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Genetic Diversity of Balbiani Ring l Gene in Two Species of Chironomus

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GENETIC DIVERSITY OF BALBIANI RING I GENE IN
TWO SPECIES OF CHIRONOMUS

by

Lola-mae Palmer

Submitted in partial fulfillment of the requirements for the degree of Master
of Science in Biology from the department of Biology of Seton Hall
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Abstract

Differences in genetic composition have been predicted among aquatic midge fly larvae called chironomids. Although several genes including microsatellites and ribosomal protein genes have been explored in our lab, the gene that has so far been studied the most is the Balbiani Ring (BR) 1 gene. There are several genes in this family, including Balbiani Ring 2.1, 2.2 and 6. These BR genes are known to contain long arrays of tandemly repeated units with a ranked repeat organization (Paulsson et al. 1992). This secretory protein gene forms a large puff located on polytene chromosome IV (Bentivegna et al., 1993). The gene codes for a protein that when secreted forms the larval tube from which these organisms feed. Organisms within the same family share similar genetic makeup, therefore understanding variations in a specific gene may prove to be invaluable. By studying this gene, it may be possible to understand the changes that it, and genes like it, undergo when exposed to environmental stressors such as toxicants.

Early study of the BR1 gene suggested that it contains tandem repeated sequences, which indicates that its genetic evolution is not conserved. This study analyzed DNA from fourth instars of *C. tentans* and *C. riparius*. Data was generated using PCR and single-stranded-conformational-polymorphism. Results indicated that double stranded DNAs (dsDNA) from both species were similar in size, between 123 and 246 base pair. The single stranded positive and single stranded minus bands of both species varied however, the minus strand showed more variation than the positive one. This variation was seen between individuals of the same species and between each species. More variation was seen in *C. tentans* than in *C. riparius*. The results based on these techniques suggested
that there are polymorphisms, genetic variation in the single strand DNA (ssDNA) of each of the species tested.
Introduction

Chironomidae is a Family of midge flies that are globally distributed and abundant in aquatic ecosystems (Armitage et al., 1995). The larval stage of these organisms is the most often studied stage of life. These larvae are often associated with sediments, where toxic substances accumulate, and are therefore commonly used in sediment toxicity studies. A few genes have been sequenced in these organisms, but there are no studies in the literature comparing their genetic variability. Many species in the Family appear morphologically similar suggesting that their gene sequences should be highly conserved. The aim of this study is to further explore the genetic variability of the Balbiani ring genes in these organisms.

There are four Balbiani ring genes (BR1, BR2.1, BR2.2 and BR6) currently known, with BR3 being a possible new member to this gene family (Paulsson et al., 1990). These genes are part of a common internal sequence organization and are built from 35 to 40 kilobase long repeated units arranged in tandem (Pustell et al., 1984). Balbiani Ring genes, in general, are active chromosomal puffs that code the major polypeptide of the salivary gland (Meyer et al., 1983). The secretory protein genes are located on the active site of the polytene chromosome.

The Balbiani Ring I (BRI) gene is responsible for the formation of a tube that larvae use for feeding. The genes are constitutively expressed, which means that their protein product is continuously produced. The gene has repetitive sequences exhibiting tandem repeats (Paulsson et al., 1992) and therefore might show variability among species.
Tandem repetitive sequences are grouped into three different classes: satellite, minisatellite and microsatellite. Microsatellite have variable numbers of repeated sequences that vary among individuals. Microsatellite (MS) are simply repetitions of the same short sequence of bases over and over again. MS consist specifically of multiple “CA” sequences, mostly located in the non-coding region (Griffith et al., 1996). Minisatellite on the other hand, are located in euchromatin subtelomeric regions. Minisatellites are well known as VNTRs (variable number tandem repeats), with repeated units of 15-100 base pair long (Griffith et al., 1996). As with MS, Minisatellites contain variable repeated sequences that vary among individuals. The last and final category is satellite, which are the largest of the repeated sequences. Satellite DNA is found to consist of multiple tandem repeats of short nucleotide sequences, stretching up to hundreds of kilobases in length” (Griffith et al., 1996). BR genes are classified as satellites. Satellites are usually located in heterochromatin regions, where meiotic recombinations have been shown to be suppressed. BR genes are different from typical satellites as they are located in the euchromatin.

To study genetic diversity of these BR genes, single-stranded conformational polymorphism (SSCP) was employed. SSCP analysis has been used to identify mutations in a variety of systems, including oncogenes and tumor suppressor genes (Fan et al., 1993). The polyacrylamide gel, which SSCP utilizes identifies subtle changes in migration due to altered 2° conformation. The nucleotide sequence determines the type and the extent of the intra-strand binding, which ultimately determines secondary conformation. Aligned bands indicate sequence similarity. However, if bands are misaligned this is usually an indication of polymorphism. PCR-SSCP method has been
established as a rapid and sensitive method for detecting polymorphisms in DNA (Nakajima et al., 1996). PCR was performed on DNA from two species, C. tentans and C. riparius. PCR primers, BP30 and BM1, had been previously established in Dr. Bentivegna's lab. The double stranded PCR products were generated from the two species and compared on agarose gels. Select bands were cut out, isolated and used to generate ssDNA. The ssDNA was then used for SSCP, which was performed in both agarose gel and polyacrylamide gel in a temperature-controlled chamber. Final analysis of composite DNA was accomplished by sequencing the two BRI fragments.

The main objective of this thesis was to compare variability among and within species of Chironomus tentans and Chironomus riparius. While the complete genomic sequence of C. riparius and C. tentans was unknown, BR genes have been sequenced in related Chiromomus species. BR I genes have been proven to contain tandemly repeated sequences, which often have genetic variations. (Hoog et al., 1988) It is these variations in the repeated nucleotides which might show polymorphisms. SSCP was employed due to its sensitivity in detecting polymorphisms. Once the genetic variations are known, future studies may then take place. Future studies may include examining the extent to which toxic agents affect genetic diversity of an organism. Other genes, such as ribosomal protein genes and microsatellites, may also benefit, as it may lead to looking at their diversity. Another reason for pursuing this thesis was in the hope of coupling these experiments with gene expression experiments, in order to view how environmental stressors change or alter genes that are passed down from generation to generation. Heterozygosity was also evaluated among the two species.
Materials and Methods

A. Animals

The organism used for the experiments was 4th instar larvae of *C. riparius*. *Chironomus riparius* were a gift from Dr. Alan McIntosh, University of Vermont. They were maintained at room temperature (20-25°C) on a 12 light:12 dark hour photoperiod. Water for the cultures was particle and carbon filtered using the Millipore machine, Millipore Corp. (CDPRM1206 and CDFCO1204). The water hardness was 136 mg/ml, with a pH of 7.3. Chiromomus larvae diet consisted of TetraDoro Green® floating sticks (Tetra, Germany). Natural play sand was provided a substrate. It was acid washed using 10 percent HCl for 3-4 hours, washing it abundantly in deionized water and oven drying it overnight.

B. RNA Isolation

For extraction and isolation of RNA, six 4th instar of *C. riparius* were placed in a 2 ml glass mortar and pestle, and homogenized in 1 ml of TriReagent (Sigma Biosciences, St. Louis, MO). The homogenate was then placed in a 2 ml micro-centrifuge tube and centrifuged at 12,000 rpm for 10 minutes at 4°C. Next the supernatant, containing the DNA, RNA and protein was transferred to a clean 2 ml micro-centrifuge tube. To this 0.2 ml of chloroform was added to remove DNA and protein. This mixture was shaken for 15 seconds then allowed to stand at room temperature for 15 minutes. Again the mixture was centrifuged at 12,000 rpm, for 15 minutes at 4°C. The RNA was now in the upper, clear layer of the supernatant. It was removed and placed in another micro-centrifuge tube. Half of an milliter of cold isopropanol was added, mixed and allowed to stand at room
temperature for 10 minutes. The mixture was centrifuged as above, then the supernatant was removed and discarded. To recover the RNA, the pellet was washed with 1 ml of 75% ethanol. The ethanol was then removed, and the RNA pellet was dissolved in 100 µl of diethyl pyrocarbonate treated water (depc-water). The optical density of the RNA was taken using a spectrophotometer (Ultraspect 3-Pharmacia/LKB).

The isolated RNA was then "messed cleaned" to remove any remaining DNA. The message clean procedure was done according to the manufacturer's instruction (GenHunter, Nashville, Tn). First, the RNA was exposed to DNase I digestion. In this reaction, 50 µl of total RNA was added to 5.7 µl 10X reaction buffer and 1.0 µl of DNase I, followed by a 37°C incubation for 30 minutes. Second, DNase was removed and RNA extracted by adding 40 µl of 3:1 phenol/chloroform mixture. This mixture was vortexed for 30 seconds, followed by samples being placed on ice for 10 minutes undisturbed. The mixture was then centrifuged at the above mentioned conditions, and the upper phase collected into a clean micro-centrifuge tube. The third step involved adding 5 µl of sodium acetate and 200 µl of 100% ethanol. Samples were left to stand for 1 hour at -70°C, then centrifuged at 12000 rpm for 10 minutes at 4°C. The supernatant was then carefully removed and RNA washed with 0.5 ml of 70% ethanol. Again the RNA was centrifuged in the tabletop eppendorf centrifuge to remove any excess ethanol. The clean and ethanol precipitated RNA was redissolved in 10-20 µl of depc water. The RNA concentration was quantified through measuring the absorbance. Finally, the RNA samples were stored at -70°C until use.
C. DNA Isolation

To prepare the genomic DNA, six fourth star larvae were homogenized in Proteinase K buffer (0.5 M NaCl, 2% SDS, 0.01 M Tris pH 7.4, 0.0125 M EDTA pH 8) and centrifuged (2000 rpm, 5 minutes) to a pellet. The supernatant was transferred to a clean tube to which 2 mg of Proteinase K (Sigma Chemical, Co., St. Louis, MO) was added. The mixture was heated at 37° C overnight. Protein and RNA were then removed simultaneously using TriReagent (Sigma Chemical, Co., St. Louis, MO) according to the manufacturer’s directions. The DNA was then resuspended in 200µl TE (10 mM Tris/HCl, 1mM EDTA, pH 7.4) and extracted with 200µl PCI (25:24:1 Tris buffered phenol: chloroform: isoamylalcohol). A second extraction was performed using 25:1 chloroform:isoamylalcohol. The DNA sample was then precipitated from the aqueous layer using 75% ethanol and 0.5 M NaCl. The DNA was then washed in 70% ethanol and rediluted in 100 µl of ddH₂O.

D. PCR

A Standard PCR kit from Life Technologies (Gaithersburg, MD) was used to perform the PCR. The following ingredients were combined for the 50 µl PCR reaction: ddH₂O, approximately 1 µg of genomic DNA, 3 µl of 2.5 mM dNTP, 5 µl 10x PCR buffer with MgCl₂, 20 pmol of each primer (BP30 and BM1) and 5 units Thermus aquaticus (Taq) DNA polymerase. The samples were placed in a Gene Amplification PCR system thermocycler from Perkin Elmer (Branchburg, NJ) and amplified under the following conditions: 94° C for 3 min then 94° C for 30 seconds, annealing temperature of 54° C for 1 min, 72° C for 1 min, for 40
cycles. The samples were then incubated at 72° C for 3 minutes and stored at 4° C. PCR products were analyzed by electrophoresis using a 2% low melting agarose gel. The amplified products were recovered and isolated using the ultrafree DA® centrifuge tube (Fisher Scientific).

E. Reamplification

Isolated genomic fragments were reamplified in triplicate 50 µl single stranded reactions: 1 µl undiluted DNA template, 3 µl of 2.5 mM dNTP, 5 µl 10x PCR buffer, 2µl 50 mM MgCl₂, 20pmol of BP30 or BM1, ddH₂O and 5 units Taq polymerase. Composites were done in triplicates and individuals were done in duplicates. The samples were then subjected to the following PCR condition: 94° C 3 minutes, then 94° C 30 seconds, 50° C 30 seconds 72° C 30 seconds for 40 cycles, then 72° C for 3 minutes and 4° C until collection. Products were electrophoresed on 2% agarose as with the genomic DNA.

F. Single-Stranded Conformational Polymorphism (SSCP)

Triplicate single stranded PCR fragments from agarose gels were combined and purified using the concert nucleic acid purification system (Invitrogen, Carlsbad, Ca). Once collected the DNA was dried on a spin column and rediluted in ddH₂O to approximately 12 ng/2 ml. Two milliters of this dilution was combined with 2 ml of formamide xylene dye in preparation for phast gel electrophoresis. The products were loaded onto a 12 % polyacrylamide gel and electrophoresed on the Phast System at 15° C for 30 minutes. In the end the phast gel was stained using silver stain from Amersham-Pharmacia-Biotech (Upsala, Sweden) and analyzed.
G. Sequencing

Composites from each species were sequenced. The single stranded products of each species were compared specifically for the possible variations in the nucleotide sequence of the BR I gene. The PCR products were sequenced with the Ampli-Taq Cycle Sequencing kit from Life Technologies (Gaitherburg, MD). This kit is based on a well-known sequencing method known as the Sanger sequencing method (Sanger et al., 1975). During PCR, synthesized DNA were labeled by random incorporation of $^{32}$P-alpha-ATP. Products were separated on a 6% polyacrylamide gel using SequinGen apparatus from BioRad (Hercules, CA). The gel was exposed to X-Ray film from Wolf X-Ray Corp. (West Hempstead, NY) for 24 hours following the drying process. The film was then manually developed.
Results

A. Amplification of BR I genes from composite DNA

Double stranded DNA products were generated using BP30 and BM1 from composite genomic *C. tentans* and *C. riparius* (Table 1). The PCR products were then electrophoresed on a 2 % agarose gel (Figure 1). The two genomic bands were shown to be the same size at approximately 180 bp. The genomic bands were then cut from the gel, isolated and subjected to another round of amplification. This time, however, single stranded (ssDNA) products were generated. Utilizing a lower annealing temperature and 35 cycles PCR, triplicates of single stranded positive and minus bands resulted (Figures 2-3). Figure 2 shows that the minus bands of *C. tentans*, although consistent with one another were larger than the positive bands. In Figure 3A, the positive bands of the *C. riparius* were consistent and similar in size to that of the *C. tentans*. Figure 3B, however, exhibited differences between the minus band triplicates. For example, lane 2 showed 2 very distinct bands measuring at 280 and 369, respectively, while lanes 1 and 3 showed only one strong band each.

B. SSCP analysis of genomic DNA

The triplicates of each single stranded product were combined and electrophoresed. The Phast system was employed, each sample was loaded onto a phast gel (Figure 4). This special polyacrylamide gel, has the ability to separate single stranded DNA with high resolution and at a rapid pace (Mohabeer et al., 1991). Both bands from each species appeared to have a major distinct band in common. This band can be seen
in Figure 4 at approximately 246 bp. Despite being quite faint, the minus band of each species showed multiple minor bands. These differences in the minor bands suggested that the minus bands showed greater variations than the positive bands of both species.

C. Sequencing of composite ssDNA

Each ssDNA product from each species was sequenced and aligned as shown in Figure 5. The amplified region of the BR I gene had a high percent of homology (81%) between the two species. *C. riparius*, however, showed a partial match to *C. tentans* at certain points indicated by the asterisks (*). The bolded letters indicated that there are 2 possible base pair at that location. For example, the W indicated a possible A or T base pair, Y indicated a C or T, and M indicated an A or C. This display of heterozygosity indicated more genetic diversity at the sequence level in *C. riparius* than *C. tentans*. 


Table 1. Established Sequence of Primer pair in BR 1 gene

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP30</td>
<td>AGAACCTGTACGGAGG</td>
</tr>
<tr>
<td>BM1</td>
<td>GATCATCTTACGACGCTTGC</td>
</tr>
</tbody>
</table>

Figure 1. PCR product from genomic DNA C. riparius and C. tentans. L was a 123 base pair (bp) ladder. Lane 1 was the double stranded (dsDNA) product generated from genomic C. tentans DNA. Lane 2 was the dsDNA product from C. riparius. Both species appeared to be a similar size, ~180 bp. Bands were generated using primers BP30 and BM1.
Figure 2. Single stranded PCR product amplified from dsDNA C. tentans. Using a lower annealing temperature and shorter PCR Cycle than the genomic dsDNA product, the ssDNA products were generated. L corresponded to the 123 bp ladder, lane 1,2,3 were triplicates of the positive ssDNA product. Lanes 4,5,6 were triplicates of the minus ssDNA products. Primers BP30 and BM1 were used to generate each band. The positive single stranded bands appear to be -246 bp in size. The minus single stranded band on the other hand, were -300 bp.
Figure 3. Single Stranded PCR product derived from dsDNA of C. riparius. Products were generated using same primers as the C. tentans, BP30 and BM1. A) L was 123 bp ladder, lanes 1,2,3 were triplicates of positive single stranded C. riparius. B) Lanes 1,2,3 were the triplicates of the minus band of the C. riparius. The positive bands (A) measured at ~246 bp while the minus bands (B) were of different sizes. Lane 1 band was ~280 bp. Lane 2 shows two different bands, one at ~280 bp the other just under 369 bp. Lane 3 band was at ~369 bp.
Figure 4. Single Stranded Conformational Polymorphism
Using the purified single stranded products SSCP was accomplished. L was the 123 bp ladder, Lane 1 was the positive band of the C. tentans, and Lane 2 was the positive band of C. riparius. Lane 3 was the minus band of the C. tentans, and Lane 4 was the minus band of C. riparius. All major bands in lanes 1-4 were ~246 bp. Lane 2 showed a distinct secondary band at ~615 bp. Lanes 3 and 4 however exhibit secondary bands that ranged in size.
Figure 5. Alignment of *C. riparius* and *C. tentans* following sequencing. The positive and minus bands were sequenced separately using positive primer BP30 and minus primer BM1, respectively. The amplified region is shown above. There was 81% homology between the *C. riparius* and *C. tentans* as indicated by “I” linking the identical base pair. The bolded letters indicate heterozygosity of the organism. The asterisk (*) indicates a partial match between the two species.
D. Amplification of BR 1 genes from individual organisms DNA

The results for PCR products from individual organisms were consistent with those from the composite study. These products were generated from genomic DNA of each individual organism using BP30 and BM1. Once amplified, all samples were electrophoresed on a 2% agarose gel (Figures 6 and 7). Figure 6 showed PCR products generated from nine *C. tentans* individuals. Lanes 1-5 of panel A and lanes 6-9 of panel B showed a common band of ~250 bp. All the individuals in Figure 6A were shown to be similar in terms of the bands present. Figure 6B also showed similarity between the individuals but there were additional bands. One band, ~650 bp, was similar to that in individual #4 in Figure 6A. Overall there seemed to be some variation between each *C. tentans* individual, and more bands were found in individuals than in the composite sample (Figure 1).

Figure 7 showed the PCR products from genomic PCR of *C. riparius* individuals. The bands were smeared but similar for each individual. Individuals in Figure 7A showed the band of interest at ~250 bp with secondary bands throughout. Figure 7B also showed a major band for all individuals of ~250 bp. Even though the smearing indicates non-specific primer binding, each set of individuals exhibited little variation. Comparing the two species, individuals of *C. tentans* appeared to show more genetic variation than *C. riparius* when double stranded PCR products are separated by agarose gel.
Figure 6. PCR product from individual genomic DNA from *C. tentans*. PCR products from gel A and B represented dsDNA from *C. tentans*. A) L was a 100 bp ladder; lanes 1-5 represented PCR products from individual *C. tentans*. All 5 individuals are seen to share a common band measure at ~250 bp. B) L was a 100 bp ladder; Lanes 6-9 represented a different set of individual *C. tentans*. Although all PCR product from each individual exhibited the band of interest at ~250, there were secondary bands visible in each individual. The 250 bp product was used in single stranded PCR.
Figure 7. PCR product from individual genomic DNA *C. riparius*. PCR products from gel A and B dsDNA from *C. riparius*. A) L was a 100 bp ladder, Lanes 1-5 were dsDNA from individuals that measure roughly -250 bp. In addition, there were secondary band visible in all 5 lanes at -350 bp and 500 bp. B) L was a 100 bp ladder, Lanes 6-9 were dsDNA of another set of *C. riparius*. All individuals share a common band slightly above -250 bp. The 250 bp product was used in single stranded PCR.
E. Amplification of duplicate single stranded PCR products from each individual dsDNA

Genomic PCR products from individual organisms were amplified into single stranded positive and minus PCR products, respectively. In order to generate enough DNA for SSCP, each single stranded product was duplicated. Figure 8A and B showed the single stranded positive primer product generated from the double stranded *C. tentans* band in Figure 6. All major bands in this figure appeared to be at approximately 250 bp. These agarose gels did not show very distinct bands, which was speculated to be due to reduced primer specificity. Figure 9 showed the minus single stranded PCR products for *C. tentans*. All individuals showed the major band at ~250 bp; however, there were secondary bands visible. Each set of duplicates for an individual was similar in band pattern, indicating reproducible results. Figure 9A lane 4 showed two distinct secondary bands approximately 300 and 320 bp. Lane 5 of the same panel also showed additional bands, at 300 and 350 bp. Figure 9B lane 6 showed secondary bands at ~300 bp, while lanes 7 showed a lower secondary band at ~225 bp. Lanes 7 and 8 showed additional bands at 300 and 350 bp.

Results for the positive single stranded product from dsDNA of *C. riparius* are shown in Figure 10. Both panels A and B of Figure 10 showed a band at ~250 bp in all individuals. The smearing present here was similar to the smearing seen in the *C. tentans* positive, which may be attributed to self-binding of the DNA strand. Figure 11A and B showed uniform band patterns for the single stranded minus primer of *C. riparius*. These band patterns were seen to be very different from those of *C. tentans*. In Figure 11A all duplicated individual showed bands between 200 and 250 bp. Figure 11B, which appeared to be more smeared, showed a band for all sets of duplicates at ~200 bp. A
secondary band also appeared above \(~250\) bp. The smearing effect prevented the individual bands to be visible separately. Overall the minus single stranded product showed more genetic variation than the positive single stranded product in *C. tentans*.

The duplicates of each individual were purified and combined for SSCP. They were then electrophoresed on the Phast system using polyacrylamide gel. This system separated the DNA at a lower temperature of \(15^\circ\) C than electrophoresis on an agarose gel (RT 21\(^{\circ}\)). Figure 12 depicted the SSCP results of the positive *C. tentans*. Figure 12A showed individual 1-5, as seen in lanes 1-5. Individuals 2, 3 and 5 faintly exhibited the band of interest at \(~250\) bp. Individuals 1 and 4 were not visible. This may be due to insufficient primer and multiple bands. There were however secondary bands \(~700\) bp in individuals 2,3,4 and 5. Figure 12B individual 6 showed the band of interest at \(~250\) bp. Individuals 7-9 however showed light bands at \(~250\) bp. All individuals also exhibited secondary bands at \(~700\) bp. Figure 13 showed the results of SSCP minus bands of *C. tentans*. Figure 13A represented individuals 1-5, which showed a primary band of \(~250\) bp with secondary bands at \(~300\) bp and beyond. Individual 5 however showed its primary band at \(~300\) bp with the subsequent band at \(~350\) bp.

Figures 14 and 15 showed the SSCP results of *C. riparius*. Figure 14 exhibited the purified positive DNA of *C. riparius*. In individuals 1-5 of Figure 14A the primary band is seen at \(~300\) bp which is consistent with 12A of the SSCP results of *C. tentans* individuals. There were also secondary bands in individual 2, 4 and 5 measuring slightly above \(600\) bp Figure 14B showed individuals 6-9, with a common band at \(300\) bp. There seemed to be a secondary band visible in all lanes at \(~600\) bp. The final figure, 15 showed the minus SSCP results of *C. riparius*. Figure 15 A showed individuals 1-5.
While barely visible, bands were seen to appear at ~250 bp, with secondary bands measuring between 500 and 700 bp. Figure 15B also exhibited barely visible band at ~250 bp. The secondary bands here as with 15A were seen between 500 and 700 bp. Overall the SSCP results for both species appeared to share similarities. The positive bands of *C. tentans*, although not as distinct as the *C. riparius* were close in size. The positive strand of both species showed more similarities, than that of the minus bands. There were multiple bands present in each individual overall. This would indicate that there were multiple conformations for bands generated by both primers.
Figure 8. ssDNA positive PCR product amplified from individual dsDNA of C. tentans. Single stranded positive DNA products were generated using primer BP30 only in 9 individual chironomids. A) L was 1 kb ladder. Each individual was duplicated. The main band in all individuals while hard to visualize measured ~250 bp B) L was 1 kb ladder. As in panel A the individuals were duplicated. Therefore individuals 6, 7, 8 and 9 were generated from the same dsDNA PCR band, respectively. The major bands in each set of duplicates were ~250 bp.
Figure 9. ssDNA minus PCR product amplified from individual dsDNA C. tentans. Using minus primer BM1 the above single stranded products were generated. A) L was 100 bp ladder, each individual was duplicated. Individuals 1, 2 and 3 showed major bands at ~250 bp. Individual 4 showed a major band at ~250 bp and a secondary band at slightly above 300 bp. Individual 5 showed a strong band at 300 bp. B) L was 100 bp ladder, individual 6 showed bands at ~250 bp and ~300 bp, individual 7 showed bands at ~225 bp and ~250 bp, individual 8 showed bands at ~200 and 250 bp, individual 9 showed bands at ~250, 300 and slightly above 300 bp.
Figure 10. ssDNA positive PCR product amplified from individual dsDNA C.riparius. Single stranded positive DNA products were generated using primer BP30 only. A) L was 100 bp ladder. Each individual was duplicated. Therefore individuals 1, 2, 3, 4 and 5 were from the same individual, respectively. The main band in all individuals was measured at ~250 bp. B) L was 100 bp ladder. As in panel A the individuals were duplicated. The major band in each set of duplicates was shown to be ~250 bp.
Figure 11. ssDNA minus PCR product amplified from individual dsDNA C. riparius. Using minus primer BM1 the above single stranded products were generated. A) L was 100 bp ladder. Each individual was duplicated. Individuals 1-5 showed major bands at ~200 and ~250 bp. B) L was 100 bp ladder. As in panel A the individuals were duplicated and were generated from the same dsDNA PCR band. Individual 6-9 showed bands at ~200 and ~250 bp.
Figure 12. SSCP results of individualss positive C.tentans.
Purified single stranded products were used to carryout SSCP on 9 individual C. tentans. A) L was 100 bp ladder, individual 1 and 4 did not exhibit the band of interest. Individual 2, 3 and 5 however showed this band which measure at ~300 bp. B) L was 100 bp ladder, individual 6 showed a band at ~250 bp, individual 7-9 faintly exhibited a visible band at ~250 bp.
Figure 13. SSCP results of ss minus individual C. tentans. Using the purified single stranded product SSCP was employed for individual larvae. A) L was 100 bp ladder, individual 1-4 exhibited a similar band at ~250 bp. Individual 5 however failed to have this common band but showed a strong band at ~300 bp. B) L was the 100 bp ladder. Mild disturbance in the gel caused individual 6-9 to shift slightly left and downward. Primary band was shown however to be ~250 bp with secondary bands at 300 bp.
Figure 14. SSCP result of individual as positive *C. riparius*. A) L was 100 bp ladder used, lanes 1-9 showed individual single stranded positive DNA. All individuals in panel A showed similar band slightly below 300 bp. Individual 1, 3 and 4 exhibited secondary band is at ~600 bp. B) L was 100 bp ladder, individual 1-4 also showed similar band pattern below 300 bp. A secondary band is also visible in each individual at ~600 bp.
Figure 15. SSCP result of individual ss minus C. riparius. A) L represented 100 bp ladder. Individual 1-5 exhibits multiple banding patterns. The end of each lane showed a faint band which measures at ~250bp. B) L was 100 bp ladder. Individual 1-4 represented individual C. riparius. There are multiple bands visible in each lanes, however the band of interest appeared sometimes faint in all lanes at ~250 bp.
Chironomids larvae are often a quantitatively important component of macrofauna communities inhabiting freshwater sediments. Therefore their distribution has been well studied and related to sediment composition and other environmental factors (Pinder 1986). While they have widely been studied, a genetic variability study such as the one described in this paper was the first of its kind. The data gathered from this study gives insight into the diversity of two species of chironomids, based on the sequence of the BRI gene. The study involved two levels of comparison, first among species and then within species. It was expected that due to the tandemly repeated sequence of the BR I gene, there would be variability among species, but less variability within each species on an individual basis. The populations used in these experiments were control populations grown in the laboratory, therefore it was expected that little variation would be shown within the species due to inbreeding. These populations offer a baseline for future studies, which will involve individuals taken from a stressed environment. The secondary interest of this study was to evaluate PCR-SSCP and its effectiveness in detecting variations. By first understanding this naïve population, comparisons can then be made with chironomids from chemically polluted environments.

The composite part of the studies showed that there was genetic diversity among *C. riparius* and *C. tentans* at the single stranded level. Single stranded analyses in agarose gels, showed that the positive bands for both species were similar in size at ~246 bp (Figures 2 and 3). The minus band showed no variation in *C. tentans* with a major
band at 246 bp and a minor band at 350 bp. However, in \textit{C. riparius} there was variation with one band (~246 bp) in individual #1, two bands (246 and 350 bp) in individual #2 and one band (350 bp) in individual #3. Further examination with SSCP using polyacrylamide gel electrophoresis (PAGE) proved that there was more variability than seen in agarose gels (Figure 4). In PAGE gels, there was a minor band (~1230 bp) for the positive strand in \textit{C. riparius} not seen in \textit{C. tentans} (Figure 4). The minus strand formed different minor bands as well. One unique to \textit{C. tentans} was ~254 bp. One unique to \textit{C. riparius} was ~369 bp. The minus strand bands were similar in both agarose and PAGE gels. However, the minor positive band of \textit{C. riparius} was only seen in PAGE gels. Sequencing of these two PCR products showed conclusive evidence of diversity between species. At the sequence level \textit{C. tentans} and \textit{C. riparius} shared 81\% homology. \textit{C. riparius} appeared more heterozygous as two different nucleotides were found several times at the same position in the sequence.

Individual studies showed varying and complicated results. Each individual species was observed at three experimental levels. The first of which was on an agarose gel following genomic PCR. These gels revealed multiple PCR products for \textit{C. riparius} and \textit{C. tentans}. While the band sizes varied among the \textit{C. tentans} individuals (Figure 6), they were relatively consistent for all nine individuals within \textit{C. riparius} (Figure 7). The second level consisted of comparing ssDNA from individuals on agarose gels. Once the individuals were separated into positive and negative bands, the similarities and differences were clearer. Positive strands among individuals were nearly indistinguishable for both species (Figures 8 & 10). The minus strand produced variable bands sizes among individuals in \textit{C. tentans} but had a consistent band pattern in \textit{C.
riparius (Figures 9 & 11). The final level was observing bands on polyacrylamide gels. The positive strands of each species showed a common band at ~300 bp, however, unlike agarose gels, secondary bands varied (Figure 12 & 14). For example in C. tentans, individual 2 (Figure 12 A) showed a secondary band at 1000 and 1100 bp and individual 5 showed secondary bands at 800 and 900 bp. In C. riparius, individuals 2, 4, 5, 7, 8, and 9 shared a common secondary band at 800 bp not seen in 1, 3, 6, and 10 (Figure 14). The minus bands of both species were quite different. C. tentans individuals showed secondary bands ranging from 700-100 bp (Figure 13 A & B). Individuals 1, 2, and 4 showed the same three similar sized bands at 700, 800 and 900 bp. Individuals 3 and 5 showed a common band at 700 bp. Minus bands of C. riparius ranged in size from 500-1000 bp (Figure 15 A & B). The secondary bands were quite similar in sizes for all individuals of C. riparius.

In SSCP there was smearing evident in both the agarose and polyacrylamide gels, which was attributed to non-specific PCR products or multiple conformations for intra-strand binding. Overall, the positive strand formed a similar band in both species that was similar among individual, within a species, the minus strand produced more bands in both species and they were more variable in size among individuals of the same species and between species. C. tentans showed more variability among individuals than C. riparius, which was different than that seen in the composite studies. The variation in C. tentans was observed at all three levels of experimentation.

Non-specific PCR products or intra-strand binding might account for the apparent heterozygosity seen in the C. riparius DNA sequence. Non-specific PCR products might
interfere with accurate reading of the gel. Intra-strand binding might interfere with the polymerase and genetic sequencing errors.

Single Stranded Conformational Polymorphism has the ability to highlight variation occurring within species in the conformation of denatured fragments (Nakajima et al. 1996). Double stranded agarose gel electrophoresis alone while informative, was not as conclusive as SSCP. However, when coupled together they proved to be very instrumental in detecting genetic variability. Hayashi and coworkers (1991) estimated PCR-SSCP analysis sensitivity as more than 99 % for 100-300 bp fragments and 89 % for 300-450 bp fragments. In comparison with other methods, SSCP has several advantages, which makes it the technique of choice to screen for minor deletions and point mutation (Bastos et al., 2001). The greatest advantage of the SSCP method comes with its relatively rapid screening method, as compared with direct sequencing that was performed in the composite phase of this study. PAGE and agarose gels seemed to similar in benefits in terms of the information provided based on this study. The bands in the PAGE gels, however, appeared more distinct and less smeared than in the agarose gel. The differences among individuals were not more apparent in either gels, but there were more bands evident in the PAGE gel.

While many studies have explored genetic diversity, there were very few that employed gene specific PCR followed by SSCP as their technique. Dalebout and coworkers (2001) used species of bottleneck whales and PCR to study genetic diversity. The variations in chironomids agree with the data presented in their paper. First both studies used species from the same Family, Chironomidae and Hyperoodon, respectively. Second, gene specific primers for the mtDNA control region were used to amplified a
434 bp DNA fragment in bottlenose whales. Although there were differences in the analysis of the data, the overall results were similar to this study. One specie, *Hyperoodon ampullatus* was less diverse than that of the other *Hyperoodon planifrons*. *C. tentans* was more diverse than *C. riparius* in this study. Both studies gave strong support that genetic diversity exists between individuals of the same species.

In another experiment, PCR and SSCP were employed in a molecular diversity study. This was conducted by Kjoller and Rosendahl (2001), in which species of glomalean fungi were compared. The procedures were similar to the one used in this study. The variability of fungi from harvested pea root lineage was monitored. Several roots of plants were used from different plots within the same region. PCR was used to amplify a specific diverse region of ribosomal DNA, using eukaryote specific primers LR1 and NDL22. SSCP was utilized to determine multiple sequence types present. Unlike the chironomid study, sequencing was used as a final test to further identify possible diversity in the *Glomus* species. The four fungi haploid types showed little genetic variation. This was speculated to be due to the low amount of molecular information extracted and could be similar to results for *C. riparius*.

Genetic diversity is important on all environmental levels. Genetic variation has allowed selection for beneficial genes, and maintaining it is crucial to continue the response to changes in environment (Blott *et al.*, 2001). Based on a genetic variability study performed using randomly amplified polymorphic DNA (RAPD), the EPA's Exposure Research laboratory reports that "genetic diversity within species of aquatic organisms is an indicator of the condition of stream ecosystems" (Silbiger *et al.*, 1998). It is also an indicator of the sustainability of a population, since populations with low
genetic diversity are less able to adapt to unfamiliar environmental stressors. The results of this study give our laboratory a basis for further studies in variability. The other genes such as ribosomal protein and microsatellite genes may now be explored in the same manner. Once the data is gathered from these other genes then the next step may be taken. Directly comparing the changes at a genetic level of species exposed to heavy metals toxics such as cadmium, we may determine how affected the genes really are. Eventually comparison of laboratory grown species may be compared with that of environmentally stressed species from a polluted lake or pond.


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