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ANALYSIS OF *EURYCEA* HYBRID ZONE
IN EASTERN MISSOURI

by

BONNIE JEAN BEASLEY

A THESIS

Presented to the Faculty of the Graduate School of the
MISSOURI UNIVERSITY OF SCIENCE AND TECHNOLOGY

In Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE IN APPLIED AND ENVIRONMENTAL BIOLOGY

2010

Approved by

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ABSTRACT

Evolutionary mechanisms are often difficult to observe in action because evolution generally works slowly over time. Hybrid zones provide a unique opportunity to observe many evolutionary processes, such as reinforcement, because of the rapid changes that tend to occur in these zones. Salamanders provide an ideal model for examining the rapid changes in populations that result from hybridization because many closely-related species lack reproductive barriers. In Missouri, a well-documented hybridization zone exists among the two subspecies *Eurycea longicauda longicauda* (long-tailed salamander) and *E. l. melanopleura* (dark-sided salamander). These salamanders inhabit caves, limestone creek beds, and abandoned mine shafts. A closely related species, *Eurycea lucifuga* (red cave salamander) also inhabits caves and mine shafts. A recent study found that *E. lucifuga* and *E. longicauda ssp.* were likely hybridizing in the Onondaga Cave system. In this study, samples were collected from three Missouri caves with the *E. longicauda ssp.* hybrid zone. Morphological analysis demonstrated significant differences in the morphology of each species and genetic analysis presented evidence of potential hybridization among these species. Because of the apparent differing degrees of hybridization occurring among the *Eurycea* species, this hybrid zone could offer a valuable natural laboratory to investigate the mechanisms of reinforcement.

ACKNOWLEDGMENTS

First and foremost, I would like to thank my advisor, Dr. Anne Maglia for her continued confidence and support throughout this project. I am extremely fortunate to have had the opportunity to work with such an incredibly kind and inspiring person. I would also like to thank my other committee members, Dr. Melanie Mormile and Dr. Jennifer Leopold. Both of them had an uncanny ability to say just the right thing at just the right time to help me manage days I was certain I was not going to survive. I would like to thank Maria Potter for unending assistance throughout this project. Adam Martin and Vanessa Kaighin of the MS&T cDNA lab are owed more than I can ever attempt to repay. My knowledge of how to get around a DNA lab was very limited, and they spent about three months teaching me tricks of the trade. I thank Pat Robinson and Gena Robertson for being such amazing volunteers. I appreciate Maduka Rupasinghe's assistance with morphological statistics. Also, a big thank you goes to the cave owners, Randy Long and Bill Allen, for allowing me into some remarkable caves. I thank the Missouri Department of Natural Resources for granting permission to conduct research in Onondaga Cave State Park, and the Missouri Department of Conservation for wildlife collector's permits to sample the salamanders in such delicate environments. This research was supported through funds from NSF grant DBI 0640053. Finally, I would like to thank my incredible family for encouraging me to follow my heart and go back to school for something I am truly passionate about. I could not have done any of this without you.

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1. INTRODUCTION

A driving force behind many evolutionary studies is the question of how the multitude of species found on this planet came to be. In the not too distant past many believed that all the species on Earth had been here since the beginning of time and those species experienced very little change over their time on Earth. In 1859, Charles Darwin transformed that thought process by introducing the concept of evolution with his book “On the Origin of Species by Means of Natural Selection”. He presented evidence for species developing from common ancestors and suggested the change among descendants was in part due to natural selection. While there was much controversy at the time of publication, Darwin’s theory of “descent with modification” is now widely accepted among biologists. An additional contribution to the study of evolution was Gregor Mendel’s model of inheritance. At the same time Darwin published his work on natural selection, Mendel was in the middle of conducting experiments with pea plants. Mendel’s work demonstrated that individual traits are inherited from parents by discrete units, which now are referred to as genes. The combination of the two theories is known today as the *Modern Synthesis*. Scientists now investigate the many mechanisms and processes involved in evolution such as what modifications or adaptations take place, how the changes take place, and why changes take place. Concepts such as isolation, sexual selection, mutations, reinforcement, sexual recombination and natural selection all have crucial roles in the evolutionary process. The combination of these processes can take millions of years to change a population enough to result in a new species, making it nearly impossible to physically observe the specific mechanisms involved in these

concepts. Fortunately, there are certain circumstances in nature that offer biologists the opportunity to witness evolutionary processes in action. In the last few decades, investigations of hybrid zones have provided such an opportunity. These investigations have fueled discussions on everything from defining evolutionary concepts to determining the mechanisms behind concepts such as reinforcement really work. Summarizing what is known and understood is a necessary first step for discussions in this area.

1.3. BACKGROUND

1.1.1. What Is A Species? What defines a species continues to be discussed by biologists from all fields of study. Because the word *species* is Latin for “appearance”, most likely the historical definition simply referred to different types of animals or plants based on visible differences as different *species*. This concept is workable for distantly related species with obvious differences in appearances. However, confusion arises when two similar species have few differences in appearance but have different life histories such as occupying different geographical regions or not interacting with one another even when sharing the same habitat. This requires a more complex definition to appropriately define what is meant by the term species. Ernst Mayr’s *biological species concept* (BSC) is the most frequently followed definition and describes a species as a group of genetically distinct individuals who may interbreed to produce viable offspring but are incapable of breeding with other populations (Mayr 1942, Coyne and Orr 2004). This definition implies the existence of reproductive barriers that inhibit two distinct species from producing offspring. Because this definition is based upon the ability to reproduce

with another organism, it does not encompass asexual organisms. Other definitions of species include both asexual and sexual organisms. For example, the *morphological species concept* is based on similarities in body size, structure, and shape, categorizing species based on similarities rather than differences (Campbell and Reece 2005). A strong disadvantage to this definition is the subjective nature of the criteria used to define the species. The *ecological species concept* defines a species based on its ecological niche or its function in the community it inhabits (Campbell and Reece 2005). The *paleontological species concept* is used for defining species based solely on fossil records of species because the reproductive qualities of those organisms cannot be evaluated (Campbell and Reece 2005). Each of these definitions is useful depending on what type of question is being investigated. When exploring speciation among sexually reproducing organisms, the biological species concept is a common and practical definition to use.

1.1.2. Speciation and Reinforcement. Speciation is the evolutionary process in which the divergence of one ancestral species results in new (distinct) species. Isolation of populations and genetic divergence are the two main factors involved in speciation (Mayr 1942). Isolation can occur through development of geographic barriers or through the development of reproductive barriers. In either case, the barriers prevent gene flow among populations. Over time, because of variations in the alleles of the populations they evolve differently. If enough variation occurs, and the genetic differences among the populations prevent successful reproduction, new species have formed.

A main mode of speciation is allopatric speciation. Allopatric speciation occurs when a parent population is divided by geographic barriers which prevent gene flow

between the two populations (Campbell and Reece 2005). Due to natural selection and the availability of different resources in the different locations, each population evolves differently. If the populations diverge enough so that they no longer successfully interbreed, separate species result. This type of speciation intuitively makes sense in that it is likely different resources are available in the different geographic regions, therefore species are going to evolve differently. Peripatric speciation is a term given to specific type of allopatric speciation in which two species ranges are separated by a physical barrier preventing gene flow, but one population is significantly smaller than the other population (Ridley 2003). Parapatric speciation is where two species form over a large geographical region due to being in different areas of the region (Ridley 2003). Although there is not a specific physical barrier preventing the individuals from meeting, the physical distance among individuals on either end of the range prevents gene flow among those individuals. Again, over time, enough genetic differences arise to prevent successful reproduction among individuals of the separate populations.

The fourth main mode of speciation is sympatric speciation. This occurs when two populations share the same geographical area but do not interbreed (Campbell and Reece 2005). The same resources are available to each population yet distinct species arise. In this case, reproductive barriers are the primary isolating mechanisms. Pre-zygotic barriers include mechanisms such as breeding at different times of the year, having different courtship rituals, or simply mechanical incompatibility. Hybridization occurs when individuals from two genetically distinct populations do interbreed (Harrison 1993). Post-zygotic barriers such as gametic incompatibility, hybrid inviability, or hybrid infertility generally prevent the two populations from merging.

Wasting valuable energy and resources on unsuccessful reproduction can be detrimental to a species' survival. To avoid this wasted effort, strengthening of reproductive barriers is promoted through a concept called reinforcement.

Reinforcement is the evolution of isolating mechanisms in areas of overlap or hybridization that promote selection against hybridization (Howard 1993, Servedio 2004). For example, if two populations diverge due to a geographic barrier for a period of time long enough to develop some unique traits and then meet again due to removal of that geographic barrier; they may still be able to physically breed. However, the offspring with mixed ancestry may be inviable or simply less fit. It is in the best interest of both species to not waste energy and resources on interbreeding so stronger pre-zygotic barriers (such as having different mating calls) develop over time to prevent this wasted effort. These barriers tend to be more intense in areas of sympatry of closely related species than in areas of allopatry. This is known as reproductive character displacement (Howard 1993). The hypothesis of this process can be credited to Dobzhansky's 1940 article which suggested stronger sexual isolation in two *Drosophila* species that occupied a region of overlap than the same two species which occupied regions that did not overlap (Howard 1993). Reinforcement is of interest to many evolutionary biologists because it serves as a link from the macroevolutionary process of speciation to the microevolutionary process of natural selection (Servedio 2004). As with all evolutionary mechanisms, there are still many unknowns about reinforcement. How often it occurs, what promotes reinforcement, and specific genetic mechanisms involved in reinforcement are some key questions needing answers for a clear understanding of this process. Because reinforcement is thought to occur in regions of overlap, a better

understanding may come from investigating specific types of overlapping regions such as hybrid zones.

1.1.3. Hybrid Zones. Any region in which two separate taxa meet, mate and produce offspring of mixed ancestry can be classified as a hybrid zone (Harrison 1990). The study of animal hybrid zones has been increasing over the past few decades due in part to the increasing ease of genetic analyses of these zones (Barton and Hewitt 1985, Harrison 1993). Hybrid zones tend to occur where the range of two closely related species meet, although they sometimes occur intermittently throughout overlapping ranges of otherwise sympatric species. Tension zones is the term given to hybrid zones that may move, when not bound by local ecological conditions, in the direction of the less adapted population until two stable populations have formed or a physical barrier prevents further movement (Key 1968, Barton and Hewitt 1985). Frequent or occasional hybridization can occur in different hybrid zones resulting from ecological variants such as habitat changes or resource availability (Harrison 1993). Hybrid zones occur naturally but also may develop because of anthropogenic activities such as introduction of non-native species or habitat alteration (Riley et al. 2003, Allendorf and Leary 1988, Rhymer et al. 1994 and Allendorf et al. 2001). Some hybrid zones have successful, fertile hybrids while others appear to select against hybridization and the resulting hybrids are inviable, infertile or simply less fit. Hybrid zones occur across a variety of taxa including birds, amphibians, reptiles, mammals, insects and fish (Grant and Grant 1992, Wake 1980, Fitzpatrick et al. 2008, Heaney and Timm 1985, Harrison 1983, and Planes and Doherty 1997, to name a few).

Hybrid zones can provide evolutionary biologists with the opportunity to witness evolution in action (Hewitt 1988, Harrison 1993). Hybrid zones can be sources for the development of new species, the extinction of species, the strengthening of selection against hybridization (i.e., reinforcement), or simply a natural setting in which biologists can observe genetically distinct populations interacting. Specifically, mechanisms of reinforcement can be investigated in hybrid zones due to either its breakdown allowing hybridization to occur, or its strengthening which prevents hybridization from occurring.

1.1.3.1 Hybrid zones of salamanders. Salamanders frequently lack the reproductive barriers necessary to prevent hybridization among closely related species (Voss and Shaffer 1996). Numerous natural and laboratory settings have shown that salamanders across many genera can produce hybrids (Brown 1974, Wake 1980, Veith 1992, McGregor et al. 1990, Voss and Shaffer 1996, Riley et al. 2003, and Alexandrino et al. 2005). The hybrid zones may occur from primary or secondary contact, within ecotones, or from anthropogenic activities. Members of the family Plethodontidae in particular have been found to hybridize frequently (Highton and Peabody 2000).

The Plethodontidae is the largest family, and includes 396 species (Frost 2010). It is a diverse group with two subfamilies and three tribes (=supergenera). All members of this family lack lungs; hence are commonly known as the lungless salamanders. They are widely distributed throughout North America and also are found in Central America and two genera in South America (Petranka 1998). The North American species tend to be associated with cool waters, forest habitats, and subsurface dwellings. Some members of this family are strictly aquatic, others are strictly terrestrial, and some maintain the

typical biphasic life history (Larson et al. 2003). Some members have developed into troglobites and others are entirely arboreal. Species from the different genera of this family can frequently be found in the same community. The ability to utilize different resources within the same habitat is a classic example of sympatric speciation. However, as mentioned earlier, members of this family can also be found in numerous hybrid zones. Typically in sympatric speciation, reproductive barriers appear to be stronger to enhance reproductive isolation thereby preventing hybridization. The occurrence of so many hybrid zones is thought to be related to rapid diversification (Highton 1995, Wiens et al. 2006).

Hybridization has also been found in other clades that experienced rapid diversification or radiation such as Galapagos finches, Hawaiian crickets, and Rift Lake cichlids (Grant and Grant 2002, Shaw 2002, Seehausen et al. 1997). All three groups have experienced recent rapid radiation, and all currently experience regular within-group hybridization. It has been hypothesized that rapid radiation may result in a time period of incomplete evolution of reproductive isolating mechanisms, allowing introgression to commonly occur (Seehausen 2004). Three specific clades within the Plethodontidae family have undergone rapid radiation in eastern North America. The subfamily Spelerpinae, supergenus *Desmognathus*, and genus *Plethodon* diverged approximately 50 million years ago. All three clades appear to have experienced rapid radiation within those groups over the last 25 million years and include many species diverging in less than 10 million years (Kozak et al. 2009). *Plethodon* have been studied extensively; they have exhibited evidence of recent diversification and have high rates of introgression (for review see Highton 1995, Wiens et al. 2006, Kozak et al. 2006). *Desmognathus* and

Eurycea (Spelerpinae genus) have not been studied as extensively but there are some cases of hybridization among species in both of these groups as well (Smith 1961, Smith 1964, Johnson 1977, Tilley et al. 1978, Sweet 1984, Guttman and Karlin 1986, Potter 2008). Most of these studies involve frequent interbreeding among sister species but studies involving two particular *Eurycea* species (e.g., Smith 1964, Potter 2008) report on rare hybridization.

1.1.3.2 Case study: *Eurycea* genus. *Eurycea lucifuga* and *Eurycea longicauda melanopleura* were examined by Smith (1964) in Foshee Cave in Arkansas. Of the approximate 200 *E. l. melanopleura* collected, over seven percent were found to have some genetic influence from *E. lucifuga*; however, the 200 *E. lucifuga* had no evidence of mixed ancestry. Female hybrids were found tight with eggs but it is unknown if they were able to produce viable offspring as the specimens were lost in a laboratory accident. Potter (2008) investigated individuals in Onondaga Cave in Missouri that had questionable morphological characteristics reflective of both species. For minimum disturbance of the cave life, individuals were opportunistically collected but sampled in the cave and released the same place they were caught. Due to time constraints and limited availability of samples, conclusive evidence of hybridization among these two species within Onondaga was not reached in this study, although questions regarding unidentifiable individuals were presented. In both cases, a few hybrids (or potential hybrids) were found over at least a year of sampling, indicating hybridization may be a rare event among these species. Examining the life history of these species may provide insight regarding the occurrence of these events.

Eurycea longicauda currently contains two subspecies, *Eurycea longicauda longicauda* and *Eurycea longicauda melanopleura*. *Eurycea guttolineata* was considered a subspecies of this group at one time, but is now considered its own species (Carlin 1997, Petranka 1998). *E. l. longicauda* occurs in the eastern United States, including southern New York toward northern Alabama and westward toward the eastern part of Missouri. *E. l. melanopleura* occurs in the central and western parts of Missouri, northern Arkansas, and the eastern edge of Oklahoma (Petranka, 1998). There is a presumed hybrid zone of the two species along the eastern side of Missouri, into the western edge of Illinois and southward in the northeastern part of Arkansas (Petranka 1998). *E. l. longicauda* is generally yellow to yellowish-brown with a cream or yellow belly. Dark, irregular blotches are found along the dorsum and at times form discontinuous lines along the sides. *E. l. melanopleura* is similar in appearance except for a dark, broad stripe which is found along both sides. These stripes tend to have scattered white flecks. The intergrades tend to have patterns consistent with both species (Smith 1961). It is worthwhile to also note that *E. l. longicauda* has been implicated in potential hybridization with *E. guttolineata* (Myers 2009). *E. longicauda* ssp. are often found in forested habitats near cool streams, under rocks or logs, and in or near caves or abandoned mines. Breeding tends to occur in autumn through early winter. Finding egg deposits has been rare, but some have been identified in subsurface habitats such as caves or mines. Incubation lasts from 4-12 weeks, depending on water temperature, and metamorphosis typically occurs in June or July. There have been some cases of overwintering of larval stage and transformation the next summer (Anderson and Martino 1966, Franz and Harris 1965, and Huheey and Stupka 1967). Adults grow to 10-20 cm

total length, and their diet mainly consists of invertebrates. As adults, their tails comprise 60-65% of their total length, resulting in the common name, long-tailed salamander.

They are most active in the early evening hours.

Eurycea lucifuga occurs primarily in limestone regions of Missouri, Kentucky and Tennessee. They can be found from the western edge of Virginia to eastern edge of Oklahoma and from northern Alabama and Georgia northward to southern Indiana. *E. lucifuga* is generally orange with round, dark spots along the dorsum. They are frequently seen in the twilight zones of caves but can also be found deep within caves or along rock walls associated with springs within forest habitats. Although not well documented, is believed that breeding tends to occur in late summer and autumn (Petranka, 1998). It is unusual to find egg deposits of this species, and those that have been identified were found deep within caves. The larval period varies for this species with some completing metamorphosis by the end of the summer and others overwintering before metamorphosing. Adults generally reach 10-20 cm in total length, and are active in the evening hours. There is some documentation of cave salamanders migrating deep within caves during autumn and winter, and then returning to the surface during late spring (Hutchinson, 1958). Cave salamanders forage on a variety of invertebrates. This species does secrete a noxious substance from the tail when attacked by predators (Brodie, 1977).

The *E. longicauda ssp.* and *E. lucifuga* have similar life histories and share similar habitats. Their ranges, as well as the *E. longicauda ssp.* hybrid zone, are displayed in Figure 1.1.

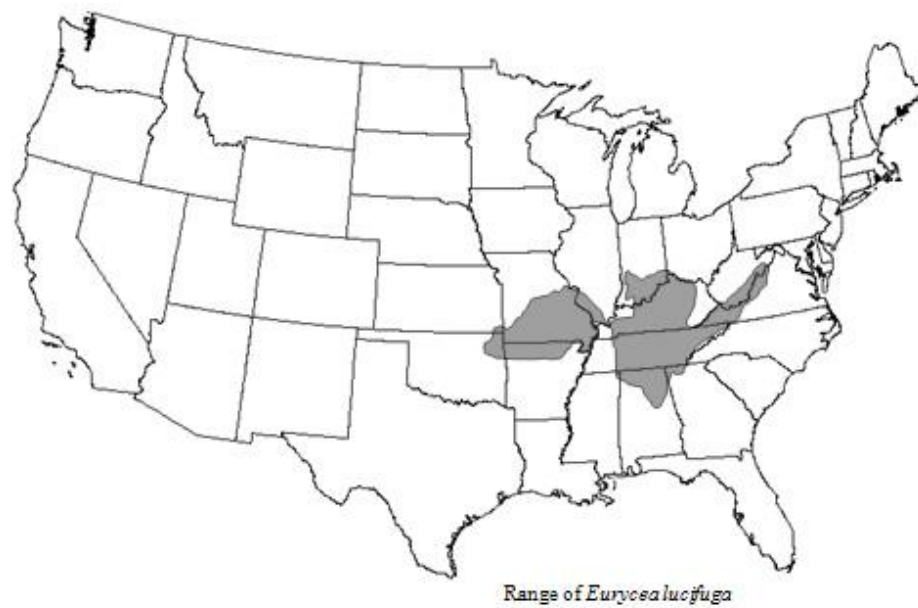
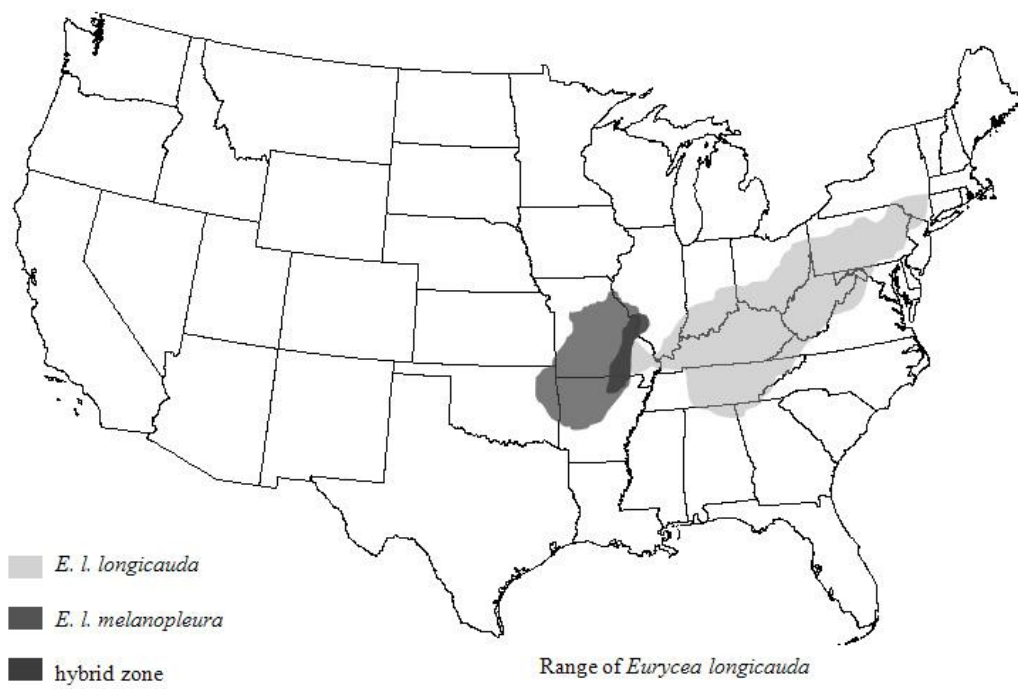


Figure 1.1 Distribution of *E. longicauda* ssp. and *E. lucifuga*. (Redrawn from Petranksa 1998)

These taxa are sister species that diverged about 10 million years ago, and in a phylogeny produced from mitochondrial and nuclear DNA, *E. l. melanopleura* and *E. lucifuga* appear to be more closely related than *E. l. melanopleura* and *E. l. longicauda* (Kozak et al. 2009). It has been proposed that there may be competition among these animals due to the negative correlation found between the numbers of each species found in Virginia caves, suggesting the use of very similar resources (Hutchison 1958, Petranka 1998). Smith discussed the significance of a slight overlap of the breeding seasons of the two species (1964). He suggested that the male *E. lucifuga* may still be present in the caves toward the end of their breeding season as the *E. l. melanopleura* migrate into the caves at the beginning of their breeding season, resulting in infrequent hybridization events. This failure of reproductive isolation may be a case of incomplete reinforcement or a breakdown of reinforcement. The combination of being such closely related species with such similar life histories along with the potential occurrence of rare hybridization events makes these two species ideal for studying speciation, reinforcement, and hybridization.

1.2. OBJECTIVES

The evolutionary mechanisms of reinforcement are vital to the process of speciation. Examining hybrid zones may provide insight to the specific mechanisms of reinforcement. The occurrence of frequent hybrid zones among members of the Plethodontidae makes them exemplary for investigating reinforcement. Missouri terrain features karst landscape throughout a large portion of the state, an ideal environment for Plethodontidae; according the Missouri Department of Natural Resources, there are over 6,000 caves and over 3,000 springs recorded in Missouri, ranking it second only to

Tennessee in number of caves. Additionally, there is forest land throughout much of Missouri. This habitat is ideal for both *E. lucifuga* and *E. longicauda ssp.*, and a known hybrid zone of *E. longicauda ssp.* is located along a narrow strip in eastern Missouri. Onondaga Cave is located within the known hybrid zone of *E. longicauda ssp.* and is the location of a recent study which discussed the potential hybridization *E. longicauda ssp.* and *E. lucifuga* (Potter 2008). Another report documented hybridization of these two species comes from a Foshee Cave in Independence County, Arkansas, which is outside the documented *E. longicauda ssp.* hybrid zone, but still geographical close to this zone (Smith 1964). The hybridization that did occur in Foshee Cave was rare, potentially indicating some isolating mechanisms at work which could be due to reinforcement. Two questions arise from these findings: 1) Does hybridization of *E. longicauda ssp.* and *E. lucifuga* occur more frequently throughout this (or near) the known *E. longicauda ssp.* hybrid zone? 2) Is reinforcement present in the form of pre- or post-zygotic barriers? The objective of my project is to determine if hybridization is occurring among *E. longicauda ssp.* and *E. lucifuga* using microsatellites for genetic markers.

2. GENETIC ANALYSIS OF HYBRID ZONES

2.1. MOLECULAR TECHNIQUES USED TO STUDY HYBRID ZONES

2.1.1. Background. Genetic analysis is a key component in contemporary investigations of hybrid zones. Before genetic analysis, scientists had to rely on morphological characteristics to distinguish hybrids. Phenotypes vary greatly within a species, so comparing specific characters based solely on morphology among different species was difficult. It was especially challenging among sister species with very similar morphologies such as in cases of field crickets, orioles, and toads (Harrison 1986, Rising 1983, Sattler 1985). With the growing ease and decreasing cost of molecular techniques, researchers can now use genotypic data along with phenotypic data to investigate hybrid zones (Selkoe and Toonen 2006). To examine genotype variations, there is an assortment of molecular techniques that can be used. Techniques using DNA sequencing or fragment analysis such as restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLP), and microsatellites have most commonly been used in hybrid zone studies (Harrison and Arnold 1982, Guttman and Karlin 1986, Howard 1986, Potter 2008).

2.1.2. Sequencing. DNA sequencing establishes the actual sequences of individuals in question using a primer and dideoxy ribonucleotides (ddNTP) in a reaction similar to PCR (DNA Sequencing ... 2002). The primer initiates DNA synthesis at a known sequence and the DNA is amplified as in PCR. During elongation, occasionally a ddNTP attaches. The ddNTP's lack a 3' OH group, which prevents another nucleotide from attaching, thereby terminating the strand. The fragment sizes then can be compared

and a sequence can be established. A popular technique using DNA sequencing for population studies involves mitochondrial DNA (mtDNA) (Zhang and Hewitt 2003). This DNA is not found within the cell nucleus (nuclear DNA) but rather in the mitochondria of the cell. Sequences in mtDNA are highly conserved across generations allowing relationships to be established within and among populations (Avisé et al. 1987, Harrison 1989, Simon 1991). Single nucleotide polymorphisms (SNPs) provide a unique identity to each individual. This process compares specific sequences of mtDNA of the individuals in question. Although this can be a useful genetic marker, it does not come without some problems. MtDNA is maternally inherited, so in hybrid zones where it is important to determine both maternal and paternal inheritance, additional comparisons of some other markers are needed. Also, there have been some examples of mitochondrial pseudogenes found within nuclear genomes of some animals; these have greatly weakened the effectiveness of mtDNA markers (Zhang and Hewitt 1996, Bensasson et al. 2001).

2.1.3. Fragment Analysis. Fragment analysis is the alternative to DNA sequencing. Rather than determining the specific order of nucleotides, sizes of fragments amplified in PCR are compared to known DNA fragment lengths established by a size standard. RFLP's have target sequences which can bind to labeled probes and are flanked with restriction sites (RFLP ...2001). Through a process described by Botstein et al. (1980), a series of bands can be compared among individuals tested by performing a Southern blot hybridization. However, this test is arduous and time-consuming making it difficult for high throughput applications (Williams et al. 1990). As a result, RAPD's were developed using arbitrary primers to perform PCR. Essentially, random primers were used to amplify unknown segments of DNA for an individual. Amplified fragment

lengths were detected using gel electrophoresis with a size standard, and scored banding patterns could then be used to for population analysis, phylogenetic studies and gene mapping (Williams et al. 1990, Micheli et al. 1994). AFLP's are a method that basically combines RFLP and RAPD by using restriction enzymes and arbitrary selected primers (reducing the difficulty of using RFLP alone) to establish banding patterns among individuals (Vos et al. 1995, Bensch and Åkesson 2005). AFLP's are useful in population genetic studies, but do have some drawbacks; perhaps most importantly is the apparent sensitivity to DNA quality (Bensch and Åkesson 2005). Any DNA degradation or presence of residual inhibitors can affect the outcome of the amplification. AFLP's also may amplify with any species, so cross-contamination can be a problem. Another type of fragment analysis involves microsatellites, which have been noted to be valuable in kinship research (Queller et al. 1993), and in turn can answer several questions regarding hybrid zone genetics.

2.1.4. Microsatellites. Microsatellites, also known as simple sequence repeats (SSR), variable number tandem repeats (VNTR), or short tandem repeats (STR), generally consist of a series of tandem repeats of 2-5 base pairs (Dowling et al. 1996, Selkoe and Toonen 2006). Microsatellites can be found primarily in the non-coding region of the nuclear genome of most species although a few are found within coding regions. They vary in length but generally range from 5 – 40 repeats flanked by a conserved region (Selkoe and Toonen 2006). For example, a specific locus may have a dinucleotide repeat such as GC that occurs 7 times, resulting in the microsatellite: GCGCGCGCGCGCGC. Mutation rates of microsatellites span 10^{-6} to 10^{-2} per locus in each generation primarily through DNA replication slippage (Schlötterer 2000). The

conserved region flanking a microsatellite designates the specific locus of a microsatellite. This allelic diversity in microsatellites provides essential information to molecular biologists interested in genetic studies and can help answer questions such as which population did a specimen come from or how many distinct populations are present in a given area (Pearse and Crandall 2004, Selkoe and Toonen 2006). Using the above example, suppose that in a diploid species each offspring has two copies of each microsatellite locus, one from each parent. The father may have the GC microsatellite with one allele having 5 repeats and the other having 10 repeats, while the mother may have this same microsatellite with 7 repeats in one allele and 9 repeats in the other allele. The offspring could then have various combinations of those alleles, such as a microsatellite with 7 repeats and 10 repeats. To coarsely categorize hybrid zones, four to five microsatellites are recommended, but for determining specific kinship relations, at least 10-20 microsatellites are needed for accurate results (Boecklen and Howard 1997, Queller et al. 1993).

Although the process of isolating microsatellite loci can be tedious, once it has been completed for a specific species, there are many advantages to using microsatellites. First, the conserved region of microsatellites allow for the design of primers that can be used in polymerase chain reactions (PCR). The use of PCR allows small tissue samples to be analyzed, alleviating the need for whole specimen samples for genetic analysis (Selkoe and Toonen 2006). This is useful for endangered species or small populations in which too much disruption could have a negative impact on the ecological community. Second, microsatellites are species specific. This basically eliminates cross-contamination concerns which can be a problem in some cases (e.g., using fecal samples,

Selkoe and Tooken 2006). Another advantage of using microsatellites is they are short--usually 100 – 300 base pairs long compared to other sequenced loci which are generally 500-1500 base pairs long. Because they are shorter, if some DNA degradation does occur, PCR can still accurately amplify the specific segments (Taberlet et al. 1999, Selkoe and Toonen). This allows researchers to use old DNA, store new DNA in undemanding preservatives such as 95% ethanol, and use quick and easy DNA extraction kits (Taberlet et al. 1999). Lastly, microsatellites represent a segment of the genome; thus, combining several single locus microsatellites can provide a “fast and inexpensive replicated sampling of the genome” (Selkoe and Tooken 2006).

2.1.5. Summary For the *Eurycea* hybrid zone examined in this study, microsatellite molecular markers appeared to be the best choice for genetic analysis. For this project, it was important to not disturb the populations of the cave ecosystem; consequently, small tissue samples had to be used. DNA would be stored in the field for several hours as well as in the lab for several months while other samples were being collected, which could have resulted in DNA degradation of the samples. It also would be valuable to assess specific kinship to assist in determining what degree of hybridization was occurring, and paternal as well maternal lineages needed to be determined. Time was also a limiting factor in this project, so fast molecular techniques were deemed most suitable for the project. Finally, even if the primers did not indicate specific microsatellite alleles, techniques similar to RAPD could be employed to compare banding patterns of the individuals.

3. MATERIAL AND METHODS

3.1. SAMPLING METHODS

3.1.1. Sampling Locations. Three cave sites were chosen within the hybrid zone of *E. longicauda ssp.* as seen in Figure 3.1 to survey for potential hybrids of *E. lucifuga* and *E. longicauda ssp.* Each cave was chosen based on either personal observation or personal communication with cave staff and cave owners confirming the presence of both species. All three caves had permanent streams. The main collection site chosen for one cave was not along the stream, but was known for frequent sightings of salamanders and had nearly constant surface seepage in some locations. All three caves consisted of mostly dolomite and typified ideal environments for both species to inhabit.



Figure 3.1 Sampling Locations. Caves within *Eurycea longicauda* hybrid zone surveyed for salamanders. Onondaga Cave is found in the northern section, Gourd Creek Cave in the central section, and Banker Cave in the southern section (hybrid zone estimated from Petranks 1998).

Onondaga Cave is located in Crawford County, Missouri and is in the northern section of the *E. longicauda ssp.* hybrid zone. This cave is state owned and operated with public tours conducted in certain sections. It is a large cave with rooms over 100 meters long and 25 meters high. A little under a mile of passages is toured by the public. Access to the cave was through the entrance used for public tours that was over a mile from the collection site. This cave is where Potter's (2008) research was conducted regarding potential hybridization among *E. longicauda ssp.* and *E. lucifuga*. The main collection site for both Potter's study and this project was the Missouri Caverns section. This section has been closed to the public for several decades. There is an old man-made entrance at one end of this section that is no longer open to the surface. Just inside this entrance is a concrete staircase enclosed by concrete walls. On those walls, along the staircase, and near this entrance is where a variety of salamanders was found. Because the cave does not have a public entrance, arrangements with the staff were necessary for each visit.

Gourd Creek Cave is located in Phelps County, Missouri, and is in the central section of the hybrid zone of *E. longicauda ssp.* This cave is privately owned but has no gate to prevent public access. On several trips, there was evidence of humans such as litter or shoeprints near the mouth of the cave. This cave has a large, modified rectangular entrance that is at least 10 meters wide and at least 5 meters tall. The inside of the cave drastically changes shape as it veered to the left. It is a single, slightly twisting passage that is approximately 250 meters deep. It is a narrow, canyon cave in which both sides can be touched at the same time throughout most of the cave, but the ceiling is usually at least five meters tall. The floor of the cave has a permanent stream and was covered by

cobble and gravel. Access to this cave is open; no arrangements with the cave owners required for each visit of this study.

Banker Cave is located in Shannon County, Missouri, and is in the southern section of the hybrid zone. This cave also is privately owned, but has a large steel gate installed to protect the bat population from public disturbance. It had been opened to the public in the past, but has been closed for at least two decades. This cave has an elliptical entrance that is approximately five meters wide and three meters tall. The cave does not change shape but does get more narrow with a progressively shorter ceiling the deeper it goes. It is single, twisting passage cave with a permanent stream running along the entire length of the cave that supplies a spring at the entrance of the cave. For the most part, the floors are smooth dolomite with little or no gravel or pebbles. There are some places along the walls that are thick with clay and other places along the ceilings and walls with numerous speleothems. Because the steel gate was locked to protect the bat population of this cave, arrangements with the cave owner were necessary for each visit of this study.

3.1.2. Preparation for Field Work. Once the locations were determined, permission to access to the sites and specimens was necessary. Verbal permission was given from both landowners of Gourd Creek Cave and Banker Cave. Permission to conduct research on state owned property required an application be submitted to the Missouri Department of Natural Resources (MoDNR). Written permission was granted for research in Onondaga Cave for one year from MoDNR as long as a wildlife collector's permit was received from the Missouri Department of Conservation (MDC). Wildlife collector's permits #14177 and #14432 authorized sampling specimens at

Onondaga Cave, Banker Cave and Gourd Creek Cave with a maximum of five sacrificed specimens and ten salvaged specimens. The original approval documents were kept on person when conducting field work as requested by MDC.

It was important to plan efficient field work in combination with minimal disturbance to the populations under study. Cave ecosystems are delicate and changes within that ecosystem can have adverse affects on the cave. The size of the populations of either *E. longicauda ssp.* or *E. lucifuga* was not known for any of the chosen caves, and removing even a small portion of them from the cave could have a negative impact on those species and/or the cave environment. These factors contributed to the decision to not remove individuals from their habitat, but rather to do all data and tissues collection on site. Small tissue samples were stored in 70% ethanol in microcentrifuge tubes and transported from the site in a common picnic cooler. Before embarking on each visit, the local conservation agent was notified of the planned field visit, as per MDC protocol.

All equipment was properly cleaned and sterilized before going to each location to prevent possible contamination in different locations. Any equipment that was safe to submerge was bathed in a 3% bleach solution for at least fifteen minutes and then rinsed with tap water. Equipment that could not be submersed was wiped thoroughly with a towel soaked in the bleach solution and placed outside, in the sunlight, for at least four hours. (This is a recommended practice by the Missouri Department of Conservation when the temperature is above 30°C allowing all water to evaporate from the equipment). At the onset of this research, there were concerns of a fungus spreading in bat populations in the eastern United States. No cases had been reported in Missouri during the time of this fieldwork, but special precautions were taken to decrease any risk of spreading the

fungus. All equipment was sterilized with bleach solution (submersed in solution if possible) and placed in sunlight for no less than four hours. Also, separate packs were used for each cave, and a different pair of shoes were worn in Onondaga Cave than in the other two caves. For both Gourd Creek Cave and Banker Cave, rubber boots were worn due to the amount of water, but the boots were cleaned with bleach solution and exposed to sunlight for at least four hours before visiting the next cave. If coats were worn, they were washed in warm water before visiting the next cave.

3.1.3. Specimen Sampling. Adults and some juveniles were captured opportunistically at the three cave sites. Salamanders were maintained in small, plastic containers with lids to prevent them from escaping. Precautions such as water being available to keep hands moist when individuals were handled, placement of specimens in moist containers while they were being evaluated, and release of specimens as soon as recovery was complete were taken in an effort to reduce impact to the individuals. The evaluation of each specimen was completed on site and the specimen was released where it was originally captured. During evaluation, each individual was placed in a .05% solution of the anesthetic Tricaine-S (tricaine methanesulfonate, MS-222). The salamanders were placed in the solution for approximately 5-7 minutes. When the individual would no longer react to being touched but before it turned over on its back, it would be removed from the MS-222 and immediately bathed in filtered water for 15-20 seconds.

While individuals were sedated, visual assessments were completed to attempt to identify the species based on coloration and types of markings present on the individual. To determine the sex of the specimen, the vent was examined for swollen testes and the

mouth for pronounced cirri. Morphological measurements (nearest 0.1 mm) were obtained using a metric dial caliper. Each individual was placed on its back to measure the snout-vent length and tail length. The individual was then turned over to measure the left femur length and head width. The costal grooves were counted three times for verification of a correct count. Filtered water was squirted along the salamander's body when it was turned over and again when it was picked up to count costal grooves. The individual was photographed from the lateral and the dorsal view. Tissue samples for genetic analysis were then obtained. A small section of the tail (approximately 5 mm) was removed using a razor blade. Forceps were used to place the tail sample in a 1.5 ml microcentrifuge tube filled with 70% ethanol. A new razor blade was used for each specimen and the forceps were cleaned with an pre-packaged alcohol swab after each use to prevent contamination of the tissue samples. Each tissue sample was stored in a separate, labeled tube and placed in a cooler with an ice pack to keep the samples cool until returned to the lab for appropriate storage. The individual's tail was then sprayed twice with Bactine[®], an anti-bacterial agent, and the individual was placed in a moist container for recovery. Salamanders would continue to be moistened with filtered water every 3-5 minutes during the recovery period. When the individual was moving on its own, it was returned to the same location that it was found. Before leaving the cave, salamanders that could still be seen were checked on to ensure they had completely recovered. Upon returning to the lab, tissue samples were stored at 4°C until molecular analysis was completed. Only one questionable specimen was taken back to the lab for further analysis and evaluation by a colleague. It was then returned to the original site of

capture as the morphology alone did not bear enough evidence to verify that it was a hybrid.

3.1.4. Samples Collected. At the completion of the field work, a total of 22 trips had been made over nine months to the three separate caves. Table 3.1 lists the number of each species found at each site. Species that could not be identified by visual assessment alone are listed as unknown specimens; these individuals had characteristics of both species. A total of 82 samples were collected with four unknown specimens. A majority of the *E. longicauda ssp.* appear to be *E. l. melanopleura*.

Table 3.1 Samples Collected. Total samples collected from each location. Individuals that could not be identified by phenotype alone are listed as unknown.

	<i>E. longicauda ssp.</i>	<i>E. lucifuga</i>	<i>unknown</i>
Banker Cave	35	3	3
Gourd Creek Cave	1	2	1
Onondaga Cave	2	35	0
Total Samples	38	40	4

3.1.5. Morphological Analysis. Statistical analyses involving the morphology of the specimens were carried out for 56 of the 82 individuals surveyed. Broken tails prior to sampling or inadequate data collection due to the anesthetic not working properly led to the exclusion of 26 individuals from the morphological analysis. MANOVA was carried out with SAS[®] software using the GLM procedure on 56 individuals for analysis of the morphological measurements (SVL, TL, FL, HW) and costal groove count. The individuals were labeled as two groups based on what species they most looked like. A

principal components analysis was conducted using SAS[®] software. Results for these analyses can be found in the morphological analysis section of the next chapter.

3.2. TISSUE SAMPLE PROCESSING

3.2.1. DNA Extraction. DNA was extracted from the tissue samples using the DNeasy[®] Blood and Tissue Kit from Qiagen. First, tissue samples were bathed in deionized water 10-15 times to remove most of the ethanol. Protocols of the kit (provided in Appendix A) were followed to extract the DNA. In short, first the tissue was lysed using proteinase K, then the lysate was loaded onto a spin column-included with the kit, and finally, through centrifugation and wash steps, the DNA was eluted in a buffer solution, also included in the kit. It was recommended by the manufacturer of the kit to repeat the final elution step to obtain maximum DNA yield. In order to prevent dilution of the first eluate, a new microtube was used for the second elution. The concentration of extracted DNA from each individual was determined using a NanoDrop[®] Spectrophotometer ND-1000 and ND-1000 v3.2 software.

3.2.2. Microsatellite Primer Selection. No literature could be found to describe microsatellite loci primer pairs developed for either of these species. The Molecular Ecology Resources includes an online database for primers that can be searched based on species, families, etc. (Molecular Ecology Resources... accessed Sept 2009). The only *Eurycea* in the database was *Eurycea bislineata*. However, some data suggest that the conserved region flanking the microsatellites are highly conserved among families even if divergence occurred several million years ago (Fitzsimmons et al. 1995). In microsatellite study regarding *Eurycea cirrigera*, microsatellite primers developed from

the genome of *Plethodon cinereus* were screened, and seven of the 13 screened could be used in DNA analyses (Connors and Cabe 2003, Boyle 2005). In another study, some of the microsatellite loci that were isolated for *Dicamptodon tenebrosus* were found to successfully amplify alleles in *Eurycea bislineata* (Curtis and Taylor 2000). In both of these studies, microsatellite loci primer pairs developed for other species were successful in *Eurycea*. Based on the success reported in those studies, primers from each were selected to be screened with *E. longicauda ssp.* and *E. lucifuga*. In addition, a few microsatellite primers, found from searching the Molecular Ecology Resources database, developed for *Ensatina eschscholtzii* were also selected to be screened (Devitt et al. 2009). The primers names with the forward and reverse sequences are listed in Table 3.2.

Table 3.2 Microsatellite Primers. These primers were originally developed for closely related species and screened for successful amplification in the *E. lucifuga* and *E. longicauda ssp.*

^a Successfully amplified in *E. lucifuga* and *E. longicauda ssp.*

^b Used for final banding pattern determination

Locus (Genbank Accession no.)	Primer Sequence (5'-3')	Published By
Dte4 (AF149305) ^{a, b}	F: [6~FAM]TGCTTCTGCCACCATAGCC R: AGAGCCAGCCTTTGTTGCG	Curtis and Taylor 2000
Dte5 (AF150725)	F: GGAGGAGTTTTTGAAGTTG R: ATTCTCCAAACATTCTCCC	Curtis and Taylor 2000
Dte8 (AF150726) ^a	F: [TAMRA]CTGCATACATTGCATCTCCG R: CCGCAAGGTCATCTTCACTAAC	Curtis and Taylor 2000
PCII14 (AY151372) ^{a, b}	F: [5HEX]AACCCACACCAGATCCACTC R: TGGTTTGCTGTCTTCTTTGC	Connors and Cabe 2003
PCXD23 (AY151376)	F: GCAAAACAGCAACAAGACAAC R: AACCTTGATGTTTGGCAAGG	Connors and Cabe 2003
ENS4 (FJ446706) ^a	F: [Amino-C6+ROX]TTCCGGGTAACAGAAAGCAT R: AAATAACTCACAGGTTGTAATCAGG	Devitt et al. 2009
ENS6 (FJ446708)	F: CTTGTTTCAGAAAGGGGACCA R: AAGTTCATCCACTGCCCAAC	Devitt et al. 2009
ENS13 (FJ446714)	F: CAATGGCCACTGTGTTTCTG R: CAGGACACCTATAGTGGTTGGA	Devitt et al. 2009
ENS15 (FJ446716)	F: CTGAGTTGCCCATTTCTGGTT R: AGGGGGATGTTACATGTTT	Devitt et al. 2009
ENS20 (FJ446718)	F: TTCACCAATGTGGTTGAACTG R: CACACCTTTCACCCAATAAACA	Devitt et al. 2009

To prevent wasting money on fluorescently labeled primers that may turn out to be incompatible with the species in this study, it was necessary to first screen the potential primers using gel electrophoresis, which does not require fluorescent labels for analysis. Unlabeled primers were ordered dry and resuspended in enough nuclease-free

water to form a concentration of 100 pmol/ul. Original solutions were stored at -20°C with 25 ul kept in a separate tube and handled as needed for the screening process.

Forward and reverse primers were combined and nuclease-free water was added to form a final concentration of 20 pmol/ul. For each set of primers, 1 ul was added to separate microcentrifuge tubes which each contained 22.5 ul of Accuprime[®] Supermix and approximately 150 ng of DNA (5 ul of eluate) of either species. Each tube was spun down for a few seconds and then placed in a Techne TC-312 Thermocycler on a 3-step, 35-cycle. There was an initial denaturing step at 94°C for five minutes, then the cycles consisted of 94°C for 30 seconds for denaturing, 50°C for 30 seconds for annealing, and finally 72°C for one minute to complete the elongation step. After the 35 cycles, there was final extension at 72°C for four minutes and the final hold was at 10°C. After spinning down all microtubes retrieved from the thermal cycler, each PCR product had 4 ul of purple dye mixed with it. These products then were run on a 3% agarose gel with ethidium bromide at 100 V for 60 minutes. A ladder was added in one lane to compare band lengths. The gels were viewed and images captured using a Photodyne base with an Olympus C-7070 camera and Foto Analyst[®] software. Primers showing some bands were ordered with fluorescent labels and ran in a capillary genetic analyzer.

Labeled, HPLC purified, forward primers were ordered and resuspended using the same methods as above. The forward primers were then combined with the reverse primers to create a final concentration of 20 pmol/ul. Because of the fluorescent labels, the primer solutions were stored in an opaque box to prevent degradation of the labels from too much light exposure. A Type-it[™] Microsatellite PCR kit from Qiagen[®] was used for PCR with labeled primers. After some experimenting with the kit, procedures outlined by

the manufactured were altered slightly for this study and are described in Appendix B. Each microtube contained 2.5 ul of primer solution, approximately 100 ng of DNA from each specimen, and 12.5 ul of 2x Type-it Multiplex Master Mix. A 3-step, 32-cycle was used in the Eppendorf Mastergradient thermal cycler, which can be used for larger sample sizes. There was an initial denaturing step at 95°C for five minutes and then 32 cycles which included a denaturing step of 95°C for 30 seconds, an annealing step at 55°C for 90 seconds, and an elongation step at 72°C for 30 seconds. There was a final extension at 60°C for 30 minutes and then 4°C final hold. Each tube was spun down after retrieval from the thermal cycler and was ready to be prepared for the capillary sequencer.

A 96-well plate was used to run the PCR product in the capillary genetic analyzer. Each PCR product was diluted at 1:10 ratio with Hi-Di™ formamide received from Applied Biosystems. GeneScan™ 1200 LIZ® Size Standard from Applied Biosystems was diluted at 1:38 ratio with the Hi-Di™ formamide. In each well 9.5 ul of the diluted size standard was added, and then .5 ul of the diluted PCR product was added. The well was spun-down at 1000 rcf for two minutes, incubated at 95°C for three minutes, and spun-down a final time at 1000 rcf for two minutes. Order of samples were programmed into Genemapper® v3.7 from Applied Biosystems and the plate was ran in the Applied Biosystems 3130 4-capillary Genetic Analyzer. Genemapper® was used to view and analyze the results of the assays.

3.2.3. Electropherograms. Results of the assays were displayed in electropherograms plotted by Genemapper®. Although the software had an automated bin process (a process of labeling significant peaks), it did not seem to work properly as

obvious peaks were not being included, so the peaks were manually labeled or binned. This was achieved by looking at 12 individuals' electropherogram plots (one for each primer, 24 total) and designating a bin at each peak that was over 200 nm in height. Bins were labeled based on fragment size and no fragments smaller than 50 or larger than 1000 were labeled. Once this was completed, all samples were analyzed against these bins. The software then listed the samples in descending order with unlabeled bins indicated by question marks in boxes between bins currently labeled. This was part of the automated binning process that seemed to work some of the time. Through examining each box marked with question marks on the given electropherograms and designating a labeled bin to those boxes, eventually all samples were listed with no question marks. A total of 114 different bins were labeled among the 73 different specimens and two primers. At first, it was understood that all peaks in those bins over 200 nm were labeled on all samples. However, upon further investigation regarding a separate issue, it was discovered many peaks were not included on several samples, even though a bin was designated for that peak. So, each sample was manually reviewed for all 114 bins between the two primers and scored on a separate Microsoft Excel[®] spreadsheet to indicate if a specific bin was present (1) or absent (0). After reviewing all of the bins, 12 pairs were combined as they were most likely allelic stutter and four removed as they were small peaks (<250) found only once on different samples. This left a total of 98 bins. The unique banding patterns were a result of the presence or absence of each bin on each individual electropherogram. These banding patterns were distinguished using the discrete characters mentioned above (present-1; absent-0).

3.2.4. Resolution of Phylogenetic Tree. To develop a phylogenetic tree, the PHYLIP package was used. Mix v. 3.69, one of the general parsimony programs which can be used to produce discrete character trees, was selected. This program allows for situations in which ancestral states are unknown (using the Wagner parsimony), different characters and lineages may evolve independently and changes from 0 to 1 are equally probable. These assumptions were necessary to build the tree because specifics about the amplified segments amplified were unknown. The default settings with the Mix program were used to produce the tree that is presented in the next chapter.

4. RESULTS

4.1. UNKNOWN SPECIMENS

Out of 82 samples collected, there were four that had characteristics of both species. Three of the unknown specimens at first glance appeared to be *E. lucifuga*, but upon further evaluation had characteristics of *E. longicauda ssp.* such as distinct vertical bars along the sides, elongated spots on the dorsum, or the more slender build typical of *E. longicauda ssp.* The fourth unknown specimen was removed from the cave for further evaluation with a colleague. Upon this review, it was determined to be a likely *E. l. longicauda* but remained listed as unknown because it of unusual markings noted on the specimen. Presented in Figure 4.1 are pictures of a typical representative of both species and in Figure 4.2 are pictures of the four unknown specimens found during this study.

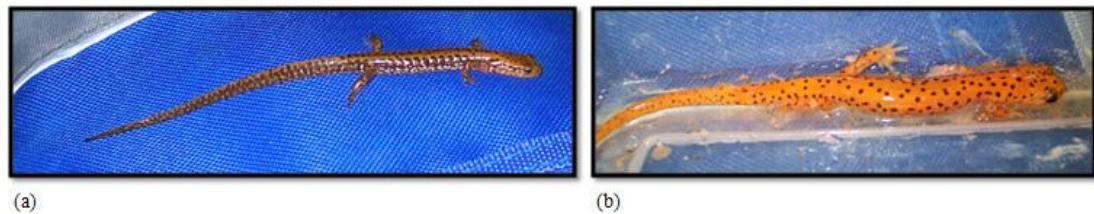


Figure 4.1 Typical Morphology of Both Species. (a) *E. longicauda ssp.*, adult, is yellow to yellow-brown with irregular, dark marks arranged in lines along dorsum, tightly arranged dark, vertical bars with interspersed white flecks along the sides, dark limbs and cream to white venter (B. Beasley) (b) *E. lucifuga*, adult, is orange with round, dark markings along dorsum and sides, orange to pale cream limbs and venter (B. Beasley)

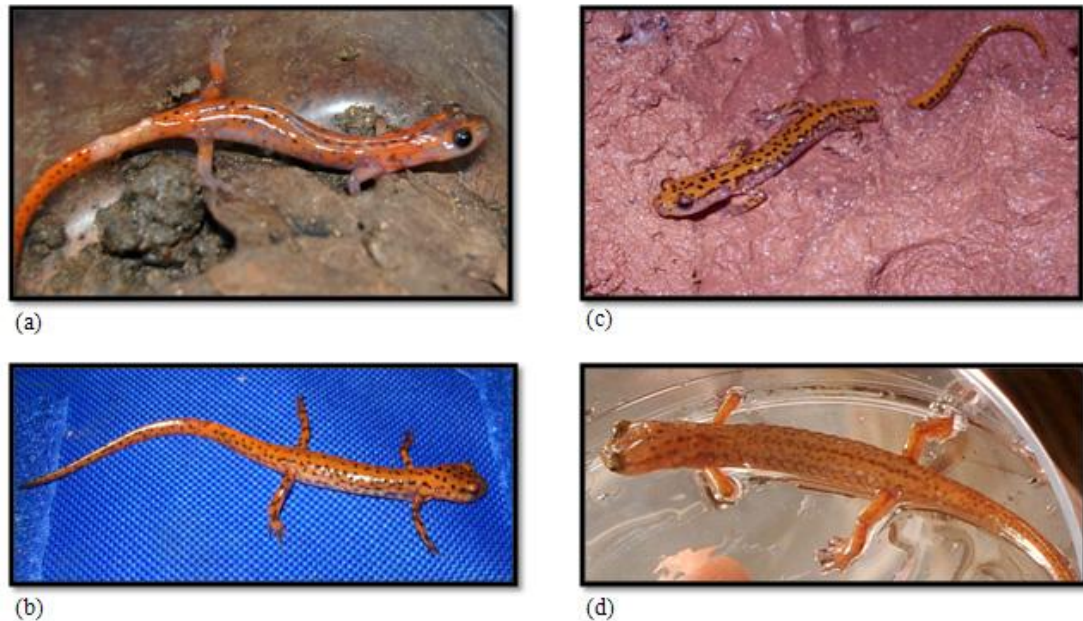


Fig 4.2 Unknown Specimens. These specimens exhibited characteristics of both species (a) Specimen B121 has coloring of *E. lucifuga* with some elongated, dark markings and dark feet similar to *E. longicauda* ssp. (B. Beasley) (b) Specimen B130 has typical coloring and marks of *E. lucifuga*, vertical bars and white flecks on sides as well as slender build similar to *E. longicauda* ssp. (B. Beasley) (c) Specimen G143 has orange coloring with round, dark marks on dorsum and robust build similar to *E. lucifuga*, vertical bars tightly arranged along sides with white flecks dispersed similar to *E. longicauda* ssp. (B. Beasley) (d) Specimen B239 has more of a robust build and pale orange-yellow venter and limbs similar to *E. lucifuga*, yellow-orange color and irregular, dark marks on dorsum and vertical bars along sides similar to *E. longicauda* ssp. (B. Rupert)

4.2. MORPHOLOGICAL ANALYSIS

MANOVA showed significant difference among the groups, so the post hoc test, Tukey's Studentized Range (HSD) Test was completed. As in Table 4.1, this test found significant differences among Group 1 (*E. lucifuga*) and Group 2 (*E. longicauda* ssp.) in all variables except tail length. *E. lucifuga* had greater snout-vent lengths, head widths, and femur lengths. *E. longicauda* ssp. had a greater costal groove count. There was a

difference in tail length, with *E. lucifuga* being greater, but it did not surpass the minimum significant difference value.

Table 4.1 MANOVA Results. Analysis of morphological measurements (mm)

Measurements (mm)	Group 1 <i>E. lucifuga</i>	Group 2 <i>E. longicauda ssp.</i>	Minimum Significant Difference
Snout-Vent Length	59.476	52.477	4.0139
Tail Length	79.597	73.445	7.6057
Head Width	9.2853	7.5227	0.556
Femur Length	5.8529	5.1045	0.3947
Costal Grooves	12.6176	13.4545	0.346

The scores of the Principal Components Analysis (PCA) were calculated as a scatter plot using SAS[®] and reproduced in Figure 4.3. In this plot, two distinct clusters are evident and are outlined by large circles. The circle on the top, left side is comprised primarily of *E. longicauda ssp.*, and the circle toward the bottom, right side is comprised mainly of *E. lucifuga*. Most of the outliers are juveniles of either species. The small circle in the middle is to simply note that one of the questionable specimens did not cluster with either group. Two components make up for 80% of the clustering exhibited in this plot.

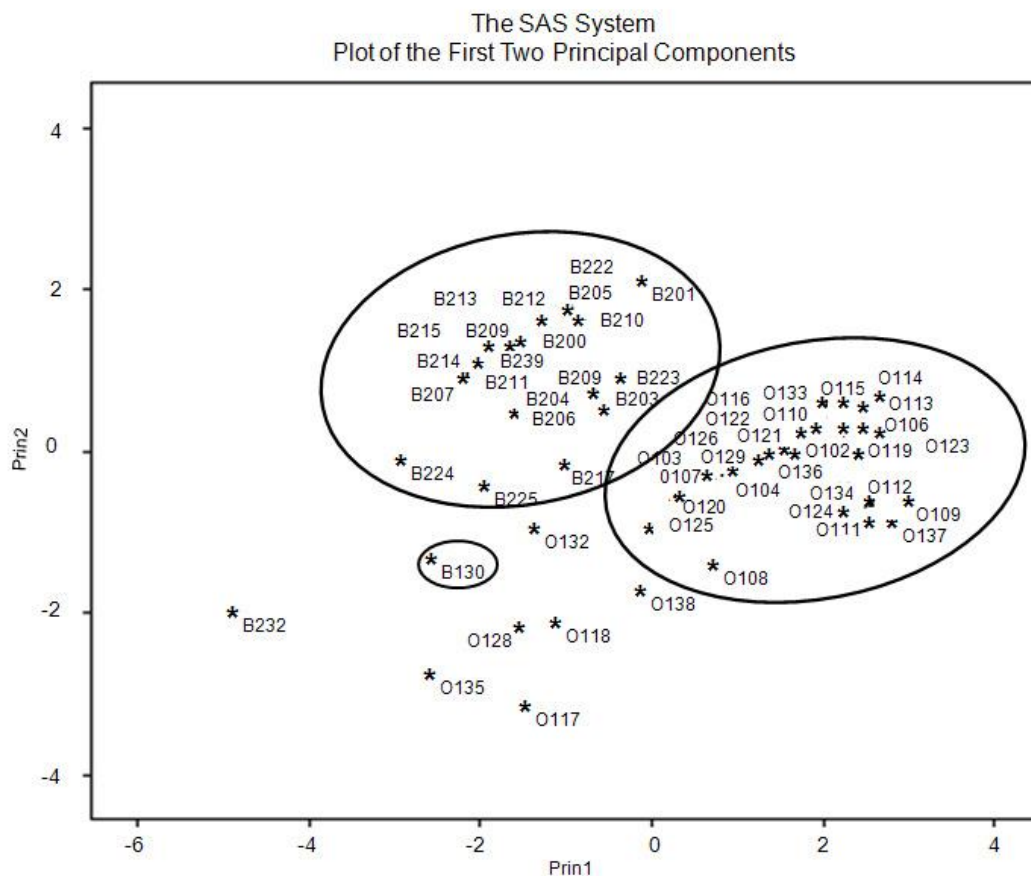


Figure 4.3 PCA Scatter Plot. Principal components analysis showing distinct clustering of two groups in scatter plot. Upper, left circle is comprised of *E. longicauda* ssp., lower, right circle is comprised of *E. lucifuga*. Outliers are juveniles of either species. Small circle in the center notes the unknown specimen does not seem to cluster with either group.

4.3. GENETIC ANALYSIS

4.3.1. DNA Extraction and Primer Results. Of the 82 tissue samples collected, DNA was extracted successfully on all but one individual. The concentrations from each sample ranged from 7.7 ng/ul to 111.4 ng/ul. Of the ten primers selected for this study, only two were used in the final run. Primers ENS6, ENS13, ENS20 did not show any quality results in screening the primers with the 3% agarose gel. Based on the amount of significant bands in the gels, Dte4, Dte8, PCII14, and ENS4 were chosen to be used as

labeled primers for the capillary sequencer. During the testing phase of the capillary analyzer, samples with multiplexed Dte4 and PCII14 had multiple peaks. Dte8 did not show up at all except for a few samples but only directly under the peaks from PCII14 (this is further discussed in the technical issues section of the next chapter); hence, it was not used in the remaining tests. ENS4 only had 1-2 medium peaks and only worked well in some samples. Because of the inconsistency and drastic difference in significant peak numbers compared to the other two primers, ENS4 was not used in the remaining experiments.

4.3.2. Capillary Genetic Analyzer Results. Of the 81 successful DNA extractions, 76 were run in the 3130 Applied Biosystems 4-Capillary Genetic Analyzer using a 96-well plate with the two primers and size standard multiplexed, allowing all reactions to be run just once. Each electropherogram plot was manually scored as no peaks (0), less than five peaks (.5), 5 or more peaks, (1) based on being able to see peaks of at least 500 nm. Two samples scored a 0 but did show the size standard, most likely indicating that the PCR did not work for those reactions. Therefore, these samples were not considered for further analysis. Two other samples also were removed from analysis because they appeared to be “super-amplified”; there were well over 20 peaks over 2000 nm when most other samples would only have at most one or two over 2000 nm (further discussion about these samples is included in the next chapter); these samples also were excluded from further analysis. This resulted in 72 specimens being used for the evaluation: 31 *E. longicauda ssp.*, 37 *E. lucifuga*, and 4 unknown specimens.

Microsatellite primers used in this study ultimately did not indicate microsatellite alleles in either of these species. Thus the original goal of analyzing the genetics of this

hybrid zone was not achieved. This was a known potential problem from the beginning of the study because microsatellite primers were used that were not specifically designed for either species. However, the individuals still could be compared based on genetic differences; using techniques similar to RAPD, a comparison of the banding patterns of all the individuals still could be accomplished.

Thorough examination of the electropherograms was completed to determine banding patterns of each individual. A sample of an electropherogram for one individual is shown in Figure 4.4. This figure includes an image of one plot with all dyes displayed. The orange peaks represent the lane standard, the blue peaks represent the fragments amplified with Dte4, and the green peaks represent fragments amplified with PCII14.

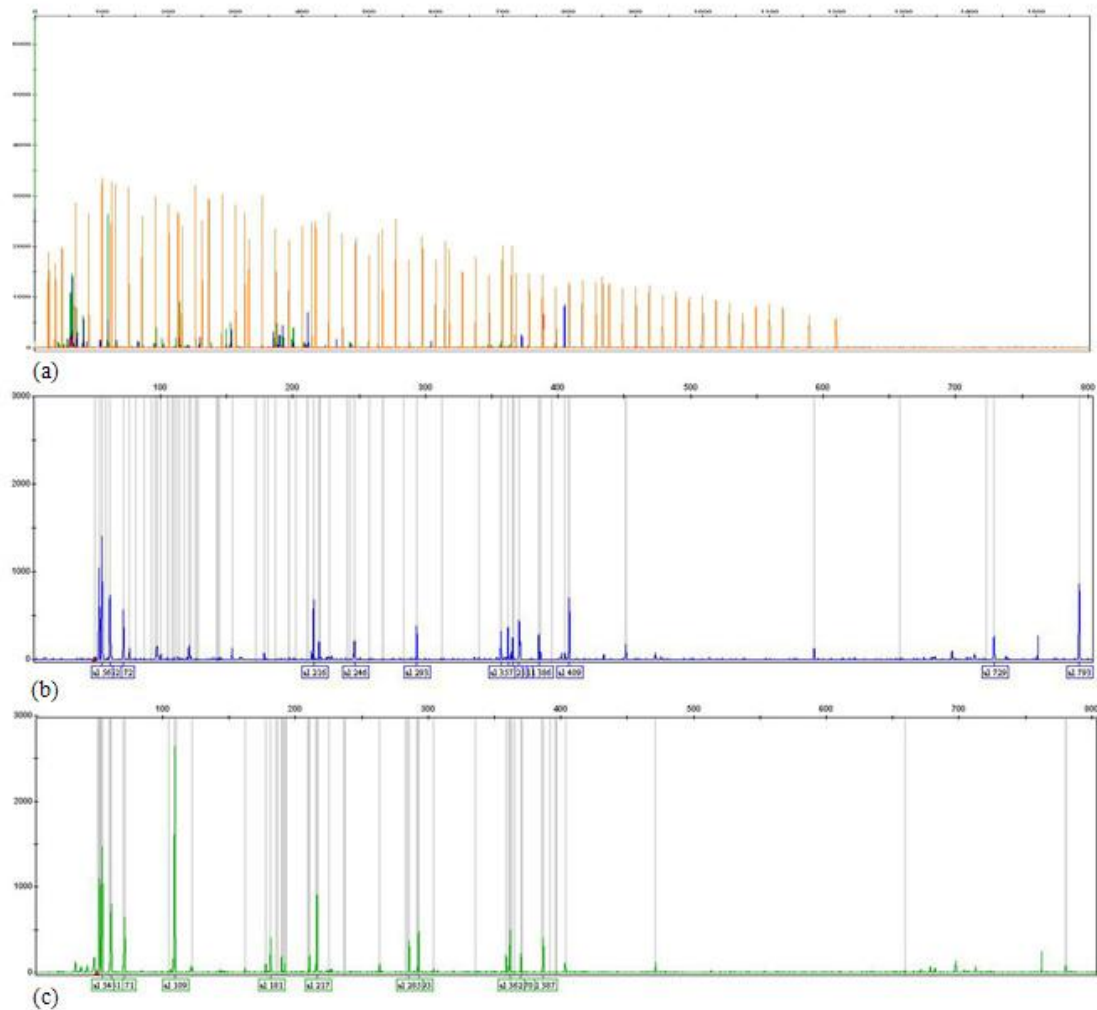


Figure 4.4 Sample Electropherogram. (a) plot showing size standard (orange), fragments amplified by Dte4 (blue), and fragments amplified by PCII14 (green) (b) plot displaying bins marked for Dte4, numbered boxes indicate significant peaks (>200nm) marked for the banding pattern of this specimen (c) plot displaying bins marked for PCII14, numbered boxes indicate significant peaks (>200nm) marked for the banding pattern of this specimen

4.3.3. Phylogenetic Tree. Drawgram v. 3.69 was the program used to plot the tree calculated by Mix. The tree is depicted in Figure 4.5.

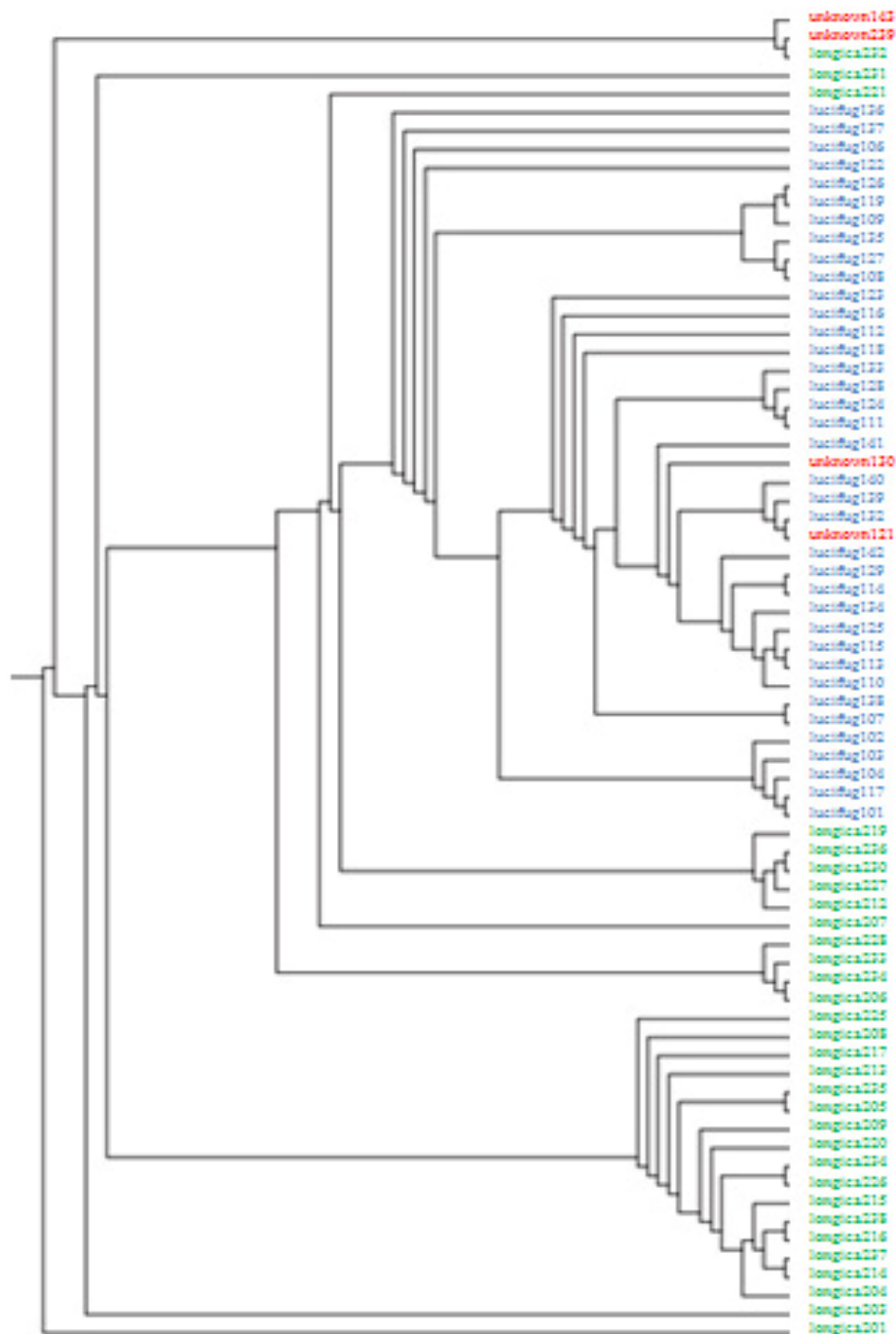


Figure 4.5 Phylogenetic Tree. Phylogenetic tree constructed from banding patterns displaying *E. lucifuga* (blue), *E. longicauda* ssp. (green) and unknown specimens (red); showing one main group for *E. lucifuga* including two unknowns, a few separate groups for most *E. longicauda* ssp., and seven outliers from the main groups (image modified from Drawgram)

5. DISCUSSION

5.1. MORPHOLOGICAL ANALYSIS

The morphological analysis did show significant differences in the morphology of the two species, and the scatter plot derived from the Principal Components Analysis also grouped most of each species in distinct clusters. The outliers were all juveniles of either species except for individual B130 (unknown130), which was listed as a questionable specimen. The morphological data of B130 is as follows: SVL = 44.4, TL = 53.0, FL = 4.3, and HW = 6.9. When comparing this information to the mean measurements calculated through the MANOVA test, this individual is physically smaller in all four measurements. It was recorded in the field notes that this individual appeared to be an adult based on the swollen testes. Combining this information with the genetic analysis and placement of B130 in the phylogenetic tree, rather than this being a hybrid of the two species, it appears more likely this individual is a small *E. lucifuga*.

5.2. GENETIC ANALYSIS

5.2.1. Primers. The primers chosen for this study ultimately did not provide the genetic markers needed for successful microsatellite analysis of the individuals in this study. However, unique banding patterns were rendered for each individual based on the numerous fragments of different lengths that did amplify with some of primers Dte4 and PCII14. The phylogenetic analysis grouped all *E. lucifuga* and two unknown individuals together. Most *E. longicauda* ssp. were grouped in a smaller group separate from *E. lucifuga*. There were seven individuals that were outliers on the tree.

5.2.2. Unknowns and Outliers. The unknowns were B121 (unknown121), B130 (unknown130), G143 (unknown143), and B239 (unknown239). B121 and B130 grouped neatly within the *E. lucifuga* sections of the tree. As discussed in the previous section, it is likely B130 was simply a juvenile *E. lucifuga*. B121 clustered with the *E. lucifuga* in the scatter plot, so it is likely this was also *E. lucifuga* with some unusual elongated markings on its dorsum.

G143 could not be included in the morphological analysis as there were no measurements taken in the field with this specimen due to the MS-222 not working properly; see the section on technical issues for further explanation. The visual assessment of this individual made it very difficult to determine which species it was because it had distinct markings of both species including round, dark spots on the dorsum similar to *E. lucifuga*, but elongated, vertical bars down both sides, similar to *E. l. longicauda*. Its coloring was a dull orange, not the typical brownish-yellow of the *E. l. longicauda* but not the bright orange of the *E. lucifuga*. This specimen's banding pattern made it distinct from all other individuals sampled. It is possible this is an *E. l. longicauda* with unusual markings because that species does exhibit some variance in coloration throughout its range. However, based on the evidence in the visual assessment and not grouping specifically with either species, it also is possible this specimen could be of mixed ancestry.

B239 was the specimen taken back to the lab for further collaboration with a colleague. This specimen clustered with *E. longicauda ssp.* in the morphological analysis. Upon seeing the specimen in the light and collaborating with a colleague, it was determined this specimen is likely *E. l. longicauda*. However, for the sake of the study, it

was left listed as an unknown because it was originally deemed an unknown in the cave and removed only because it was potentially a hybrid. Based on genetic analysis, this individual grouped with G143 and B232 (*longicauda*). It is possible this individual is an *E. l. longicauda* or an *E. longicauda* intergrade but because of some of the unusual markings, and its grouping with another unknown specimen, it also is possible this specimen is of mixed ancestry with *E. lucifuga*.

In the field notes, B232 was recorded as juvenile *E. longicauda ssp.* with a single question mark. This indicated there were some concerns with the identification but nothing specific enough to warrant it an unknown specimen. This specimen appears as an outlier in the scatter plot of the morphological data, which is likely because this individual is a juvenile. However, based on the genetic analysis, it is also an outlier in the tree and grouped with two other unknown specimens (B239 and G143). Because B239 could have been an *E. l. longicauda*, and B232 was recorded as an *E. longicauda ssp.*, it is possible this entire group is *E. l. longicauda*. They could be separate from the main groups because the other *E. longicauda ssp.* were primarily *E. l. melanopleura*. Or, it is possible that all three of these specimens are of mixed ancestry to some degree.

The remaining outliers include: B201, B203, B221, and B231. Visual assessment of all four individuals indicated these were *E. l. melanopleura*. B201 and B203 were clustered with the *E. longicauda ssp.* based on the morphological analysis. Because B231 was listed as a juvenile in the field notes, it was believed this was the cause of it being an outlier in the scatter plot. B221 was another specimen for which measurements were not obtained due to MS-222 not working properly and no successful photograph was obtained due to humidity in the cave. There were no field notes indicating questions

about identity. As with all of the outliers it is possible these are *E. longicauda* intergrades or they are of mixed ancestry with *E. lucifuga*, creating a banding pattern as to not group with either groups.

After manually reviewing the banding patterns of the outliers, certain patterns were noted. All six did not have 16 bands present in all or most *E. lucifuga*. The three outliers which grouped together had four bands present; no other outliers had this characteristic, and it was only sporadically present throughout the main two groups.

5.2.3. Genetics Summary. The banding patterns could only indicate when there were strong similarities among individuals' genetics. If there were not enough similarities to be included in a group, individuals became outliers to those groups. Unfortunately there is no way to determine the species identity of the outliers from banding patterns alone. This particular set of samples is difficult to analyze for banding patterns because of the likely *E. longicauda* intergrades present in this region. However, the genetic results at least do present some evidence of individuals with banding patterns outside the typical representative of either species. These unusual banding patterns could be due to some type of hybridization occurring, resulting in mixed ancestry.

5.3. TECHNICAL ISSUES

5.3.1. Equipment Issues. It is worth mentioning a few difficulties both for explanation of missing data in this study and as guidance for those planning similar research in the future. The primary issues were equipment malfunctions. Deep within the caves surveyed for this study a wet and humid environment was found. Taking pictures became very difficult at times due to the humidity fogging up the lens. Even if the lens

was wiped clean, a clear picture of the salamander was not possible because of the thick moisture in the air between the camera and the salamander. Because of this, some photographs of individuals did not provide clear views of the salamanders in question. Another problem occurred when the anesthetic, MS-222 did not work properly. In general, the solution seemed to work better on *E. lucifuga* than *E. longicauda ssp.* as it worked faster and they recovered more quickly. However, some days, the MS-222 simply did not anesthetize the salamanders. Different things were attempted, such as making a new solution for each visit, using the same solution for each cave, making a slightly stronger solution, and even leaving the salamanders in the solution for more than seven minutes. It is possible that using the same solution more than a few times could cause the solution to become diluted with dirt from the salamanders, thereby affecting its efficacy. When the salamanders were left in the solution for longer than seven minutes, it took them much longer to recover. This raised concerns of the long-term effects on the salamanders from the solution. The stronger solution did not seem to make a difference, and it was important not to make it too strong as this same substance is used to euthanize specimens in stronger concentrations. A brand new bottle of MS-222 was used and this did not make a consistent difference. Whatever the cause of the MS-222 difficulties, it resulted in nearly a third of the salamanders not being measured, including a few outliers of the genetic analysis. This likely had some impact on the results of this study.

5.3.2. Primer Issues. There were no existing primers specifically designed for either species, so an attempt was made to find primers developed for closely related species that were successful with closely related species (Curtis and Taylor 2000, Boyle 2005). Ten primers were screened and four of them appeared to be good candidates. Four

has been shown to be an adequate number to determine relationships among different species (Boecklen and Howard 1997). Had all four primers worked effectively as microsatellite primers, conclusive evidence likely would have been collected regarding the identity of questionable specimens.

Primer Dte8 did not appear to work properly when run in the capillary genetic analyzer. The labeled primer Dte8 only appeared to peak a few times in the very same places as PCII14 even though it did not when screened as unlabeled in agarose gel. These primers were different sequences, so the chances of the same fragments being amplified each time only in some places, with no amplification anywhere else is very unlikely. What may have caused this problem is unknown. It is possible the primer was not properly manufactured or an error occurred while preparing in the lab. Either way, it was unfortunate because it was a strong candidate for a successful microsatellite primer when the agarose gels were carried out. This was discussed with the DNA lab supervisor, and it was recommended that this primer not be used in the study because it would most likely misrepresent that primer. Also, it would not have resulted in any additional bands in the final set that was used to compare banding patterns. More markers were needed than the two primers that were used in the final analysis for any conclusive genetic evidence of hybridization, and it would have been useful for this primer to have worked effectively. Due to time constraints and funding concerns, this primer was not re-ordered to see if it might work differently.

A final issue with the primers likely did not impact the study, but is still worth mentioning. Two samples were removed from final analysis due to “over-amplification”. This is the best way to describe what happened with these two samples. When the

electropherograms were examined, it was noticed there were numerous peaks (>50) binned on both samples. Most peaks were over 2000 nm with the noise registering many small peaks. It was difficult to discern which peaks were significant and which ones were not, so the samples were removed from the final analysis. The cause of this is unknown. Many factors are involved in the final analysis of these products. It is possible there was a lab error while preparing the PCR, the capillary genetic analyzer may have had an error, or something else entirely may have been the source of the problem.

5.4. ENHANCEMENTS FOR THE PROJECT

5.4.1. Increase Sampling. More conclusive evidence also may have been uncovered if more samples had been included in this study. A few issues may have impacted the success of collecting samples in this study. Approval for a wildlife collector's permit from the Department of Conservation took longer than expected, so the initial field work was started later in the year than originally planned. Also, due to medical issues of the field investigator, no field work was done for two very wet months that were likely the ideal to find salamanders. Additionally, the coldest February in decades occurred during the span of the approved collection time, which may have inhibited the movement of the salamanders even within the cave systems; it also could be the reason that so few salamanders were found in that month. Only one breeding season was including in the time the field work was conducted. During the first breeding season, most of each species were collected, so perhaps including another breeding season would have increased the sample size. Gourd Creek Cave was full of small cobble and gravel, giving the salamanders numerous places to hide and escape from capture. These

conditions made it difficult to spot and catch salamanders, which is possibly the cause of fewer samples being collected in this cave (rather than the cave having a significantly smaller population). Also, permission to use this cave for research was received later than the other two caves, so surveying the cave commenced later than the other caves. Asking for permission on permits to sample a salamander not closely related to these species would have also been valuable to the study. This was not originally done because the microsatellites were meant to be specific to the species. However, early in this investigation there was consideration that if the microsatellites did not work, a banding pattern could likely be assessed; that plan should have included the need for a control group.

5.4.2. Improve Genetic Analysis. Effective microsatellite primers would have improved greatly the genetic analysis of this study. Even with this small sample size, more conclusive evidence may have been available through effective microsatellite primers. Only ten primers were selected to screen for potential success, and upon later discussion with an expert in microsatellites, it was learned that at times, many more primers (i.e. >50) are sometimes screened in this type of process. It might have helped to spend more time optimizing the primers that were chosen for the capillary genetic analyzer to see if they were successful at amplifying only the microsatellite segments.

5.5. FURTHER RESEARCH

Based on the visual assessments, morphological analysis, genetic analysis, and related literature gathered in this study, there are numerous opportunities for further research. A glaring need is microsatellite primer development for either or both of these

species. As mentioned earlier, *E. l. longicauda* have been reported to sometimes include another subspecies or to potentially hybridize with that species (*E. guttolineata*) in addition to its subspecies, *E. l. melanopleura*, and *E. lucifuga*. Microsatellites are invaluable in assessing kinship, so for this species in particular, the primer development would prove useful in hybrid zone studies and taxonomic distinctions. To enhance this particular study, more samples from these caves and other caves inside the hybrid zone as well as outside the hybrid zone would be useful. As control groups, even with the microsatellites, samples from *E. longicauda ssp.* populations whose range does not overlap with the *E. lucifuga* also would be a worthy addition. If evidence of hybridization is conclusive, the next steps could be to determine fitness of the hybrids in a natural setting, determine the degree of hybridization and backcrosses, and even do laboratory controlled testing of crosses among the two species. Additionally, a long-term study could also be conducted regarding reinforcement mechanisms comparing the success of hybridization of the *E. longicauda ssp.* compared to the hybridization success *E. longicauda ssp.* with *E. lucifuga*.

6. CONCLUSION

The world has an enormous variety of species. What makes each species so different from each other and how they developed throughout time is still a driving force behind many evolutionary studies of today. As evolution can be a series of slow processes, it is often impossible to witness the process. However, hybrid zones can provide a rare opportunity to see evolutionary processes in action. The processes involved in speciation are of particular interest within hybrid zones as this is where there is an apparent breakdown of barriers that prevent the interbreeding of species. The mechanisms of reinforcement support the advance of reproductive isolating mechanisms among closely related species, and thereby may be able to be studied within hybrid zones in which different degrees of interbreeding occur.

Salamander hybrid zones are quite common due to frequent lack of pre- or post-zygotic reproductive barriers. Members of the Plethodontidae are regularly used in hybrid zones studies due in part to the rapid diversification that occurred within subfamilies and genera in this family. In Missouri, a known hybrid zone exists among *E. longicauda ssp.* Within and near this zone have been a few reports of interbreeding of sister taxa, *E. longicauda ssp.* and *E. lucifuga*. There is no clear documentation of whether this hybridization is occurring sporadically throughout the ranges of these species or along a narrow zone, similar to the *E. longicauda ssp.* hybrid zone, to what degree the hybridization may be occurring, and how fit the hybrids are. Determining any or all of this information may provide some insight into the nature of reinforcement mechanisms within these species.

The objective of this study was to determine if hybridization of *E. longicauda ssp.* and *E. lucifuga* was occurring throughout the already established hybrid zone of *E. longicauda ssp.* This was attempted through surveying three cave sites found within the known hybrid zone. Morphological analysis showed significant differences among the two species, with *E. lucifuga* exhibiting more robust features but *E. longicauda ssp.* having more costal grooves. Microsatellites were chosen as the DNA markers to perform the genetic analysis. Although the primers chosen did not successfully amplify microsatellite alleles, banding patterns were produced to provide unique identities to each individual. A total of six outliers were plotted on the phylogenetic tree constructed from these banding patterns. Three of the outliers that grouped together included two unknown specimens. It is possible that all three in this group are *E. l. longicauda* rather than the typical *E. l. melanopleura* that was found. It also is possible that all of the outliers are of mixed ancestry, which is why none of them group with the two main groups. More definitive DNA evidence is needed before a valid conclusion can be made.

Although this study did not result in conclusive evidence of hybridization, it did bring to light some topics for further research---i.e., more extensive laboratory studies should be completed to gather evidence of hybrid fitness and microsatellite primers need to be developed for these species. This would allow for more intense assessments of relatedness of individuals. If hybridization is occurring, but to a lesser degree than with *E. longicauda ssp.* hybrids, then this hybrid zone may provide an ideal natural setting in which to examine mechanisms of reinforcement.

APPENDIX A

PROCEDURE FOR DNA EXTRACTION FROM ANIMAL TISSUE

APPENDIX A

Procedure for DNA Extraction from Animal Tissue

1. Allow tissue samples to thaw to room temperature (15-25°C).
2. Bath tissue sample in deionized water to remove most of the ethanol. This was completed by filling a 1.5 ml microcentrifuge tube with the deionized water, placing the tissue sample in the tube, and shaking vigorously for 5-10 seconds. After removing the tissue sample, fresh water was placed in the tube and the process was repeated. This was done 10-15 times for each sample.
3. All tubes were labeled before putting tissue samples and different mixtures in the tubes.
4. Buffer AW1 and Buffer AW2 from the DNeasy[®] kit were supplied as concentrates, so 25 ml and 30 ml of 100% ethanol were added, respectively, as instructed on the bottle label.
5. Following DNeasy[®] Blood and Tissue Handbook, the following steps were taken to extract the DNA:
 - a. Tissue sample was cut in half to aid in more efficient lysis as suggested by this handbook. The tissue was placed in a 1.5 ml microcentrifuge tube and 180 µl of Buffer ATL (provided in the kit) were added.
 - b. 20 µl of proteinase K (provided in the kit) was added and the mixture was thoroughly mixed by vortexing for 5-10 seconds.
 - c. Tissue sample was incubated at 56°C in thermal cycler for 4 hours. After the first 2 hours, the sample was vortexed for 5 seconds to aid in the lysis, and then placed back in the thermal cycler for 2 more hours.
 - d. At the end of the four hours, the sample was vortexed for 15 seconds. 200 µl of Buffer AL was added and the sample vortexed again for 5 seconds. 200 µl of 100% ethanol was then added and the sample vortexed again for 5 seconds.
 - e. The mixture was pipetted into a DNeasy Mini spin column (provided in the kit) that was placed in a 2 ml collection tube (provided in the kit). The tube was then centrifuged for 1 minute at 8000 rcf.

- f. Spin column was placed in new 2 ml collection tube (provided in the kit), 500 μ l of Buffer AW1 added, and tube centrifuged for 1 minute at 8000 rcf.
 - g. Spin column was placed in new 2 ml collection tube (provided in the kit), 500 μ l of Buffer AW2 added, and centrifuged for 5 minutes at 16,100 rcf (adjusted from handbook protocol suggesting 3 minutes at 20,000 rcf because the centrifuge used had 16,100 maximum rcf).
 - h. Spin column placed in 1.5 ml microcentrifuge tube and 200 μ l of Buffer AE pipetted onto the spin column membrane. Incubated at room temperature for 1 minute and then centrifuged for 1 minute at 8000 rcf.
 - i. As suggested by the DNeasy handbook, for maximum yield, this step was repeated with new 1.5 microcentrifuge tube.
6. The concentration of each eluate was then measured with the NanoDrop[®] Spectrophotometer ND-1000 and ND-1000 v3.2 software, recorded and the sample frozen at -20°C until ready to complete PCR.

APPENDIX B
PROCEDURE FOR MICROSATELLITE PCR

APPENDIX B

Procedure for Microsatellite PCR

1. Template DNA and the Type-it™ Microsatellite PCR kit (Qiagen) were thawed to room temperature (15-25°C).
2. Microcentrifuge tubes containing DNA were spun-down, 0.5 ml microcentrifuge tubes labeled for PCR.
3. 12.5 µl of 2x Type-it Multiplex PCR Master Mix (provided by kit), 2.5 µl of Dte4 primer and 2.5 µl of PCII14 primer, and approximately 100 ng of DNA (4 µl of eluate) added to the 0.5 ml microcentrifuge tube. Mixture was gently mixed by shaking tubes and then spun-down.

Note: This kit suggests mixture be brought to a final volume of 25 µl by addition of RNase-free water (provided by kit). Through some experimenting, better amplification was found without this addition for these primers.
4. PCR tubes were placed in an Eppendorf Mastercycler thermal cycler programmed for a 3-step, 32-cycle. An initial step of 5 minutes at 95°C to activate the HotStar Taq Plus DNA Polymerase was followed by the 3-step cycling: denaturing for 30 seconds at 95°C , annealing for 90 seconds at 55°C (adjusted from 60°C as recommended by kit), and elongation for 30 seconds at 72°C. After 32 cycles, there was a final extension step of 30 minutes at 60°C and then a final hold at 4°C.
5. Samples were spun-down and then prepared for capillary genetic analyzer the same day.

BIBLIOGRAPHY

- Allendorf FW, Leary RF. 1988. Conservation and distribution of genetic variation in a polytypic species: the cutthroat trout. *Conserv Biol* 2:170-184.
- Allendorf FW, Leary RF, Spruell P, Wenburg JK. 2001. The problem with hybrids: setting conservation guidelines. *Trends Ecol Evol* 16(11):613-622.
- Alexandrino J, Baird SJE, Lawson L, Macey JR, Moritz C, Wake DB. 2005. Strong selection against hybrids at a hybrid zone in the *Ensatina* ring species complex and its evolutionary implications. *Evolution* 59(6):1334-1347.
- Arntzen JW, Wallis GP. 1991. Restricted gene flow in a moving hybrid zone of the newts *Triturus cristatus* and *T. marmoratus* in western France. *Evolution* 45:805-826.
- Barton NH, Hewitt GM. 1985. Analysis of hybrid zones. *Ann Rev Ecol Syst* 16:113-148.
- Bensch S, Åkesson M. 2005. Ten years of AFLP in ecology and evolution: why so few animals? *Mol Ecol* 14:2899-2914.
- Boecklen WJ, Howard DJ. 1997. Genetic analysis of hybrid zones: numbers of markers and power of resolution. *Ecology* 78(8):2611-2616.
- Bogart JP, Licht LE. 1986. Reproduction and the origin of polyploids in hybrid salamanders of the genus *Ambystoma*. *Can J Genet Cytol* 28:605-617.
- Bogart JP, Ellinson RP, Licht LE. 1989. Temperature and sperm incorporation in polyploidy salamanders. *Science* 246:1032-1034.
- Bogart JP. 2003. Genetics and systematics of hybrid species. In: Sever DM, editor. Reproductive Biology and Phylogeny of Urodela. Enfield (NH):Science Publishers. p. 109-134.
- Boyle M. 2005. Genetic distinctiveness of a peripheral Illinois population of the southern two-lined salamander (*Eurycea cirrigera*) [thesis]. [Chicago (IL)]: Chicago State University. p. 2-20.
- Carlin JL. 1997. Genetic and morphological differentiation between *Eurycea longicauda longicauda* and *Eurycea guttolineata* (Caudata: Plethodontidae). *Herpetologica* 53:206-217.
- Connors LM, Cabe PR. 2003. Isolation of dinucleotide microsatellite loci from red-backed salamander (*Plethodon cinereus*). *Mol Ecol Notes* 3:131-133.
- Coyne JA, Orr HA. 1998. The evolutionary genetics of speciation. *Phil Trans R Soc Lond B* 353:287-305.

- Curtis JM, Taylor EB. 2000. Isolation and characterization of microsatellite loci in the Pacific giant salamander *Dicamptodon tenebrosus*. *Mol Ecol* 9:107-118.
- DNA Sequencing. [2002]. Madison (WI): University of Wisconsin; [cited 2010 April 30]. Available from:
http://bioweb.uwlax.edu/genweb/molecular/theory/dna_sequencing/dna_sequencing.htm
- Fitzpatrick BM, Placyk JS, Niemiller ML, Casper GS, Burghardt GM. 2008. Distinctiveness in the face of gene flow: hybridization between specialist and generalist gartersnakes. *Mol Ecol* 17:4107-4117.
- Frost DR. 2010. Amphibian Species of the World: an Online Reference. Version 5.4 (8 April, 2010). Electronic Database accessible at
<http://research.amnh.org/vz/herpetology/amphibia/>
 American Museum of Natural History, New York, USA.
- Grant P, Grant R. 1992. Hybridization of bird species. *Science* 256(5054):193-197.
- Guttman SI, Karlin AA. 1986. Hybridization of cryptic species of two-lined salamanders (*Eurycea bislineata complex*). *Copeia* 1:96-108.
- Harrison RG. 1986. Pattern and process in a narrow hybrid zone. *Heredity* 56:337-349.
- Harrison RG. 1993. Hybrids and hybrid zones: historical perspective. In Harrison RG, editor. *Hybrid Zones and the Evolutionary Process*. New York: Oxford University Press. p. 3-12.
- Heaney L, Timm R. Morphology, genetics, and ecology of pocket gophers (genus *Geomys*) in a narrow hybrid zone. *Biol J Linnean Soc* 25:301-317.
- Hewitt GM. 1988. Hybrid zones-natural laboratories for evolutionary studies. *Trends Ecol Evol* 3(7):158-167.
- Highton R. 1995. Speciation in eastern north American salamanders of the genus *Plethodon*. *Annu Rev Ecol Syst* 26:579-600.
- Highton R, Peabody R. 2000. Geographic protein variation and speciation in salamanders of the *Plethodon jordani* and *Plethodon glutinosus* complexes in the southern Appalachian Mountains with the description of four new species. In: Bruce RC, Jaeger RG, Houck L, editors. *The Biology of Plethodontid Salamanders*. New York(NY): Kluwer Academic/Plenum Publisher. p. 31-94.
- Hutchison VH. 1958. The distribution and the ecology of the cave salamander, *Eurycea lucifuga*. *Ecol Monograph* 28(1):2-20.

- Kozak KH, Blaine RA, Larson A. 2006. Gene lineages and eastern North American palaeodrainage basins: phylogeography and speciation in salamanders of the *Eurycea bislineata* species complex. *Mol Ecol* 15:191-207.
- Kozak KH, Weisrock DW, Larson A. 2006. Rapid lineage accumulation in a non-adaptive radiation: phylogenetic analysis of diversification rates in eastern North American woodland salamanders (Plethodontidae: *Plethodon*). *Proc R Soc B* 273:539-546.
- Kozak KH, Mendyk RW, Wiens JJ. 2009. Can parallel diversification occur in sympatry? Repeated patterns of body-size evolution in coexisting clades of North American salamanders. *Evolution* 63(7):1769-1784.
- Lanyon W. 1979. Hybrid sterility in meadowlarks. *Nature* 279:557.
- Larson A, Weisrock DW, Kozak KH. 2003. Phylogenetic systematics of salamanders (Amphibia: Urodela), a review. In Sever DM, editor. *Reproductive Biology and Phylogeny of Urodela (Amphibia)*. Enfield, New Hampshire: Science Publishers, Inc. p. 31-108.
- Myers K. 2009. Identification of *Eurycea* using cytochrome *b* [thesis]. [Lynchburg (VA)]: Liberty University. p. 3-32.
- Micheli MR, Bova R, Pascale E, D'Ambrosio E. Reproducible DNA fingerprinting with the random amplified polymorphic DNA (RAPD) method. 1994. *Nucleic Acids Res* 22(10):1921-1922.
- Moore W, Koenig W. 1986. Comparative reproductive success of Yellow-shafted, Red-shafted, and hybrid flickers across a hybrid zone. *The Auk* 103:42-51.
- Pearse DE, Crandall KA. 2004. Beyond F_{ST} : analysis of population genetic data for conservation. *Conser Genet* 5:585-602.
- Petranka JW. 1998. Salamanders of the United States and Canada. Washington DC/London: Smithsonian Institution Press. p. 157-158, 254-261.
- Planes S, Doherty PJ. 1997. Genetic and color interactions at a contact zone of *Acanthocromis polyacanthus*: a marine fish lacking pelagic larvae. *Evolution* 51(4): 1232-1243.
- Potter M. 2008. AFLP fingerprint analysis of hybrid salamanders in the Missouri caverns section of Onondaga cave [thesis]. [Rolla (MO)]: Missouri University of Science and Technology. p. 1-111.
- Queller DC, Strassmann JE, Hughes CR. 1993. Microsatellites and kinship. *Trends Ecol Evol* 8:285-288.
- Ridley M. 2003. *Evolution*. Oxford: Blackwell Publishing.

- Riley S, Shaffer HB, Voss RS, Fitzpatrick BM. 2003. Hybridization between a rare, native tiger salamander (*Ambystoma californiense*) and its introduced congener. *Ecological Applications* 13(5):1263-1275.
- Rising JD. 1983. The progress of Oriole hybridization in Kansas. *Auk* 100:885-897.
- Sattler PW. 1985. Introgressive hybridization between the spadefoot toads *Scaphiopus bombifrons* and *S. multiplicatus* (Salienta: Pelobatidae). *Copeia* 1985:324-332.
- Seehausen O, van Alphen JJM, Witt F. 1997. Cichlid fish diversity threatened by eutrophication that curbs sexual selection. *Science* 277:1808-1811.
- Seehausen O. 2004. Hybridization and adaptive radiation. *Trends Ecol Evol* 19(4):198-207.
- Selkoe KA, Toonen RJ. 2006. Microsatellites for ecologists: a practical guide to using and evaluating microsatellite markers. *Ecology Letters* 9:615-629.
- Servedio M. 2004. The what and why of research on reinforcement. *PLoS Bio* 2(12):2032-2035.
- Schlötterer C. 2000. Evolutionary dynamics of microsatellite DNA. *Chromosoma* 109:365-371.
- Smith PW. 1961. The amphibians and reptiles of Illinois. *Bul Illinois Nat His Sur* 28:1-298.
- Smith C. 1964. The problem of hybridization of the red cave salamander, *Eurycea lucifuga* (Raf), and the long-tailed salamander, *Eurycea longicauda melanopleura* (Green). *Arkansas Academy of Science Proceedings*, 18: 59-62.
- Sweet S. 1984. Secondary contact and hybridization in the Texas cave salamanders *Eurycea neotenes* and *E. tridentifera*. *Copeia*, 1984(2):428-441.
- Taberlet P, Waits LP, Luikart G. 1999. Noninvasive genetic sampling: look before you leap. *Trends Ecol Evol* 14:323-327.
- Tilley S, Merritt R, Wu B, and R Highton. 1978. Genetic differentiation in salamanders of the *Desmognathus ochrophaeus* complex (Plethodontidae). *Evolution*, 32:95-115.
- Voss SR, Shaffer HB. 1996. What insights into the developmental traits of urodeles does the study of interspecific hybrids provide? *Int J Dev Biol* 40:885-893.
- Wake DB, Yang SY, Papenfuss TJ. 1980. Natural hybridization and its evolutionary implications in Guatemalan Plethodontid salamanders of the genus *Bolitoglossa*. *Herpetologica* 36(4):335-345.

- Wiens JJ, Enstrom TN, Chippendale PT. 2006. Rapid diversification, incomplete isolation and the “speciation clock” in North American salamanders (Genus *Plethodon*): testing the hybrid swarm hypothesis of rapid radiation. *Evolution* 60(12):2585-2603.
- Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res* 18(22):6531-6535.
- Zhang D, Hewitt GM. 2003. Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. *Mol Ecol* 12:563-584.

VITA

Bonnie Jean Beasley was born on June 9, 1980. She was raised in rural Texas County, near Houston, Missouri. She attended Houston High School where she graduated as valedictorian in May 1998. She attended the University of Missouri in Columbia, Missouri and received a Bachelor of Arts degree in Psychology in May 2002. After graduating, she spent five years working in social work with the elderly and disabled. Although her work in this field was rewarding, she knew her true passion was in the field of science. Because of her interest in science, she decided to enroll in the Applied and Environmental Biology Master's program at Missouri University of Science and Technology (Missouri S&T) in January 2008. In addition to her thesis, she worked with a team of biologists and computer scientists on an amphibian ontology project. Through this project, she was introduced to the field of bioinformatics and participated in various workshops where she assisted in teaching experts in the herpetological community about the ontology project. In addition to participating in a variety of new academic challenges during this time, she also gave birth to her wonderful child, Hannah. Bonnie received a Master of Science in Applied and Environmental Biology in August 2010. She hopes to share her enthusiasm of science through teaching.