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Identification of Eurycea Using Cytochrome *b*

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Abstract

Genomic sequencing is a powerful tool that has many applications for research, one of which is in the field of taxonomy and the identification of species. This thesis discusses the mitochondrial gene cytochrome *b* and its utility in population genetics and identification of larval amphibians. The development of the Polymerase Chain Reaction and primers are an integral part of the modern DNA sequencing process. The Polymerase Chain Reaction is used to amplify a target DNA sequence, and the protocol for this procedure must be optimized for the specific sequence of target DNA. Primers must also be designed and modified for a selected portion of the DNA to be copied. This thesis also discusses the application of these techniques in a current study of a population of *Eurycea*. Three species of *Eurycea*, the Cave Salamander (*E. lucifuga*), Long-tailed Salamander (*E. longicauda longicauda*), and Three-lined Salamander (*E. guttolineata)* were discovered in an abandoned mine shaft near Riverville, Amherst County, Virginia in 1999. The population is unusual, as *E. longicauda longicauda* and *E. lucifuga* are outside their normal distribution and this was the first syntopic occurrence in Virginia of *E. guttolineata* and *E. longicauda longicauda*, the species usually indigenous to the Piedmont physiographic region. In addition, there is possible hybridization between *E. guttolineata* and *E. longicauda*. The larvae of these species are difficult to identify morphologically; so the paper discusses the use of cytochrome *b* sequencing for species identification among this population of *Eurycea*.

Identification of Eurcyea Using Cytochrome *b*

Introduction

Not only does DNA contain the instructions for life-enabling metabolic processes, but this molecule also contains the information determining the characteristics unique to each individual species. At a molecular level, sequence analysis allows for a better understanding of the function and shape of the protein for which a gene encodes and at the macroscopic level, information concerning the phylogeny, phylogeography, and maternal ancestry can be derived. DNA sequencing is a powerful tool that has made enormous advancements in the last twenty years. Currently more than 260,000 organisms have at least some section of their genome sequenced and available for comparison in on-line gene Databases (Benson et al., 2008). This has made it possible for researchers across the globe to conduct comparative studies fairly easily and has had dynamic impacts in a number of fields, including population ecology and taxonomy. Another application of this technology is the identification of species from tissue samples, as discussed in this paper.

DNA sequencing has been particularly useful in the identification of larval amphibians. Amphibians have complex life cycles that include a larval stage that is radically different in morphology from the adult animal (Vences et al., 2005). The larvae of many amphibians are difficult to identify because many species are conservative in their anatomy and display close similarities in the larval stage (Parmelee, Knutson, & Lyon, 2002). Larval morphology can also vary geographically and with developmental stages and the immediate physical and biotic environment can influence the anatomy of

the larvae as well (Parmelee et al.). Genomic sequencing provides an alternative method of identification and can be a valuable asset in the correct identification of larvae species.

Mitochondrial DNA

The variability in DNA sequences can be considered on the level of individual genes or whole genotypes (Sunnucks, 2000). Mitochondrial DNA has many advantages as a molecular marker because it evolves faster than nuclear DNA, mostly likely due to inefficient replication repair (Kvist, 2000). While nuclear DNA is inherited from both parents, mitochondrial DNA is only inherited maternally and has a lower $N_{e,}$ or effective population size, meaning that it is subject to greater genetic drift than nuclear markers, so variants consequently have become a more rapid diagnostic of taxa (Sunnucks). Different regions of the mitochondrial genome evolve at different rates which allows for the selection of a region suitable for the question under investigation (Kvist). Mitochondrial DNA is also ideal for identification of species because it can be used in degraded tissue samples as MtDNA is present in the cell in a much higher copy number (=1000) than nuclear DNA, and short fragments, ranging from 200-250 base pairs, can be used (Teletchea et al., 2008). MtDNA is a sensitive indicator of population level processes, and analysis of its divergence can reveal geographic clusters of related individuals or matrilineal genealogies and can even be used to trace historical events such as bottlenecks or hybrid zones (Kvist). MtDNA can also divulge phylogenetic relationships between two closely related taxa (Kvist).

Animal mitochondrial DNA is composed of about thirty-seven genes and is a small circular molecule consisting of 15-20 kilobase pairs (Kvist, 2000). Arranged very efficiently, the mitochondrial genome lacks introns, has small intergenic spacers, and

even has occasionally overlapping reading frames (Kvist). Twenty-two of the thirty-seven genes in the mitochondria code for transfer RNAs, there are two ribosomal RNAs, and thirteen messenger RNAs coding for proteins involved in the electron transport chain and oxidative phosphorylation in the mitochondria (Kvist, 2000). MtDNA is made up of a light strand and a heavy strand, the heavy strand being differentiated by a higher proportion of thymine and guanine residues giving it a larger mass (Lu et al, 2007). The heavy strands codes for the two rRNAs and all the structural proteins except one. The mtDNA also contains a non-coding region called the control region that carries the light and heavy strand promoters, in addition to the displacement loop and replication elements (Lu et al.).

Mitochondrial genes are inherited by a non-mendelian mechanism called cytoplasmic inheritance (Alberts et al., 2008). Cytoplasmic inheritance is the passage of traits, such as mitochondrial genes, to daughter cells through the division of the cytoplasm in the parent cells (Alberts et al.). In lower organisms, such as yeast, both haploid cells are equal in size and donate equal amounts of mitochondrial DNA to the zygote, but in higher animals the egg cell is much larger than the sperm and contributes much more cytoplasm to the zygote (Alberts et al.). MtDNA rarely recombines, though some recombination events have been recorded (Kvist, 2000). This type of inheritance results in each molecule of mitochondrial DNA being comprised of a single genealogical history though the maternal lineages (Kvist, 2000), which means a cross between animals results in the offspring only inheriting mitochondrial DNA from the female (Alberts et al.).

Cytochrome b

The Mitochondrial gene cytochrome *b* is one of the most extensively sequenced genes among vertebrates making it useful for comparative studies (Johns & Avise, 1998). The evolution dynamics of this gene and the biochemistry of its protein product are also better characterized that most of the other molecular systems (Johns & Avise). Furthermore, the level of genetic divergence typically associated with sister species, congeners, and confamilial genera are in a range at which the cytochrome *b* gene is phylogenetically informative and unlikely to be compromised by saturation effects from superimposed nucleotides substitutions (Johns & Advise) making this mitochondrial gene one of the most suitable markers for vertebrate identification (Telechea et al., 2008). The cytochromes are a family of colored proteins that are related by the presence of a bound *heme group* and carry one electron at a time through the inner membrane of the mitochondria (Aberts et al., 2008). Cytochrome *b* contains eight transmembrane helices which are connected by extramembrane or intramembrane domains (Kvist, 2000).

Even though this gene evolves slowly in terms of non-synonymous substitutions which cause different amino acids to be coded in the protein, cytochrome *b* gene is excellent for phylogenetic work because the rate of evolutions in the silent positions which do not change the amino acid sequence of the translated protein is relatively fast (Kvist, 2000). The cytochrome *b* gene also contains large regions of interspecies sequence diversity with little or no intraspecific variation, as well as several regions that are conserved, allowing for short fragments along its entire length to be amplified using *trans*-vertebrate primers (Telechea et al., 2008). This is extremely helpful when dealing with tissue that has been degraded. Cytochrome *b* is thought to be conserved enough to

clarify deeper phylogenetic relationships while still being variable enough for population level dynamics (Kvist). The range at which cytochrome *b* is phylogenetically informative encompasses the levels of genetic divergence typically associated with confamiliar genera, congeners, and sister species because the gene is not likely to be severely compromised by saturation effects involving superimposed nucleotide substitutions (Johns and Avise, 1998). Some parts of the gene are more conserved than others as a result of functional limitations, resulting in evolutionary constraints but the variable regions of the gene evolve at a high enough rates to cause detectable divergence (Kvist). The coding regions of transmembrane domains and the amino- and carboxy-terminal ends appear to be the most variable positions (Kvist).

Cytochrome *b* has gained the status of a universal metric because of its world-wide use, allowing for easy comparison of studies conducted at differing times and locations (Kvist, 2000). Differences in nucleotide sequences reflect varying amounts of divergence depending on the phyla being analyzed. For example, when comparing the mean genetic distances between species in the same genus for each of the five major vertebrate classes, fish, reptiles, and amphibians show larger variances between species than do birds (Johns & Avise, 1998). The values of mammals lie intermediate to the two extremes (Johns & Avise). Amphibians and reptiles also have significantly larger genetic distances between genera within a family than do birds and mammals at that same taxonomic rank (Johns and Avise).

Statistical analysis of frequency distributions in cytochrome *b* of sister species, or monophyletic pairs, among vertebrate classes reveals a tendency for larger genetic distances between sister species of mammals than for those of birds or fish (Johns &

Avise, 1998). The genetic distances between taxa increase as the taxonomic rank increases; there are larger distances for confamilial genera than for congeneric species, and for congeneric species than for sister species (Johns & Avise). This is to be expected if the cytochrome *b* genetic distances is not overly truncated by saturation effects at these levels (Johns & Advise). These differences in genetic variation between classes must be taken into account when interpreting cytochrome *b* data in order to arrive at accurate conclusions.

The frequencies for the nucleotide bases adenine (A), guanine (G), cytosine (C), and thymine (T) in cytochrome *b* are similar among mammals, birds, reptiles, amphibians, and fish, although birds show a higher percentage of cytosine than the other groups at all sites, and reptiles show higher frequencies of guanine in third-codon positions (Johns & Avise, 1998). The genetic similarities expected in randomized sequences of a given base composition are equal for the five vertebrate classes which suggests that the effects of saturation dynamics are not largely different (John & Avise). Strong biases in the bases composition of a class could theoretically lead to faster saturation and thus give lower percentages of sequence divergence for a given period of time, but the amount of base compositional differences observed in the vertebrate families does not suggest that this is the case (Johns & Avise).

Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) has arguably been one of the most important developments for advancing genomic sequencing. This revolutionary method was developed by Kary Mullis in 1983 and is a highly sensitive method of amplifying minute quantities of specific DNA (McPherson & Møller, 2000). Before the invention of

PCR, the amplification of specific DNA segments was accomplished by inserting the target DNA into a vector to be expressed by bacteria, a process that required weeks. Now PCR can make millions of copies of a sequence in a matter of hours. PCR is accomplished in three steps: denaturation, annealing, and elongation.

In the denaturation step, the reaction mixture containing the DNA to be copied is heated to 94 degrees Celsius. At this temperature the DNA double helix melts separating the two strands. The annealing step follows denaturation and primers pair up with corresponding segments of DNA at a temperature around 54ºC (McPherson & Møller, 2000). Primers are short segments of single-stranded nucleic acids that correspond to segments of known sequence on the opposite strands of the template DNA and the 3' end of each primer points towards the other primer. They bind to the template DNA strand to give the DNA polymerase the starting point it requires to initiate synthesis. The annealing temperature can vary depending on the base content of the DNA. DNA that is G-C rich is more difficult to pull apart because of the greater stability between the base pair due to three hydrogen bonds between the two bases, as compared an A-T base pair which only has two hydrogen bonds. Following the annealing step is the elongation step in which DNA polymerase attaches at the primers and copies the DNA template in both directions using the bases adenosine, guanine, cytosine, and thymine present in the PCR reaction mixture. These three steps can be repeated 25-40 times depending on the specific purpose for the amplified DNA. PCR uses Taq polymerase, which works optimally at approximately 72ºC because it is from *Therumus auquaticus,* a bacterium native to hot springs (McPherson & Møller).

With each cycle of PCR, the number of DNA copies is doubled, resulting in an exponential increase of the target sequence DNA (Figure 1) (McPherson & Møller, 2000).The specificity of the primers allows PCR to replicate DNA that is not pure or concentrated, and that may even be degraded, allowing for numerous uses in research, species identification, population ecology, the food industry, and forensics.

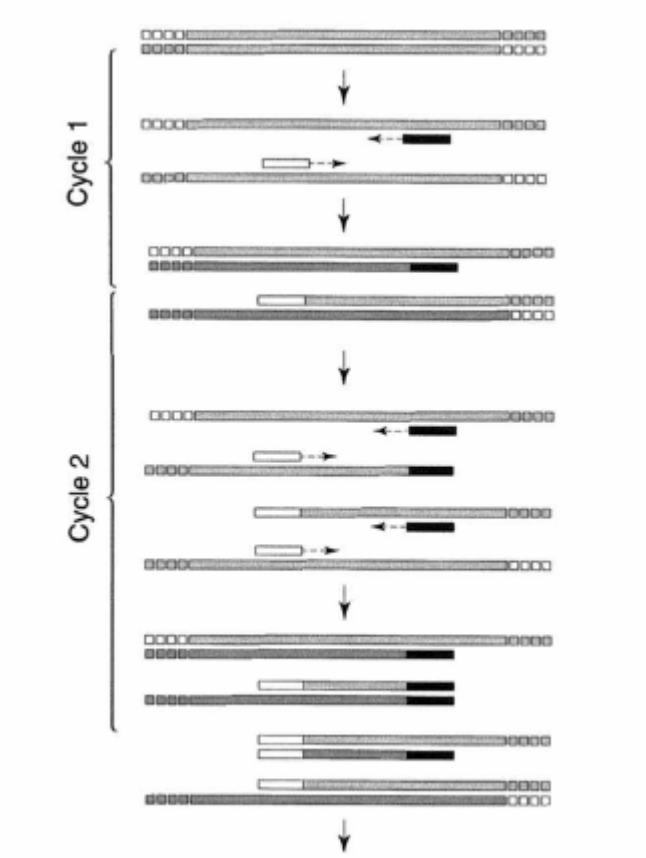

Figure 1. Exponential amplification of a single template molecule of DNA during PCR. Each cycle consists of a denaturation, annealing, and elongation stage (McPherson & Møller, 2000).

The polymerase reaction mixture consists of six components; DNA, primers, Taq Polymerase, dNTPs, Magnesium Chloride, and Potassium Chloride. These ingredients must be used in specific proportions for optimal yield of product, and these proportions must also be adjusted depending on the characteristics of the specific DNA to be

sequenced. As a general rule, low concentrations of primer, target DNA, Taq and nucleotides are recommended in order to produce a cleaner product and lower background (Altshuler, 2006).

There are two types of DNA in a PCR reaction; the target sequence to be amplified and the non-target DNA, which is also known as "burden" DNA. It is possible to have too much total DNA in the reaction, which causes poor DNA synthesis because the Taq polymerase is obstructed by the densely packed DNA. In addition, the concentration of the target DNA should be balanced with the number of cycles in the reaction. An elevated concentration of target DNA with a normal to high number of cycles may cause the rapid accumulation of nonspecific products. When the total amount of the DNA in the PCR reaction is extremely small, there is a higher likelihood of loss due to chemical or enzymatic degradation, and a small amount of target DNA also has an increased risk from contamination from impurities that may come into contact with the DNA solution. The DNA diluents, dust in the air, exhalations or skin particles carry DNA and DNA-degrading substances such as nucleases. Nucleases are a major source of DNA degradation and are ubiquitous, as they are abundant on the human skin and can be found everywhere else as well (Altshuler, 2006).

The concentration of dNTPs should range from 200 µM to 400M each, as excessive dNTPs and suboptimal concentrations will cause premature termination of DNA synthesis or incomplete primer elongation. Primers should be present in concentrations between 0.1 µM and 1.0 µM. Higher concentrations of primers lead to the formation of primer-dimers if the primers are capable of forming dimers, or if the primers do not form dimers then non-specific primer binding may occur and spurious, non target PCR

products will be created. The only exception is when amplifying a short sequence of DNA. When the target sequence is short, such as 100 base pairs in length, a larger number of PCR product molecules are necessary to provide the target yield of amplified DNA (Altshuler, 2006).

Optimal Taq concentration is 1 unit of Taq enzyme per 25 µl of reaction. Too little Taq may result in incomplete primer elongation or premature termination of PCR product synthesis, and excessive Taq can produce a high amount of unwanted background DNA fragments that will cause a smear on gel, and an enormous excess will can the reaction to fail completely (Altshuler, 2006).

Magnesium in the PCR mixture works as a cofactor to stabilize the double stranded DNA and thus raises the melting temperature which is required for thermostable DNA polymerases. The concentration of the magnesium ions controls the specificity of the reaction because a suboptimal concentration results in a low yield of PCR product and excess ions increase non-specific products and misincorporations which appear as smears or ladders on gels. The concentration of $MgCl_2$ should usually be between 1 mM and 4 mM in the reaction mixture, although changes in dNTP and KCl-based buffer concentrations, or other mixture components may require a slight adjustment (Altshuler, 2006). The concentration of potassium chloride is usually 50 mM for the reaction mixture, although a concentration of 70-100 mM is sometimes recommended for target DNA in the range of 100-1000 bp. Generally, the amplification of longer sequences is more efficient at a lower salt concentration, and the amplification of short products works better at higher concentrations (Altshuler).

Primers

Primer sequence may be the most critical factor in successful amplification. Primers should range between 18 and 30 nucleotides long, with the optimal length being 20-25 nucleotides (McPherson & Møller, 2000). In addition, the G-C content of the primer should be 40-60% and the optimal melting temperature between 55ºC and 75 ºC. Primers that are self-complementary and can form more than four consecutive bonds with itself or eight bonds total are problematic primers because they can self-hybridize and form dimers. Also, primers that are A-T rich at the 3' end tend to be more specific in action (McPherson & Møller). Usually a pair of primers is chosen that amplifies a target sequence in opposite directions with the target sequence in between the two primer sites. The direction the primers amplify is often designated either "H" for heavy strand or "L" light strand. Heavy strand primers amplify in the opposite direction of light strand primers (Jackman, Applebaum, & Wake, 1997).

Highly conserved primer sequences are beneficial because they can amplify conserved sequence sites among diverse taxa, although primers for highly conserved regions also have the potential for amplifying DNA from contaminants rather than the target DNA (Goebel, Donnelly, & Atz, 1999). Primers do not work on some taxa because of gene rearrangements and some DNA regions of interest do not have conserved segments of a size appropriate for primers (Goebel et al.). The utility of primers for use in a taxon, such as amphibians, can be estimated by comparing the sequence data from other species within that taxa, as well as within more distant taxa (Goebel et al.). There are many conserved primer sites among both amphibians and distant vertebrates such as humans or mice (Goebel et al.). Utility can also be estimated by identifying all taxa in which primers are presently successful (Goebel et al.). The primer with the greatest utility

will be one that is useful within many taxa which are contained in the smallest, but still most inclusive phylogenetic rank within which primers have been useful to the present (Goebel et al., 1999).

Maps of all published primers are useful for identifying new combinations of primers originally mapped in alternate sources (Goebel et al., 1999). Cytochrome *b* is 5' to the control region in all amphibians whose mitochondrial gene arrangement is known (Goebel et al.) and is amplified by the primers Cyt-b2 and MVZ-16 in a wide variety of vertebrates (Jackman et al., 1997). Primers MVZ-15, -18, and -25 were designed to match sequences from *Ensatina* but also amplify DNA in other amphibians (Jackman et al.). Other primers that amplify cytochrome *b* in amphibians include, but are not limited to DB1-L, CB2-H, CB3-H, CB1Xen-L, CB2Xen-H, CB3Xen-H, CytbAR-H, CytbA-L, ControlW-H, and Ctyb18R-L (Figure 2) (Goebel et al., 1999).

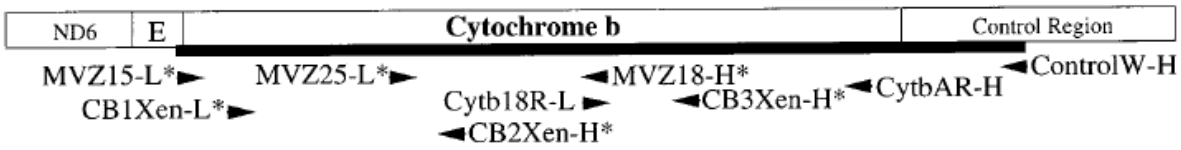

Figure 2. Published primers for the Cytochrome *b* gene (Goebel, Donnelly, & Atz, 1999).

The primers used in this study of *Eurycea*, MVZ 15 (5'-GAACTAATGGCCCACACW WTACGNAA-3') and MVZ 16 (5'- AAATAGGAART ATCAVTCTGGTTTRAT-3') (Mayhew, 2008), were designed by Moritz, Schneider, and Wake (1992) to evaluate the evolutionary relationships within the Salamanders in the *Ensatina eschscholtizii* complex. These primers amplify up to 784 base pairs of

cytochrome *b* as a single fragment (Jockusch & Wake, 2002) and have been used with success in numerous studies of many taxa within the family *Plethodontidae* such as *Bolitoglossa*, *Batrachoseps*, *Hydromanaates*, and *Ensatina* (Jackman Applebaum, & Wake, 1997). This made MVZ-15 and -16 an ideal choice for the amplification of cytochrome *b* in *Eurycea*, which are also in the subfamily *Plethodontidae*.

Current Research Using Cytochrome b Sequencing

Cytochrome *b* sequencing is currently being used for species identification in three species of *Eurycea* discovered in an abandoned mine shaft near Riverville, Amherst County, Virginia in 1999. The three species of salamanders were Cave Salamanders (*E. lucifuga*), Long-tailed Salamanders (*E. longicauda longicauda*), and Three-lined Salamanders (*E. guttolineata*). The discovery of the Long-tailed Salamander and Cave Salamander in this region extended the distribution of these species 60 km from the usual Ridge and Valley and Blue Ridge Mountain physiographic region into the Piedmont physiographic region (Reichenbach, LeMon, & Hinson, 2006). In addition, this was that first syntopic occurrence in Virginia of Long-tailed and Three-lined Salamanders, a species usually indigenous to the Piedmont physiographic region (Reichenbach et al.).

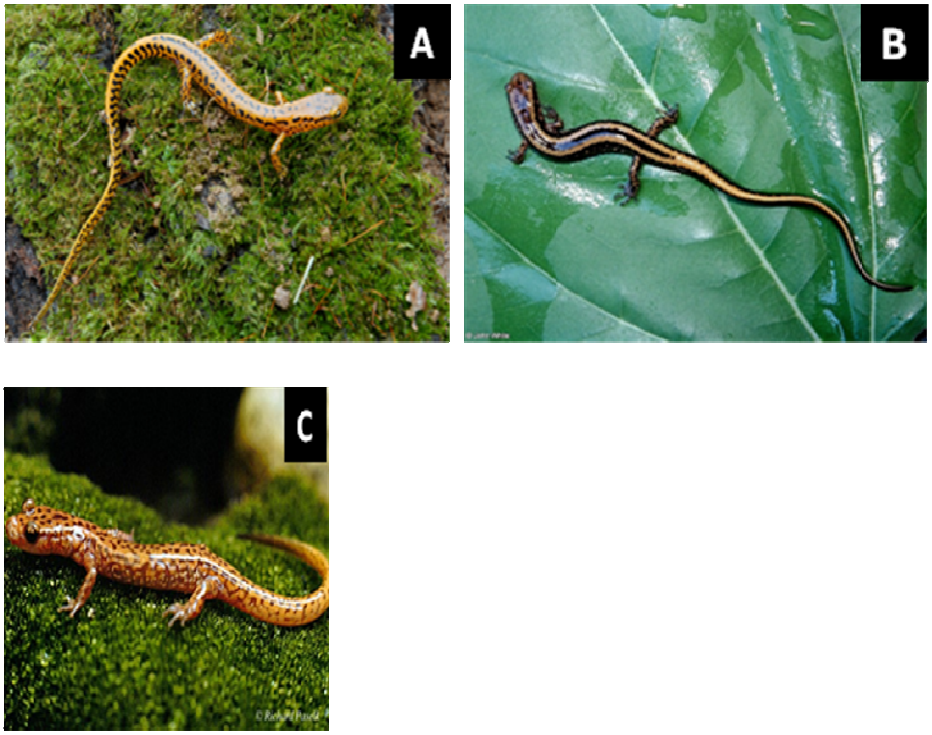

Figure 3. (A) – *Eurycea l. longicauda* (Long-Tailed Salamander); (B) *Eurycea guttolineata* (Three-lined Salamander); (C) *Eurycea lucifuga* (Cave Salamander)

Eurycea longicauda (Figure 3: A) is a slender yellow to orange/red species with many round black spots on the back and sides and vertical dark bars on the sides of the tail (Martof, Palmer, Bailey, & Harrison III, 1980). It is the only yellowish salamander with vertical black markings on the tail, which can frequently vary from the herringbone or "dumbbell" theme (Conant, Stebbins, & Collins, 1992). The ventral surface is unmarked and the tail is often two-thirds of the total length in large adults, from which the name of the species is derived (Martof el al.). The young are yellow and the tail is relatively short. The range of this species extends from the eastern bank of the Mississippi from Louisiana to the north reaching through all of Mississippi and Tennessee on into Kentucky. The range also extends eastward to Georgia, South Carolina, western and central North Carolina and eastern Virginia (Lannoo, 2005). In

Virginia, the Long-tailed salamander is found West of the Blue Ridge; and, in North Carolina, this species is known in the Watauga, Nantahala and Little Tennessee river basins (Martof et al.). *Eurycea longicauda* can be found along rocky streams and is usually associated with limestone and shale substrates, in addition to commonly inhabiting damp caves (Martof et al.). The Long-tailed salamander lays eggs in late fall. Females will deposit an average of ninety eggs in an underground site attached to rocks or logs suspended above or in the water. The eggs hatch during the winter and metamorphosis takes place by early summer (Wilson, 1995).

Eurycea guttolineata, known as the Three-lined salamander, resembles the Long-tailed salamander in size and shape (Figure 3:B) (Martof et al., 1980). In fact, three-lined salamanders were considered a subspecies of the long-tailed salamanders until recently. In 1998, Carlin elevated *E. guttolineata* to full specific status with morphological and genetic data (Carlin, 1997). The three-lined species has a black median stripe with tan stripes on the sides (Martof et al.). The sides of the salamander are dark with a light streak between the limbs (Martof et al.), and the ventral surface is mottled with yellow to greenish gray pigmentation (Conant, Stebbins, & Collins, 1992). This salamander hybridizes with Long-tails in certain areas, including northern Gerogia, Alabama and Mississippi (Martof et al.). The hybrid between the three-lined salamander and the long-tailed salamander has an intermediate morphology of the two salamanders, with three dorsal lines, characteristics of a three-lined salamander, and also a light ventral color and herring-bone marks on the tail, characteristics of long-tail morphology. The range of the Three-lined salamander extends from Virginia to extreme western Tennessee and south to northern Florida (Wilson, 1995). *Eurycea guttolineata*, like the Long-tailed salamander,

also inhabits river and creek bottoms, seepage areas at springs, swamps and the undersides of rocks in small creeks. Three-lined salamanders can also be found substantial distances apart from standing water as long as it is in a damp environment, as would be the case in many caves (Wilson). Breeding for this species occurs in the winter, and eggs are deposited in early spring. The young undergo metamorphosis in four to five months and attain sexual maturity the following summer (Wilson).

The Cave Salamander has coloration which ranges from dull yellow to orange and bright orange-red (Figure 3:C) (Conant, Stebbins, & Collins, 1992). Black spots, which are usually irregularly scattered, can also form 2 to 3 longitudinal rows (Conant et al.). The tail of the cave salamander is long and lacks the vertical markings of the Long-tailed (Martof et al., 1980). The ventral surface is unmarked and ranges from colorless to yellow (Smith, 1978). It also has a much broader head with bulging eyes (Martof et al.). This species range includes extreme western Virginia, to Georgia and Alabama, north to southern Indiana and areas of Illinois, Missouri, Kansas and Oklahoma (Wilson, 1995). Information is sparse concerning the breeding habits of the Cave salamander, although a study in southern Illinois showed they deposited eggs from fall through early December and the larval period is estimated to be six-eighteen months (Ringia & Lips, 2007). The study recorded a peak in number of larvae in November with their numbers gradually falling off as larvae leave the pools into streams until few were left in the pools in June (Ringia & Lips). Females are known to deposit up to sixty eggs on the undersides of rocks in pools or streams, and may lay multiple clutches (Wilson, 1995) (Ringia & Lips).

The larvae of these species, especially the three-lined and Long-tailed salamander are very difficult to distinguish morphologically, and before a certain age are virtually

impossible to classify with certainty. The Cave Salamander larvae have dark pigmentation on the throat and feet as a distinguishing feature, but the other two species are highly difficult to distinguish with certainty before metamorphosis. This is where genomic sequencing discussed previously may prove helpful. Currently cytochrome *b* sequences for the Cave and the Long-tailed are in the online CoreNucleotide gene bank, but none have been submitted for the three-lined salamander.

An ecological study of this abandoned Virginia mine at Riverville was conducted by Norm Reichenbach and the Liberty University ecology class throughout year 2000. Twenty-one trips were taken during this year, with at least one trip per month. Thirty-one total salamanders were found in the mine during the study (Table 1), and they were collected, measured for total length, uniquely marked and released at their capture location with effort made to minimize disturbance to their habitat. Approximately 50% of the thirty-one salamanders were three-lined (fifteen out of thirty-one total salamanders found). There were only six cave salamanders and four long-tailed salamanders observed throughout the year. Two more species of salamanders were also observed living in the mine, including five *Pseudotriton r. ruber* and one *Plethodon cylindraceus.*

Table 1. *Number of salamanders (n = 31) observed during 2000 in an abandoned mine in Amherst County, Virginia* (Reichenbach, 2000).

Species	Number Observed
Eurycea lucifuga	6
Eurycea l. longicauda	4
Eurycea guttolineata	15
Pseudotriton r. ruber	5
Plethodon cylindraceus	1

Reichenbach's study showed the populations of *E. lucifuga* and *E. l. longicauda* appeared to be sparse without recruitment. Field studies showed that the number of salamander larvae peaked during the month of March (Figure 4), after which the number of larvae steadily declined until none were observed in the month of November. Mortality of the larvae could be due to predation by crayfish or the large *P .r. ruber* larvae observed in the cave. Starvation could also be a factor since common prey items for larval *Eurycea* were never observed in the mine pools throughout the year. The total lengths of the salamanders are all within the adult size range for these species and there was a lack of recently metamorphosed individuals. The lack of recruitment was hypothesized to be due to larval mortality as evidenced by the declines in the number of *Eurycea* larvae seen over time. This study cast doubt as to how long the sparse populations of the cave salamander and the long-tailed salamander could continue in the mine without recruitment (Reichenbach, 2000).

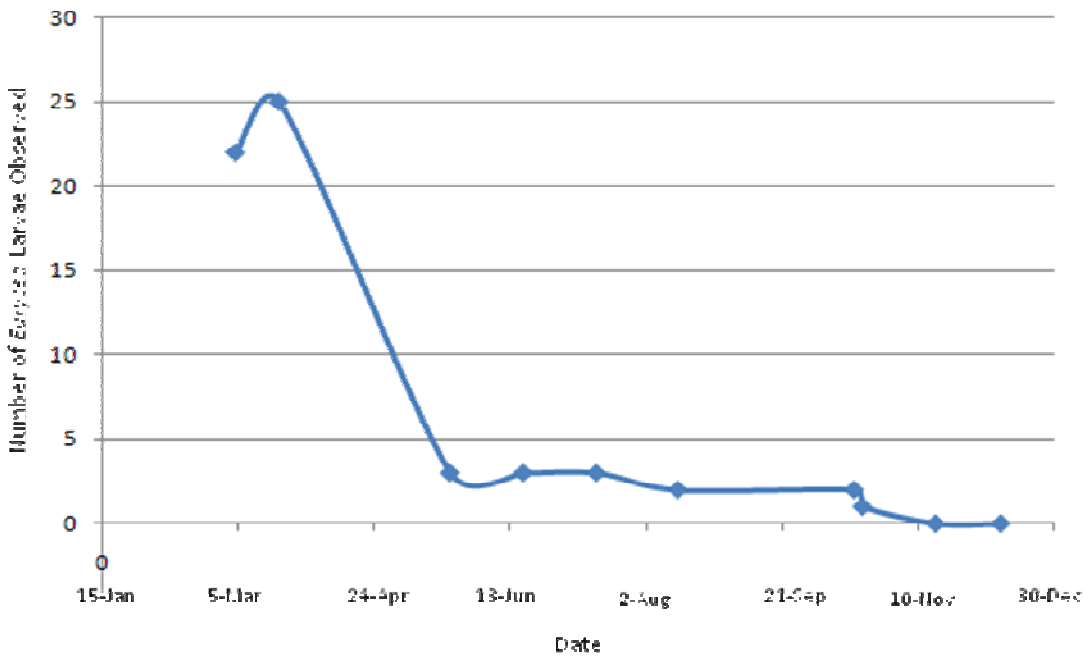

Figure 4. Number of larval Eurycea found between March and December 2000 in an abandoned mine in Amherst County, Virginia. (Borrowed from Reichenbach, 2006)

Thirty-one larvae were collected from the mine during the months of February and March in 2007. Their morphology was characteristic of either a Long-tailed or Three-lined Salamander based on Downs' and Pfingsten's (1989) "Key to the Larval Salamanders of Ohio." The salamanders collected had very sparse pigmentation in the anterior portion of the throat, if any at all, so none of the larvae were classified as Cave Salamanders which are darkly pigmented in this area. Unfortunately only two of the larvae samples collected survived long enough to undergo metamorphosis, dying soon afterwards. One of the salamanders that underwent metamorphosis appeared to be a Three-lined Salamander, and the other a Long-tailed Salamander, although the morphology was not distinct enough to make definitive classification. In 2008, Jonathan Mayhew from Liberty University worked on a Senior Thesis project to optimize a

technique using MtDNA that would enable species identification of larval salamanders collected from the mine. His protocol used Polymerase Chain Reaction to amplify a target region of the cytochrome *b* gene from larval salamanders collected from the mine to compare with gene sequences of reference animals for identification. The cytochrome *b* gene was sequenced from ten of the unknown larvae, along with the two salamanders that survived to metamorphosis and two Three-lined Salamanders from Pittsylvania County used as positive controls.

Mayhew's fourteen samples were processed at the University of Michigan DNA Sequencing Core. Eleven of the twelve unknown salamanders from the mine had similar cytochrome *b* sequences with 99.7% pairwise similarity and 99.0% identical bases and a consensus sequences was determined using Geneious Pro 3.5.6 (Figure 5) (Mayhew, 2008). These included the ten larval salamanders and the salamander that went through metamorphosis, which appeared to be a Long-tailed Salamander.

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1        10        20        30        40        50
|        |         |         |         |         |
TTAGGAATTTGCCTAATCACACAAATCTTAACAGGGTTATTTCTTGCAAT  (1-50)
GCATTATACTGCAGACACTGCCTCCGCATTCTCCTCTGTTGCCCACATTT  (51-100)
GCCGAGACGTTAACTATGGTTGACTTGTGCGCAACATTCATACCAACGGA  (101-150)
GCCTCTATCTTCTTCATTTGTATTTATCTTCATATTGGTCGTGGCCTATA  (151-200)
TTATGGCTCATACATATTTAAAGAAACCTGAAACATTGGGGTTATTCTAC  (201-250)
TATTTTTAGTAATAGCAACAGCATTTGTAGGGTATGTCCTACCATGAGGA  (251-300)
CAAATATCTTTTTGAGGAGCAACCGTTATCACAAATCTATTGTCCGCAAT  (301-350)
TCCATATTTGGGAGACACACTTGTTCAGTGAATTTGAGGCGGCTTCTCAG  (351-400)
TAGACAAAGCAACATTAACCCGATTTTTTGCCTTTCATTTTATTTTACCC  (401-450)
TTTATAATTGCTGGTGCCAGCATCGTCCATCTGCTTTTTCTACATGAAAC  (451-500)
AGGATCAAACAACCCAACAGGACTTAACTCTAACCCAGACAAAATTCCAT  (501-550)
TTCACCCATACTACTCCTATAAAGACTTACTAGGACTCCTGCTTATATTA  (551-600)
TTGTTATTAATTATGATTTCACTCTTAACACCCAACCTTTTAGGAGACCC  (601-650)
AGAAAACTTTACTCCAGCTAATCCACTTATTACACCACCACATATTAAAC  (651-700)
CAGATTGATATTTCCTATTT                                (701-720)
```

Figure 5. Consensus sequence of eleven salamander larvae exhibiting high levels of similarity (99.0%).

This consensus sequence was compared to sequences in the CoreNucleotide database for *E. lucifuga* (Zigler & Harlan, 2006) and *E. l. longicauda* (Bonett & Chippindale, 2004) because no cytochrome *b* sequence is currently available for *E. guttolineata*. A consensus sequence of ten Cave Salamanders from Franklin County, Tennessee listed in CoreNucleotide was determined using Geneious Pro 3.5.6 (Figure 6) and the cave salamander sequences had 99.5% pairwise similarity and 97.9% identical bases (Mayhew, 2008).

```
1        10        20        30        40        50
  |       |         |         |         |         |
  TTAGGAATTTGCCTAATTACACAAATCTTAACAGGGTTATTTCTTGCAAT  (1-50)
  GCATTATACTGCAGACACTGCCTCCGCATTCTCCTCTGTAGCCCACATTT  (51-100)
  GCCGAGACGTTAACTATGGTTGACTTGTGCGCAACATTCATACCAACGGA  (101-150)
  GCCTCTATCTTCTTCATTTGTATTTATCTTCATATTGGTCGCGGCCTATA  (151-200)
  TTATGGCTCATACATATTTAAAGAAACCTGAAACATCGGGGTTATTCTAC  (201-250)
  TATTTTTAGTAATAGCAACAGCATTTGTAGGATATGTCCTACCATGAGGA  (251-300)
  CAAATATCTTTTTGAGGAGCAACCGTTATCACAAATCTATTGTCCGCAAT  (301-350)
  TCCATAYTTGGGAGACACACTTGTTCAATGAATTTGAGGCGGCTTTTCAG  (351-400)
  TAGACAAGGCAACATTAACCCGATTTTTTGCCTTTCATTTTATTTTACCA  (401-450)
  TTTATAATTGCTGGTGCCAGCATCGTCCATCTGCTTTTTCTACATGAAAC  (451-500)
  AGGATCAAACAACCCAACAGGACTTAACTCTAACCCAGACAAAATTCCAT  (501-550)
  TTCACCCATACTACTCATATAAAGACTTACTAGGACTCCTGCTTATATTA  (551-600)
  TTATTACTAATTATGATTTCACTCTTAACACCCAACCTTTTAGGAGACCC  (601-650)
  AGAAAACTTTACTCCAGCTAATCCACTTATTACACCACCACATATTCAAC  (651-700)
  CAGAGTGATACTTCTTATTT                                (701-720)
```

Figure 6. Consensus sequence of ten *E. lucifuga* from Franklin County, Tennessee. (Zigler & Harlan, 2006).

The *E. l. longicauda* only had one sequence available in the database (Figure 7), because the second sequence of 335 nucleotides was too short for comparison.

```
1        10        20        30        40        50
|        |         |         |         |         |
TAGGAATTTGCCTAATCACACAAATCTTAACAGGATTATTTCTTGCAATACAT (1-50)
TACACTGCAGACACTACCTCCGCATTCTCCTCTGTAGCCCACATCTGCCGAGA (51-100)
CGTTAATTATGGTTGACTAGTGCGCCAGATTCATACCAACGGAGCTTCTATAT (101-150)
TCTTTATTTGCATTTATCTTCATATTGGACGAGGCCTATACTATGGCTCATAC (151-200)
ATGTTCAAAGAAACCTGAAACATTGGAGTTATTTTACTATTTTTAGTGATAGC (201-250)
AACAGCATTTGTAGGATATGTTCTCCCATGAGGACAAATATCTTTCTGAGGAG (251-300)
CAACCGTCATCACAAACCTATTATCCGCAATCCCATATTTAGGAGACACACTT (301-350)
GTTCAATGAATTTGAGGTGGCTTCTCAGTGGATAAAGCAACACTAACCCGATT (351-400)
TTTTGCCTTTCATTTTATTCTACCATTTATAATTGCTGGCGCCAGCATTGTTC (401-450)
ATCTACTTTTCCTTCACGAAACAGGATCAAACAACCCAACAGGACTTAACTCT (451-500)
AACCCAGATAAAATCCCATTTCATCCATACTATTCTTATAAAGATCTACTAGG (501-550)
ACTCCTGATTATGCTACTTCTGTTAATCACTATTTCACTCTTAACACCAAACC (551-600)
TACTAGGAGATCCAGAGAACTTTACCCCAGCCAATCCACTAATTACACCACCA (601-650)
CATATTCAACCGGAGTGATATTTCTTATTT (651-680)
```

Figure 7. E. l. longicauda sequence from CoreNucleotide Database (Bonett & Chippindale, 2004).

Unexpectedly the larval sequences from the mine corresponded more closely with the cytochrome *b* consensus sequence of the Cave Salamanders than the Long-tailed Salamander sequence in the online CoreNucleotide. The Cave Salamander consensus sequence and the larval consensus sequence exhibited 97.6% pairwise similarity and 97.6% identical bases. The Long-tail sequence and the larval consensus sequence only exhibited 89.7% pairwise similarity and 89.6% identical bases (Mayhew, 2008). It was surprising that all sequences of the larvae more closely identified as Cave Salamanders because none of the larvae displayed dark pigmentation on the throat and feet, which are distinct characteristics typical of Cave Salamander morphology.

The salamander from the mine that survived to metamorphosis appearing to be a three-lined salamander (Figure 8) exhibited 90.0% pairwise similarity and 90.3% identical bases with the Cave Salamander consensus sequence, and 96.9% pairwise similarity and 96.9% identical bases with the Long-tailed salamander sequence from the

online Data Base (Mayhew, 2008). This sequence only exhibited 88.9% pairwise similiarity and 73.8 % identical bases with the consensus sequence from the larvae from the mine. Currently, there are no cytochrome *b* sequences in online databases for *E. guttolineata* for comparison, but this salamander has a high probability of being a Three-lined salamander based on morphology and because the gene sequences are expected to be similar for closely related species like *E. guttolineata* and *E.l.longicauda*. The sequences from the two Three-lined positive controls from Pittsylvania County were ambiguous because they showed no similarity to the cytochrome *b* gene of *Eurycea* salamanders BLAST (nucleotide collection database). This could be due to amplification of the wrong PCR product, or to the incorrect gene product insertion into the plasmid sent into the University of Michigan DNA Sequencing Facility. The restriction digest with EcoRI on a 1.0% agarose gel showed that these two samples (JM-3L+1 and JM-3L+2B) had slightly different plasmid inserts than the rest of the samples, a possible indicator that the wrong gene product was inserted (Figure 9).

```
1        10        20        30        40        50
  |       |         |         |         |         |
  GAACTAATGGCCCACACTTTACGTAAGACTCACCCTATACTTAAGATTAT  (1-50)
  TAATAACTCCTTTATTGATCTCCCCGCCCCATCAAGCTTATCCTACCTAT  (51-100)
  GAAACTTTGGATCCCTCTTAGGAATTTGCCTAATCACACAAATCTTAACA  (101-150)
  GGATTATTTCTTGCAATACATTACACTGCAGACACTACCTCCGCATTCTC  (151-200)
  CTCTGTAGCCCATATCTGCCGAGACGTTAATTATGGTTGACTAGTGCGCA  (201-250)
  GCATTCATACTAATGGAGCTTCTATATTCTTTATTTGCATTTATCTTCAT  (250-300)
  ATTGGACGAGGCTTATACTATGGCTCATACATGTTCAAAGAAACCTGAAA  (301-350)
  CATTGGAGTTATTCTACTATTTTTAGCGATAGCAACAGCATTTGTAGGAT  (351-400)
  ATGTTCTCCCATGAGGACAAATATCTTTCTGAGGAGCAACCGTCATCACA  (401-450)
  AACCTATTATCCGCAATCCCATATTTAGGAGACACACTTGTTCAATGAAT  (451-500)
  TTGAGGTGGCTTCTCAGTGGATAAAGCAACACTGACCCGATTTTTTGCCT  (501-550)
  TTCATTTTATTCTACCATTTATAATTGCTGGCGCCAGCATTGTTCATCTA  (551-600)
  CTTTTCCTCCACGAAACAGGATCAAACAACCCAACAGGACTTAACTCTAA  (601-650)
  CCCAGATAAAATCCCATTTCATCCATATTATTCTTATAAAGATCTACTAG  (651-700)
  GACTCCTGATCATGCTACTTCTATTAATCACTATTTCACTCTTAACACCA  (701-750)
  AACCTACTGGGAGATCCAGAGAACTTTACCCCAGCCAATCCACTAATTAC  (751-800)
  ACCGCCACATAT                                        (801-812)
```

Figure 8. The cytochrome *b* sequence of the salamander that survived to metamorphosis appearing to be a Three-lined Salamander (Mayhew, 2008)

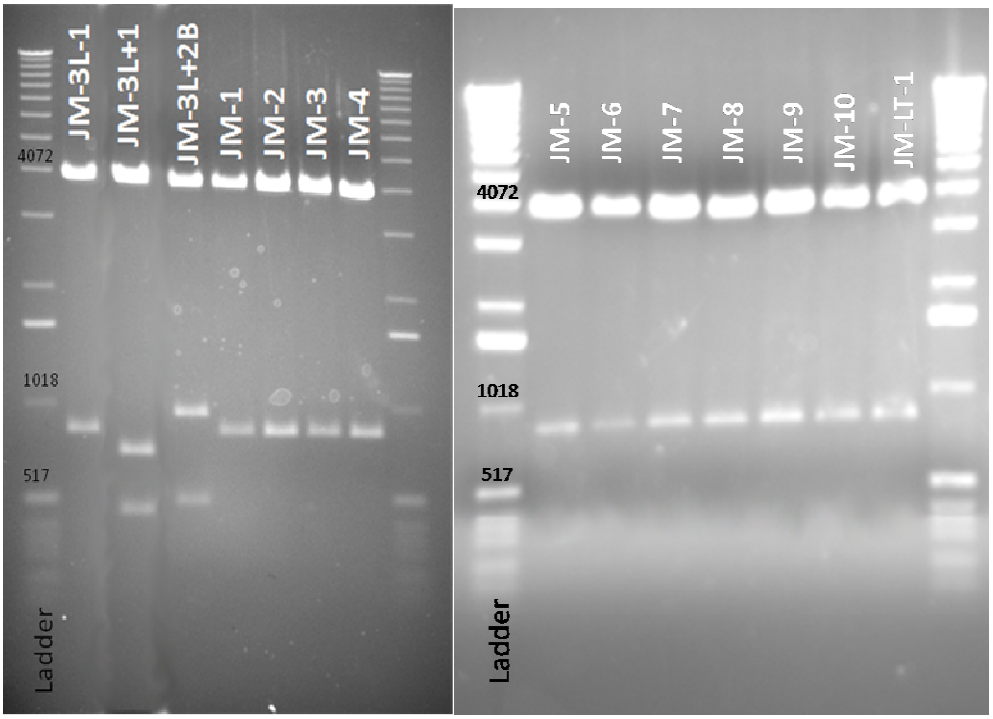

Figure 9. Restriction digest with EcoRI on a 1.0% agarose gel. (Mayhew, 2008)

Materials and Methods

Frozen larval tissue samples from the mine were collected May 14, 2008 and were stored at -20ºC. 20-25 mg of tissue samples were purified using DNeasy® Blood and Tissue Kit (50) according to protocol. The last elution step with 200 ul buffer AE was performed twice for maximum yield as recommended in the protocol. The cytochrome b gene was then amplified in the purified DNA using the polymerase chain reaction using the primers MVZ 15 (5'- GAAC TAATGGCCCACACWWTACGNAA-3') and MVZ16 (5'- AAATAGGAART ATCAV TCTGGTTTRAT-3') designed to amplify a region approximately 800 nucleotides long in the cytochrome *b* gene of the salamander *Taricha tarosa* (Moritz, Schneider & Wake, 1992). The PCR reaction mixture consisted of 0.5 ul of purified DNA, 1 ul dNTPs, 1.6 ul primer MVZ 15 and 1.6 ul primer MVZ 16, 1.5 ul $MgCl_2$, 5 ul of 10X PCR buffer and 36.3 ul ultrapure Distilled Water (Gibco) and 0.5 ul Taq Polymerase for a final volume of 50 ul for each sample. The specifications for the polymerase chain reaction for these samples was 38 cycles of denaturation for 1 minute at 94 ^{o}C, annealing for 1 minute at 50^{o}C, and elongation for 1 minute at 72 ^{o}C using a PTC-100 Peltier Thermal Cycler.

Gel Electrophoresis was used to verify the presence the PCR products. 10 ul aliquots of amplified DNA was electrophoresed in 1.0% agarose (1 X TAE) gel stained with Ethidium Bromide (EtBr) in TAE buffer (0.04 M Tris base, 0.02 M glacial acetic acid, 0.001 M EDTA, pH 8.0) for two hours at 75 V. The DNA band in the gel were then assessed under UV light using EpiChem^3 Darkroom (UVP BioImaging Systems) to verify the presence of the 800 bp fragment indicating successful amplification of the Cytochrome *b* gene.

TA cloning was used to clone the amplified gene product using the TOPO TA clonging® kit according to protocol. Individual colonies of transformed cells were selected and the plasmids were purified using QIAprep® Spin Miniprep Kit according to protocol. The purity of the DNA was spectroscopically analyzed using the absorbance ratio of the sample at 260 nm and 280 nm and the presence of the plasmid was accessed with electorphoresis using 1.0% agarose (1 X TAE) gel stained with Ethidium Bromide (EtBr) in TAE buffer (0.04 M Tris base, 0.02 M acetic acid, 0.001 M EDTA, pH 8.0) for two hours at 75 V and visualized under UV light.

The purified plasmid DNA from the samples was then digested with EcoR1 to verify the subcloning of the cytochrome *b* gene segment into the vector. EcoR1 is a restriction endonuclease that cleaves the vector on either side of the location of the insert. Gel electrophoresis was confirmed the presence of a ~800 bp fragment, which indicated the insertion of the correct DNA. The samples were then sent to the University of Michigan DNA Sequencing Facility for Sequencing and analyzed using BLAST.

Discussion

The identification of the larval species from the mine was not definitive due in part to a lack of a comparison sequence available for the Three-lined salamander. Figure 10 below illustrates a diagram showing the sequence similarities between the species under study based on available data. A Three-lined positive control sequence could reveal that the larvae from the mine are more similar to a Three-lined salamander than a cave salamander, although this is not likely due to the close taxonomic rank between *E. longicauda* and *E. guttolineata*. A Three-lined positive control would also be able to verify the identification of the Three-lined metamorph from the mine. Furthermore, there

was only one Long-tail sequence available for comparison with the larvae on the online CoreNucleotide database, thus providing a very limited variety for assessment. All three species of salamanders examined here have highly similar DNA because they are from the same genus, *Eurycea*, so even slight differences will be important for indentification. More accurate and definitive identification will require more positive controls for comparison, and a larger sampling of specimens from the cave.

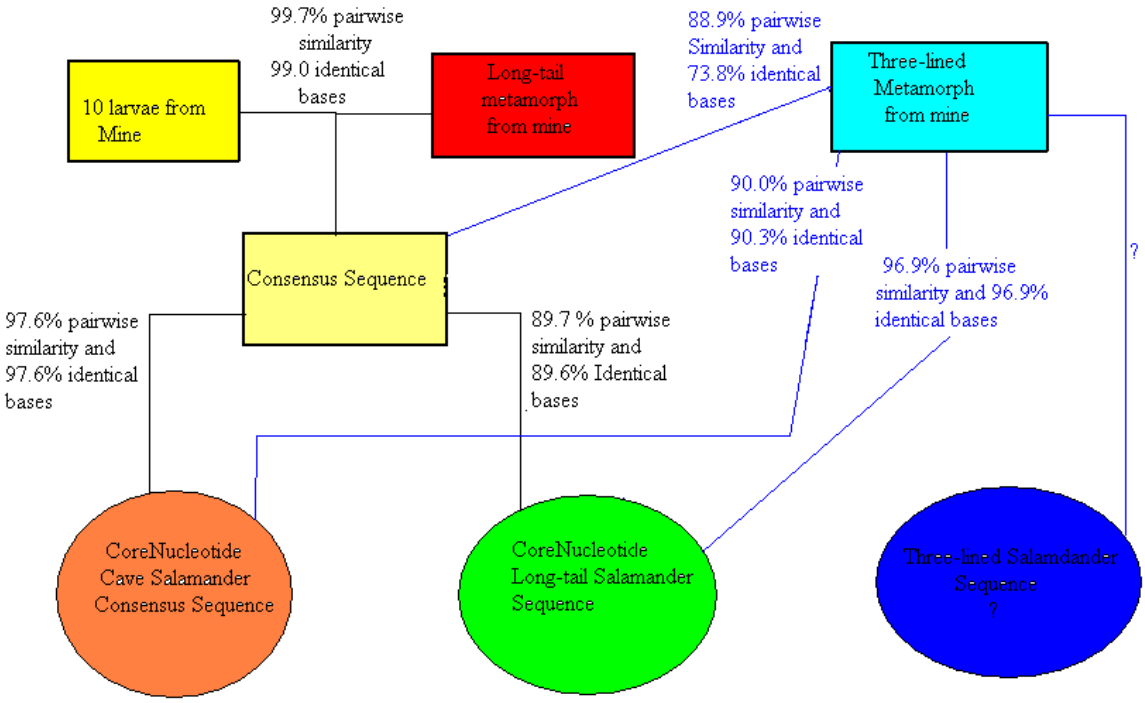

Figure 10. Diagram showing Sequence similarities from available data.

Currently samples of Three-lined and Long-tailed salamanders have been obtained from Pulaski County, Virginia, and Breaks Interstate Pack, Virginia. These samples should be sequenced and compared to the salamander sequences from the Amherst County mine. There will be differences in the sequences due to geographic

separation of the populations but comparison could still prove to be beneficial for identification. Additionally, there are four additional specimens from the mine, two of which are thought to be Long-tailed Salamanders and one a Three-lined salamander, based on morphology. The salamanders are juveniles, so the identifications based on morphology are not definitive. The fourth specimen was obtained from the mine is an adult Three-lined salamander and its morphology clearly revealed three-lined characteristics. These should be sequenced in addition to six larvae salamanders of unknown species collected from the mine in May of 2008. This data could prove to be highly beneficial in the species identification of this population of *Eurycea* in the mine.

Conclusion

Genetic sequencing can be a highly useful tool for the purpose of species identification. Only a small tissue sample is needed to perform the analysis as a result of the amplification technique of PCR, and in some cases the tissue can be degraded or even from a fossil. Sequencing allows for the comparison of individuals at the genomic level, where mutations not resulting in morphological differences are made apparent, thus more detailed knowledge of the true divergences of species is made available.

Although genetic sequencing is a useful tool, many problems can arise when attempting to carry out the sequencing process. A region of a gene must be selected to analyze and compare that will have mutations at a rate that will be phylogenetically informative. As discussed above, the mitochondrial genome is often a prime choice, although genetic material in many species is only passed down though the maternal lineage. After an applicable gene is selected, primers must be developed that will efficiently and correctly amply the chosen gene. This can be difficult because a primer

sequence must be chosen that will be conserved enough to amplify the variety of targeted individuals, but that will not also amplify contaminates in the PCR reaction. The primers must also have the correct C-G content, and lack the ability for self-hybridization and the formation of dimers. Also the PCR reaction must be optimized, with variable concentrations of Taq Polymerase, Primers, Target DNA, dNTPs, Magnesium Chloride, and potassium chloride so that a minimum amount of background DNA is produced with maximum yield of target DNA. Often containments can be a problematic when attempting to amplify and specific segment.

Despite the difficulties, the value of the information gained from genomic sequencing is clear. Genetic markers can be highly informative, not only for correct species identification but also concerning the population dynamics, the phylogeny, phylogeography, and matrilineal ancestry of a species. Furthermore, correct species identification of larvae is an important indicator of population vitality, as the presence of larvae denotes successful reproduction (Parmelee, Knutson, & Lyon, 2002). Information gained from this research can be used to better understand complex population dynamics like those of the *Eurycea* in the Riverville mine.

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