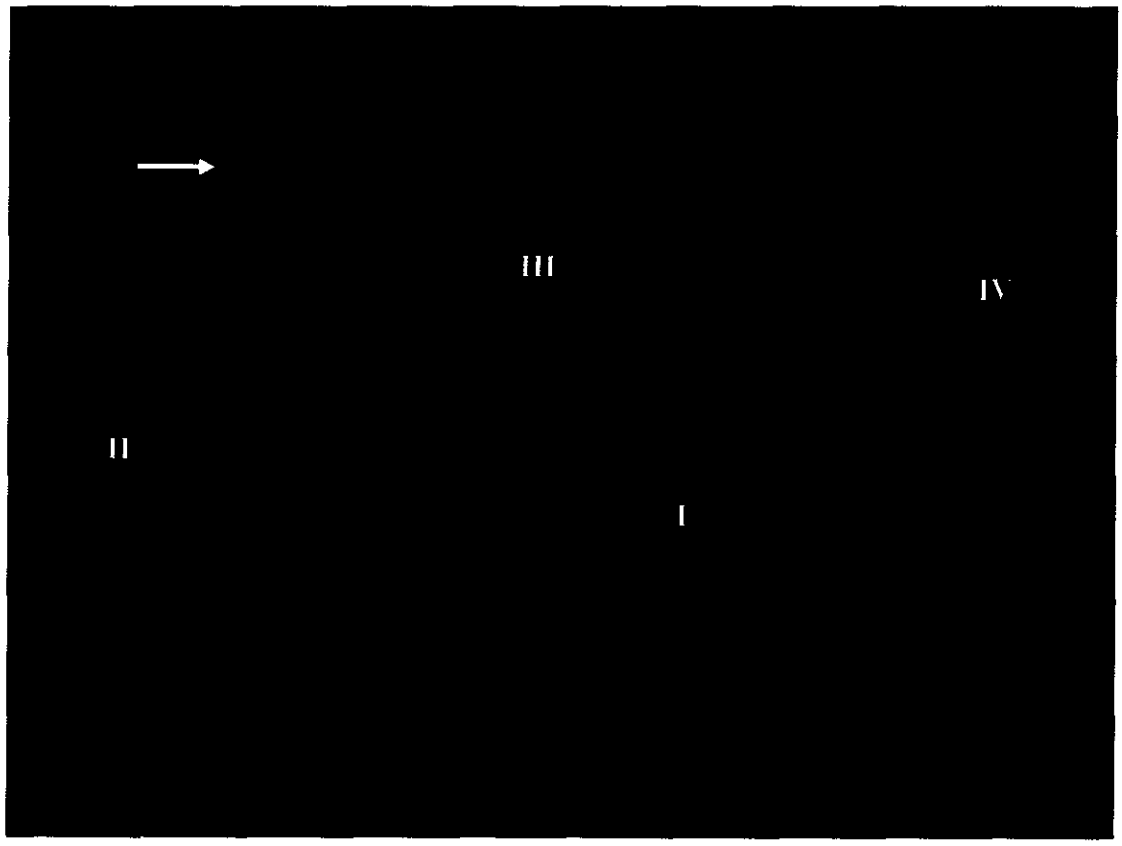

Figure 12: *In situ* hybridization of salivary polytene chromosomes of C. *riparius* with homodimeric Hb-13 and Hb-7 probes. The Hb-13 probe labeled with Alexa 488 was visualized in green under GFP/513852 filter, and the Hb-7 probe labeled with Alexa 568 was visualized in red under N 2.1 -513832 filter. The arrow points to the locus containing Hb-13 and Hb-7. Results showed Hb-13 and Hb-7 reside closed to each other on chromosome II, arm D.

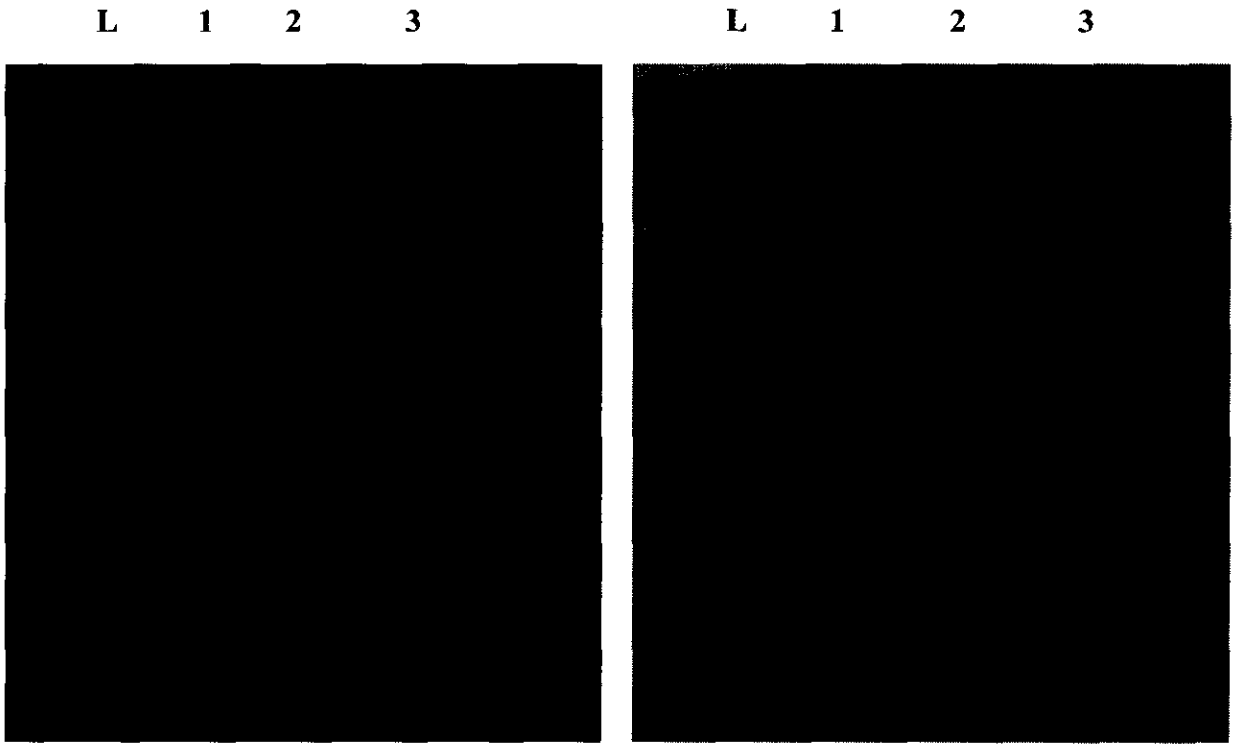

Figure13: The left panel was an electrophoresis gel of PCR amplification products from genomic DNA of C. *riparius* visualized with ethidium bromide. L: ladder (100 bp), Lane 1: Hb-13, Lane 3: Hb-7. The DNA in the gel was transferred to a nitrocellulose membrane, and then the membrane was exposed to the Hb-7 probe labeled with Alexa 488. The right panel was a Southern Blot of the same gel scanned using a fluorescent red laser diode (635 nm). Only the band in lane 3 was visualized. This proved that the Hb-7 probe bound specifically to the Hb-7 and not the Hb-13 sequence.

Discussion

The mechanisms of evolution including natural selection and genetic drift work with the random variation generated by mutation. Transposons, sometimes simply considered junk DNA, are mutagens. Once a mutation happens, it may shut off the functionality of a certain gene, and the rest of the code in DNA remains silent. (Han et al, 2005) In addition, transposable element sequences in the genome can modulate gene expression by serving as promoters, enhancers, silencers, sites of epigenetic modification, and alternative splicing sites (Han et al, 2005).

In this study, the results revealed a mutation in Hb- 13 gene of our laboratory C. *riparius* population. There was an insertion of SINE-CTRT1 into Hb-13 sequence. This was supported by the size of the HB-13 nucleotide fragment (~650 nucleotides) being roughly equal to the combined lengths of SINE-CTRT1 (~380 nucleotides) and Hb-13 of C. *tentans* (~270 nucleotides). The insertion of SINE was not found in other chironomid populations such as C. *tentans* and *Chironomus* species from Kearny Marsh, NJ. Therefore, this mutation was recent and happened after splitting the genus into different species. These results supported the previous publication that an exon of Hb-13 of

C. thummi (species name was revised to *riparius)* was interrupted by SINE (Bergtrom, 2002). The occurrence of uninterrupted Hb-13 in *C. tentans* and *Chironomus* from Kearny Marsh has not been previously published.

The hybridization signals of the SINE probe were widely dispersed on all four chromosomes (Figure 4). The distribution of SINE-CTRT1 all over the genome of C. *riparius* could provide high potential of mutation. The numbers of SINE loci found in the lab population of *C. riparius* (68-70) were comparable to those found by other

investigators: *C. thummi thummi* (100–110), C *thummi piger* (50-60) and C. *tentans* (70-80) (G. Bergtrom *et al,* 2000). These differences may result from different transposition efficiencies in each species or from transposition at different times in each species, where fewer SINE loci represent earlier episodes of mobility.

Not all mutations are necessarily dangerous for animals. Some mutations have no noticeable effect, while a small proportion of mutations may be actually beneficial (Fisher *et al,*1999). Over the long term, mutation can be a selected adaptive process which contributes to evolution and helps organisms adapt to changing climates and environments while others died out (Hall, 1982). When inserting itself into a gene, transposable elements can move or duplicative transpose on the chromosome to form another gene at a different location that has the same function (Tomoka, 1984). During cell divisions, small variations or mutations in the sequence of nucleotides start to appear and can change the function of this gene. After all, a multi-gene family can be formed from a single ancestor gene (Tomoka, 1984). The insertion of SINE into Hb-13 provides evidence of how an ancestral single-function hemoglobin molecule gave rise to descending molecules with varied functions or, in this case, the loss of function.

Studies have found that chromosomes with more gene variants have higher fitness (Tachida et al, 1998). This can explain why there are so many members of hemoglobin family on chromosomes of chironomids. Since a duplicated gene can acquire a new function while other copies retain the original function, gene duplication and subsequent differentiation are considered to be very important for adaptive evolution of organisms (Ohno, 1970; Ohta, 1988). However, as they become large, the approximation underestimates gene numbers, especially the number of pseudogenes. The following was

found from a previous study: (1) Gene redundancy measured by the average number of redundant genes decreases as advantageous selection becomes stronger. (2) Copy number selection drastically decreases the number of pseudogenes. (Tachida et al, 1998).

Hybridization of SINE-CTRT1 and Hb-13 probes revealed that they co-localized on arm D of chromosomes II (Figure 4). This was consistent with the sequencing results (Figure 2) and earlier observations that most of the homodimeric hemoglobin genes including Hb-13 are located in the F2b3 locus of arm D (Schmidt *et al.* 1988). Apparently, the insertion of the *CTRT1* into Hb-13 has destroyed the gene, even though Hb-13 retains all the features of a viable gene. It can be explained that not all hemoglobin genes in the insect are adaptively selective, and Hb13 was a dispensable member of a large, redundant multi-gene family. According to Tachida and coworkers (1998), this result indicated that adaptive selection of hemoglobin favors mechanisms of gene amplification and relocation that ensure high production levels of these environmentally relevant gene products.

The hybridization of Hb-13 and Hb-7 at the same location on chromosome II, arm D, indicated that they are neighbor genes and supported the theory of gene amplification (Figure 13). A previous study has found that Hb-9 and Hb-11 also are located at the F2b3 locus of arm D (Kao W-Y *et al.*1994). In this case, they were functionally related since they both belong to the homodimeric hemoglobin family gene and may share the same regulatory element and promoter on genomic DNA. Moreover, another study has found evidence of expansion of the *C. thummi* globin gene family in general and of the Hb-7B subfamily in particular when compared with C. *piger* (Trewitt, 1995). They confirmed that increased copy number of Hb genes has been positively selected as a mechanism to achieve a high Hb protein concentration in the larval hemolymph. Taken together, the

results presented here and in the literature showed the mechanism for the spread of the hemoglobin family favored the amplification of genes located in primarily one locus on chromosome II. The loss of function of Hb-13 due to SINE interruption could have been compensated by the expression of its neighbor genes, such as Hb-7.

The *in situ* hybridization results showed the existence of two different chromosomal loci containing hemoglobin genes. Both Hb-13 and Hb-7 were localized on chromosome II- arm D; however, the site for the monomeric Hb-4 gene was localized near the end of chromosome IV arm E (Figure 11). This was similar to a previous publication that showed hemoglobin genes could be distributed away from one locus (Hankeln, 1988). *In situ* hybridization of Hb-4 with SINE-CTRT1 did not show any co-localization, suggesting that SINE-CTRT1 was probably not involved with Hb-4's alternative location (Figure 9). To our knowledge, this is a new contribution to the literature. There was also no overlapping between SINE-CTRT1 and a single gene Ecdysteroid I-18C (Figure 10). The ecdysteroid responsive gene has not been associated with a multi-gene family (Lezzi, 1989). Its sequence has only been associated with the single I-18C location on polytene chromosomes. This gene was intended to be a negative control and was therefore not expected to overlap with SINE. However, the lack of association between SINE and Hb-4 indicated that the SINE transposon may not be responsible for the relocation and thereby expansion of the hemoglobin family. This did not preclude SINE involvement with the relocation of other genes given its wide distribution in the *Chironomus* genome.

In future studies, the co-localization of SINE-CTRT 1 and hemoglobin family genes on polytene chromosomes can be studied in heavy metal adapted populations such

as chironomids from Kearny Marsh, NJ. The relationship between other TEs and hemoglobin can also be investigated.

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