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AFLP FINGERPRINT ANALYSIS OF HYBRID SALAMANDERS IN THE MISSOURI CAVERNS SECTION OF ONONDAGA CAVE

by

MARIA LOUISE POTTER

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

2008

Approved by

Anne Maglia, Advisor
Ronald Frank
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ABSTRACT

Two species and one subspecies of salamander in the genus *Eurycea*, two species of *Plethodon* and one species of *Typhlotriton* (recently considered to be *Eurycea)* salamander currently reside in an area of Onondaga Cave known as the Missouri Caverns section. Due to the presence of two known interbreeding subspecies of salamanders, *Eurycea longicauda* (Long-tailed salamander) and *Eurycea longicauda melanopleura* (Dark-sided salamander), the possibility may exist for interbreeding of one or all of these taxa. Specifically, it is hypothesized that the two species of *Eurycea longicauda* may interbreed with *Eurycea lucifuga* (Cave salamander). Through visual assessment and phenotypic analysis, all known species were identified. Tissue samples were used to identify any undiagnosed specimens through DNA fingerprinting, also known as Amplified Fragment Length Polymorphism (AFLP), a method measuring genotypic differences. This information was used to support evidence of hybridization among the co-existing species. Evidence of hybridization may indicate that the removal of human disturbance in this area may have had a prominent impact on multiple salamander species and their willingness to compete for food and other precious resources.

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First I have to thank my advisor, Dr. Anne Maglia. She has been one of the most helpful and courteous individuals that I have ever encountered. Returning to school later in life provided me an opportunity to deal with situations from a better time management perspective and see things from a much more adult point of view. However, returning to campus twenty years later was a little intimidating to say the least. Dr. Maglia never hesitated to help me find what I needed, whether it was resources on campus or just answering what one might have been considered to be superfluous questions. She took a genuine interest in my project and, 100 percent of the time, pointed me in the right direction. I feel significantly more intelligent for having been exposed to her expertise. Thanks to Dr. Frank for stepping in "at the last minute." Dr. Niyogi was an original committee member, but having taken a position at another university just prior to my graduation, he was no longer available to see this through to fruition. A big thank you goes to James Vandike, a current colleague in my employment who also happens to be a professor at the university. Being able to converse with him both during classes and at work has been an asset. I must also say thank you to Dr. Aronstam, who at every encounter, never hesitated to ask me how my work was going. I say thank you to my computer savvy colleagues, Tiffani Martin and Ben Miller. And, Adam Martin the cDNA lab supervisor; I'm certain I was one of his most remedial students. And last but not least, I have to thank my husband for listening to me ramble on and on each time I learned about a new biological process. If it weren't for his encouragement, I may not have gotten this far.

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1. INTRODUCTION

1.1. BACKGROUND

Until recently, caves and their inhabitants have not necessary been of genuine interest to individuals other than actual cave enthusiasts. Researchers are beginning to realize that underground habitats might be of some scientific significance. Therefore, management practices are turning more toward resource stewardship. In the past, caves were considered to be quite literally, a hole in the ground. What could possibly be living in them that would be advantageous to know about, study or understand? After a few serious cave enthusiasts/researchers began to find unusual species surviving in such extreme environments, investigations of these microbes prompted the development of precautions to minimize the impact on microbial communities (Werker, 1997). Since then, several unusual species as well as useful bacteria have been found. And, studying this extreme environment is leading to a better understanding of surface water contamination, hydrology, and urban runoff.

The purpose of this project focus was twofold. First, to provide an overview of caves and cave ecosystems and discuss how caves are formed, outline their uses, in the past and present, describe the human impact on these systems and provide an introduction to my study, focusing on the potential hybridization of salamanders living in a section of Onondaga Cave that is no longer impacted by humans (and has not been for nearly sixty years). Second, to focus on determining if hybridization of the salamanders in the Missouri Caverns section occurred. I detail the use of AFLP, Amplified Fragment Length Polymorphism, and phenotype analysis

for determining if hybridization has occurred. I show supporting evidence that reversing human impact removes the stressors that might limit the reproduction of a species of amphibian that, like so many other amphibians, is an indicator species for the quality of the environment in which they live.

Caves and cave systems have been around for millions of years. Until recently, the potential as a natural research facility for groundwater movement, unique habitats and unusual and endangered species was not fully realized. Missouri currently has more than 5,700 mapped cave systems deep beneath its many terrestrial habitats. These cave systems boast more than 800 recorded animal species, 64 of which are truly troglobitic (permanent cave dwellers) vertebrates and invertebrates (MoDNR, 2002).

The recent past has brought with it a few cave explorers that wanted to learn more about this important natural resource. Many of these species were stumbled onto while mapping a new cave system. Without the genuine interest of a few individuals, the biology and geology associated with Missouri cave systems would be far less understood.

Missouri is one of the few Midwestern states that can claim magnificent variationin its habitats and natural divisions. Where one state lays claim to just one type of habitat across their entire expanse, Missouri has six different natural divisions housing within them nine different natural communities (Nelson, 2005). Each harbors a massive array of native plants, animals and microorganisms. These assemblages of biota occupy different ecological regions that subsequently shape the structure and composition of natural communities. The diverse communities that make up

Missouri's landscape range from the Glaciated Plains to the North, the Big Rivers section encompassing the Missouri and Mississippi Rivers and the Ozark border region located on either side of the river systems in the center of the state. The Ozark Highlands area is located in the central and southern portion of state and the Mississippi Lowlands comprises the boot heel section of Missouri. While the geographical, ecological and geologic aspects of these areas could be discussed in detail indefinitely, this paper focuses on one natural community or ecological subsection of the Ozark Highland region, the cave ecosystems.

1.1.2. Overview of Cave Ecosystems. Most of Missouri's caves developed in dolomite and limestone and range from the Mississippian to the Ordovician geologic time period, approximately 310-500 million years ago. The majority of solution caves can be found in what is referred to as the karst topography of the Ozark region. This type of landscape is usually characteristic of soluble rock found at or near the ground's surface and is unusually susceptible to water flow. Both the dolomite and limestone of this region are prime examples.

Karst can be deceiving. Ground water percolates swiftly through this type of topography. The integrity of the ecosystem can be quickly violated by any type of contaminant finding its way into the ground water. Gasoline or other volatiles permeating the cave environment could be devastating to the fragile organisms that reside there.

Note the timeline illustrated in Figure 1.1. Over past millennia, erosion has removed rock, leaving layers 500 to 280 million years old exposed at the surface. The oldest exposed rocks are in the bottom of Onondaga Cave - the Eminence Dolomite.

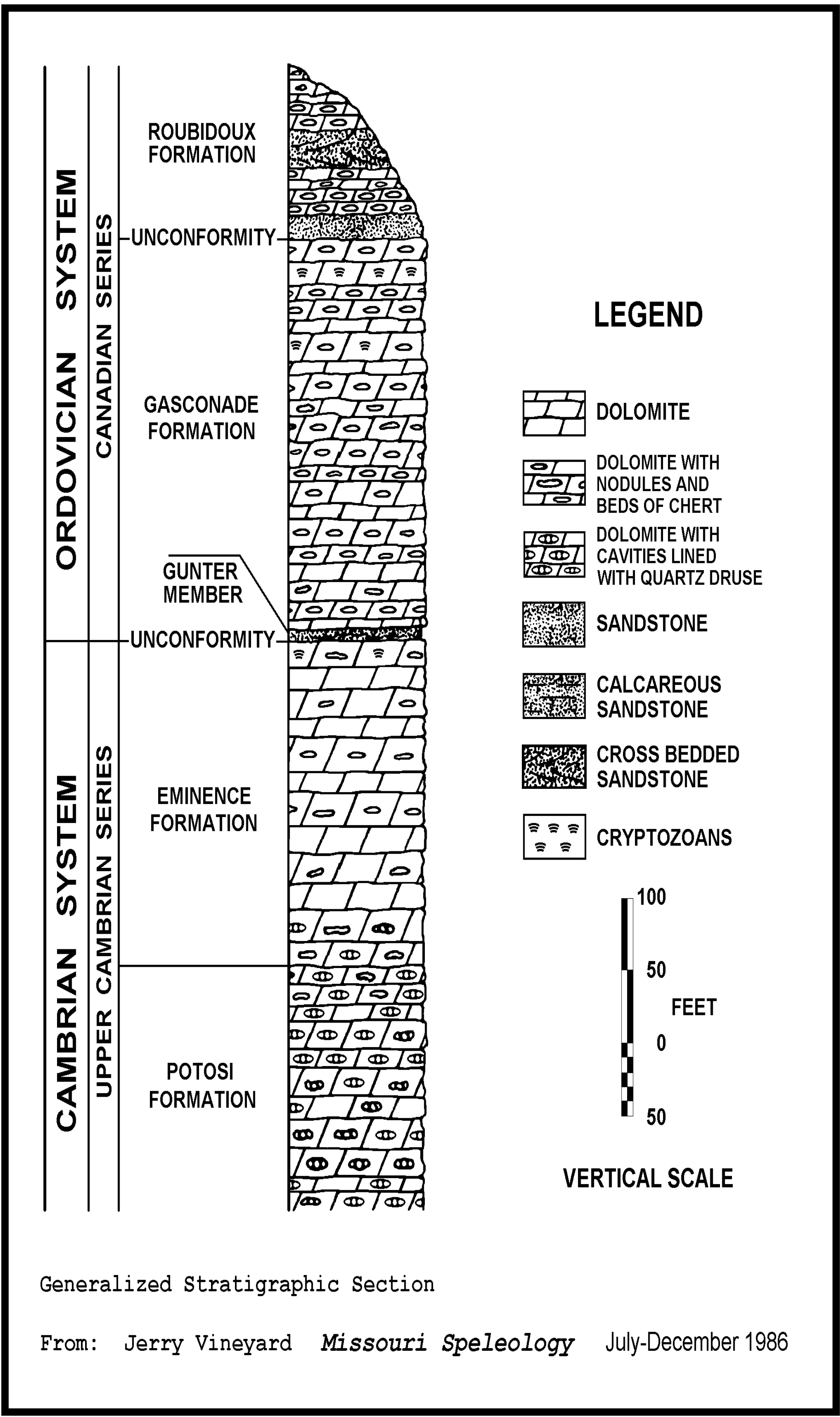


Figure 1.1. **Geological Stratigraphic Section**

All caves located within the confines of Onondaga Cave State Park lie entirely within the Ordovician Gasconade Dolomite formation. The Gasconade Dolomite is medium to coarsely crystalline dolomite with numerous interspersed chert beds throughout the unit (Thompson, 1995). The Gasconade formation is commonly separated into three somewhat distinct portions; the Upper Gasconade, the Lower Gasconade, and the Gunter Sandstone. Each of these portions of the formation varies based on grain size, percentage of chert, and mineral composition as in the case of the Gunter Sandstone. The Gasconade formation is overlain unconformably by the Roubidoux formation, a predominantly sandstone unit with many interbeds of dolomite and chert.

What actually controls the development of these caves? Because the porosity of Dolomite is less soluble than Limestone, chemical composition, solubility of the limestone and deposition of materials all play an important role in how cracks and crevices first develop to eventually form a cave. A chemical reaction in effect occurs that first dissolves the limestone to develop these cracks and crevices. This chemical reaction occurs when rainwater passes through the organic material on the ground's surface picking up carbon dioxide. The carbon dioxide reacts with the water and creates weak acidic water. This solution dissolves the minerals locked within the Limestone. The cracks and crevices enlarge and become water filled. Once an opening appears, either by water cutting or perhaps a sinkhole, the water drains out and a cave is formed. This same acid water or carbonic acid carrying minerals in solution, can now deposit them inside the cave opening. Note Figure 1.2. below.

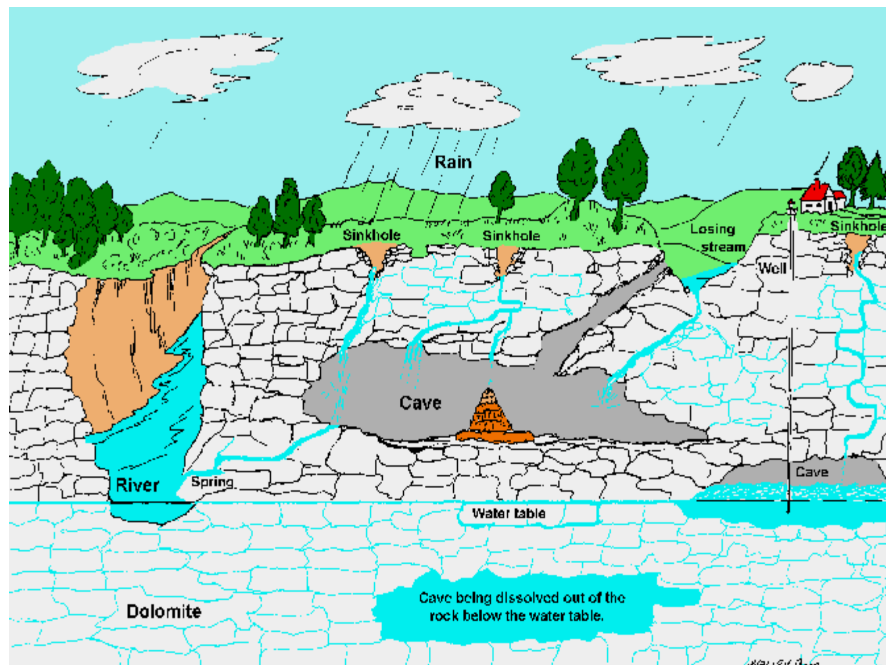


Figure 1.2. Karst and Cave Formation

Figure 1.2 depicts how water percolates through the ground's surface making its way to the water table. As it makes its decent, the chemical reaction mentioned previously $CaCO3+ H+HCO3^{-}>Ca^{2} +2HCO3^{-}$ dissolves the limestone and dolomite, increasing the minerals in solution producing acidic water. This acidic water has the ability to eat away at the bedrock and create sinkholes and caves. For this reason, it is imperative that substances introduced on the ground's surface be nontoxic.

1.1.2.1. Caves as an ecological subsection. Caves share a specific symbiotic relationship with the soil above them and the groundwater that passes through them. They serve as conduits for our drinking water. But most importantly, they provide a unique habitat for a very distinctive group of species that rarely utilize any of the

other communities that Missouri has to offer. Since caves offer a moist, dark and humid environment, many unique species call this exclusive habitat home. There are more interestingly "exclusive" habitats within the cave ecosystem than one might think. These elusive species make use of walls and ceilings, cracks and crevices, tight holes and even bat guano as their domicile.

1.1.2.2. Salamanders and cave ecosystems. Missouri is home to a variety of amphibians and reptiles. Several species of frogs, toads, salamanders, lizards and snakes utilize all of Missouri's habitats but may not always be seen frequently by the passer by. And, sometimes people are scared of things they know little about especially when they have grown up with the adage that "slimy creatures are scary creatures." Salamanders in particular are a species not commonly seen out in open areas and are fairly docile during the winter months. Because of this, it requires the diligent student or scientist to take a needed interest in them in order to better understand them and convey their knowledge for improved resource management practices. While salamanders can inhabit both terrestrial caves and aquatic caves, they require water to reproduce and lay eggs. The aquatic cave environment is more conducive to the salamander's entire life cycle. They utilize fresh water pools and very moist areas of the cave in order to lay the eggs and thus start the larvae cycle.

1.1.2.3. Extreme environments: studying cave biota. What is the future existence on a planet that its inhabitants have so greedily taken advantage of? Where would we go from here if there were no sustainable resources tomorrow? What kinds of research can be done now to benefit human kind in generations to come?

The air we breathe and the water we drink are resources that are *not* endless. Today's trend looks not only for alternative approaches to conserving our resources but also alternative environments in which to live. Research must focus on finding reliable water sources and life forms (or the ability for life to form) on other planets and planetary bodies. To that end, research efforts can focus right here Earth, examining some of this planet's extreme environments.

Studying the extreme environments that exist on Earth all around us could provide a prelude of options with a plethora of opportunity to answer questions about our continued existence. Cave ecosystems specifically provide interested researchers a living experiment within a living laboratory to investigate processes such as interbreeding, hybridization, competition, stress factors, etc.

1.1.3. Onondaga Cave History. Missouri hosts 20 show caves, open for guided tours. Quite often, these caves offer the visiting public a wealth of knowledge about the cultural history of the area and the colorful people that once lived there. Fortunately, the quest for further knowledge associated with the natural resource has prompted cave owners to allow students to do a myriad of research projects to gain a better understanding of the relationships between the surface environment and the sensitive environment beneath our feet.

Onondaga Cave has become one such show cave. Accommodating the many facets of ongoing research plays a key role in obtaining new and interesting insight to the prehistoric, geologic, and biological world that has amused visitors for so many decades. Much of what is seen on the surface in a show cave doesn't come close to what "lies beyond the walkway." Passages of absolute darkness become home to

many creatures never exposed to the public. Because many have lost their eyes as a result of adaptation, they rely on their auditory and sensory systems to navigate the terrestrial and aquatic worlds deep within the cave. Water systems meandering through these areas increase orientation barriers that vertebrates and invertebrates alike must overcome.

Onondaga Cave has passed through the hands of many owners and developed a storied history over the course of the last 125 years. First discovered in 1886 by way of boat, its discoverers attempted to become the first owners by quietly purchasing all of the property above the cave. Their intent was to develop the cave for commercial tours.

Unfortunate for them, they spent all of their funds buying property. As a result, they were unable to execute their plan and had to sell out to individuals actually interested in mining the calcite as a building material for structures at the 1904 World's Fair. After running into issues with finances and calcite extraction, it was discovered that the calcite, once exposed to the outside environment, became very brittle and was essentially worthless as a building material.

So, in an attempt to recoup some of their losses, the owners opened the cave for tours at the World's Fair. They held a cave naming contest and assigned the winning name, and the commercial dwelling known as Onondaga Cave(named for the Onondaga Indians) was born. An interesting side note: The Onondaga Indians are a Northern New York tribe that has never been known to reside in Missouri. Refer to Figure 1.3 below.

Figure 1.3. Natural Divisions of Missouri

Figure 1.3 shows Onondaga Cave (still operating as a show cave) located at Onondaga Cave State Park in Crawford County specifically at Leasburg, Missouri. Crawford County is located in the Northeastern portion of the Upper Ozark Highlands. This state park houses 27 known caves on its 1,316 acres. Several are significantly smaller in size than Onondaga but aid in the documentation of Karst topography and densities for the geographic region. Just slightly west of the park is the Huzzah Wildlife Conservation Area owned by the Missouri Department of Conservation. Within a 5-kilometer radius of the park and the conservation area, over

60 caves with length ranging from 15.2 meters to more than 2,743 meters in length have been documented. Note Figure 1.4 below.

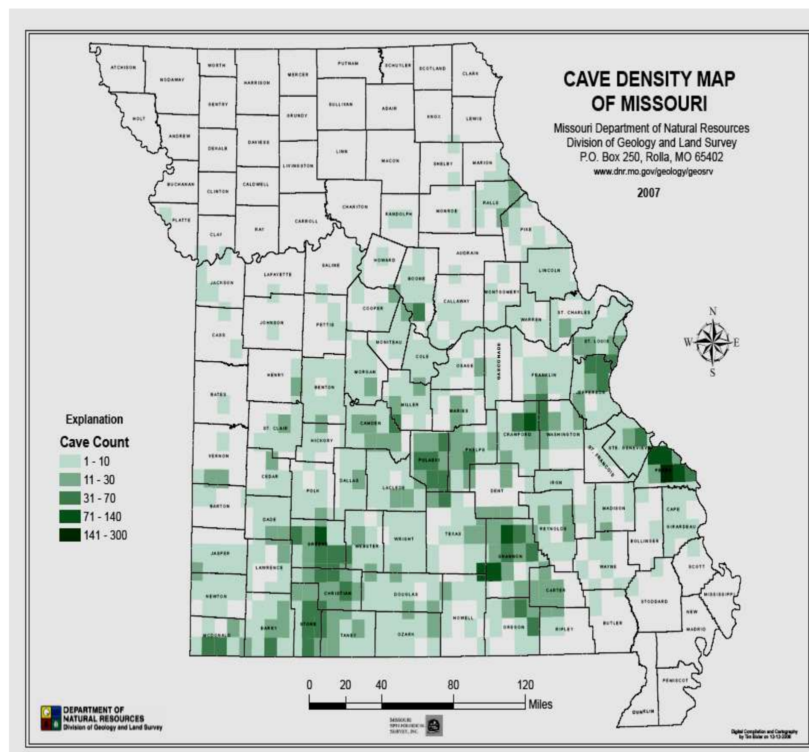


Figure 1.4. Missouri Cave Density Map

In comparison to other counties, Figure 1.4, Crawford ranks as one of the higher in cave density for any county in the state. Onondaga Cave is noted as a cave system containing superior water quality and exceptional biodiversity. Onondaga Cave is currently owned by the Missouri Department of Natural Resources and is administered by the department’s Division of State Parks.

Because of its exceptional water quality, it is used as a control cave by the

Missouri Department of Natural Resources Division of Environmental Quality when testing water quality across the state. The water quality is near pristine, assisting the cave in its rich biodiversity, see Table 1.1. Because this unspoiled foundation has been laid, six different species and one subspecies of salamanders regularly inhabit the confines of Onondaga Cave.

Table 1.1. Biodiversity

Onondaga Cave State Park Cave Dwelling Species

Scientific Name	Common Name	Class
Amphibians		
Eurycea longicauda melanopleura	Dark-sided Salamander	Amphibia
Eurycea lucifuga	Cave Salamander	Amphibia
Eurycea longicauda longicauda	Long tail Salamander	Amphibia
Notophthalmus viridescens	Central Newt/Red Eft	Amphibia
Plethodon glutinosus glutinosus	Slimy Salamander	Amphibia
Plethodon serratus	Southern Red-backed Salamander	Amphibia
Rana palustris	Pickerel Frog	Amphibia
Typhlotriton spelaeus	Grotto Salamander	Amphibia
Birds		
Saornis phoebe	Eastern Phoebe	Aves
Stelgidopteryx serripennis	Northern Rough-winged Swallow	Aves
Cathartes aura	Turkey Vulture	Aves
Mammals		
Eptesicus fuscus	Big Brown Bat	Mammalia
Myotis grisescens	Gray Bat	Mammalia
Myotis leibii	Small-footed Bat	Mammalia
Myotis lucifugus	Little Brown Bat	Mammalia
Myotis septentrionalis	Northern Long-eared Bat	Mammalia
Myotis sodalis	Indiana Bat	Mammalia
Neotoma floridana	Eastern Wood Rat	Mammalia
Peromyscus leucopus	White-footed Mouse	Mammalia
Peromyscus maniculatus	Deer Mouse	Mammalia
Pipistrellus subflavus	Eastern Pipistrelle	Mammalia
Procyon lotor	Raccoon	Mammalia
Snails and Slugs		
Fontigens aldrichi	Cave Snail	Gastropoda
Glyphyalinia indentata	Snail (trogloxene)	Gastropoda
Megapallifera ragsdalei	Snail	Gastropoda
Mesodon inflectus	Snail (troglophile)	Gastropoda
Pallifera sp.	Snail (troglophile)	Gastropoda
Zonitoides arboreus	Land Snail	Gastropoda

Table 1.1. Biodiversity (cont.)

Insects		
Arrhopalites pygmaeus	Springtail (troglophile)	Hexapoda
Atheta sp.	Rove Beetle (troglophile)	Hexapoda
Cantharis sp.	Soldier Beetle	Hexapoda
Ceuthophilus gracilipes	Camel Cricket (trogloxene)	Hexapoda
Ceuthophilus seclusus	Camel Cricket (trogloxene)	Hexapoda
Ceuthophilus silvestris	Camel Cricket	Hexapoda
Ceuthophilus unleri	Camel Cricket (trogloxene)	Hexapoda
Ceuthophilus williamsoni	Williamson's Camel Cricket	Hexapoda
Chlaenius brevilabris	Ground Beetle	Hexapoda
Dicaelius ambiguus	Ground Beetle (troglophile)	Hexapoda
Dicyyrtoma marmorata	Springtail	Hexapoda
Folsomia candida	Springtail (troglophile)	Hexapoda
Halpalus fulgens	Ground Beetle (troglophile)	Hexapoda
Hesperus baltimorensis	Rove Beetle (trogloxene)	Hexapoda
Lepidocyrtus sp.	Springtail	Hexapoda
Insects Cont.		
Macrocera nobilis	Webworm	Hexapoda
Onychiurus encarpatus	Springtail (trogloxene/troglophile)	Hexapoda
Onychiurus reluctus	Springtail (troglophile)	Hexapoda
Onychiurus sp.	Springtail (troglophile)	Hexapoda
Philonothus sp.	Rove Beetle (troglophile)	Hexapoda
Ptomaphagus cavernicola	Round Fungus Beetle (troglophile)	Hexapoda
Ptomaphagus sp.	Round Fungus Beetle	Hexapoda
Quedius erythrogaster	Rove Beetle (troglophile)	Hexapoda
Rimulincola divalis	Rove Beetle (troglophile)	Hexapoda
Sinella caeca	Springtail (troglophile)	Hexapoda
Staphylinus cinnamopterus	Rove Beetle (troglophile)	Hexapoda
Tachyura ferrunginea	Ground Beetle (troglophile)	Hexapoda
Tomocerus elongatus	Springtail (troglophile)	Hexapoda
Tomocerus flavescens	Springtail (troglophile)	Hexapoda
Centipedes		
Lithobius atkinsoni	Centipede	Chilopoda
Scutigera coleoptrata	Common Scutigera	Chilopoda
Millipedes		
Cleidonogona sp.	Millipede (troglophile)	Diplopoda
Pseudopolydesmus sp.	Millipede	Diplopoda
Tingupa pallida	Cave Millipede	Diplopoda
Crustaceans		
Armadillidium nasatum	Pillbug	Malacostraca
Caecidotea antricola	Isopod	Malacostraca
Caecidotea fustis	Cave Isopod	Malacostraca
Crangonyx forbesi	Amphipod	Malacostraca
Gammarus fustis	Scud (trogloxene/troglophile)	Malacostraca
Gammarus minus	Scud	Malacostraca
Gammarus pseudolimnaeus	Scud (trogloxene)	Malacostraca
Orconectes punctimanus	Spothanded Crayfish	Malacostraca
Orconectes luteus	Golden Crayfish	Malacostraca
Stygobromus gardneri	Gardner's Amphipod	Malacostraca

Table 1.1. Biodiversity (cont.)

Spiders and Mites		
Stygobromus onondagaensis	Onondaga Cave Amphipod	Malacostraca
Cicurina cavealis	Funnel Weaver	Arachnida
Hesperochernes occidentalis	Troglobitic Pseudoscorpion	Arachnida
Nesticus pallidus	Cave Spider	Arachnida
Rhagidia whartoni	Mite	Arachnida
Vonones ornata	Harvestmen	Arachnida
Annelids		
Lumbricidae (family)	Earthworm	Clitellata

1.1.4. Missouri Caverns History

Missouri Caverns, the site of the project, is actually part of the Onondaga Cave system. This section was only given its own name after a land feud that occurred during the 1930's. For a brief time, there were two private owners of the cave offering separate cave tours to the public through two separate entrances. For a number of reasons from both a cultural and natural history perspective, this section of the cave is very important to providing clues about the past as well as a glance at the future direction of show cave management practices.

Typically, most environmental cave studies today focus on human impact mainly because of an increasing interest in the restoration of cave habitats (Lewis, 1993). In most cases, pressure on plant and animal species force them to change and adapt to that changing environment. Unbeknownst to them at the time, by closing this section, the previous cave owners may have removed the impediment that the many species of salamanders living there were once exposed to on a regular basis. Now, the directions they take in adaptation and survival become quite different.

Many years ago, this passage was teeming with daily visitors as they toured Onondaga Cave. The feud amongst the owners of the cave along with World War II

placed a damper on tourism and travel into this section was thwarted. Eventually, that portion of the cave was no longer open to the public.

Now, more than 60 years has passed since the footprints of regular visitors have touched the floor of the passage. Due to this unusual circumstance, it is reasonable to question whether removing the human impact might in some way reverse the environmental pressures caused by disturbance.

Many commercial caves currently exist in Missouri that have been used for many years. Thousands of visitors each year pay a fee to see these unique underground wonders. Each visitor brings with them a myriad of contaminants that define human disturbance as a serious threat to the ecosystem. Lint from clothing, hair follicles, skin cells, foreign material transported in on shoes and bodily fluids all become an “introduced” food source that attract invertebrates and other small organisms into this ecosystem that they wouldn’t normally inhabit otherwise. The devastation that this impact can cause sometimes easily goes unnoticed to the untrained layman. Entire populations of invertebrates can be wiped out just by disturbing the area they inhabit. New exotic populations can result when those introduced food sources mentioned previously are left behind. Those new populations, in turn, can decimate the native populations and take over their habitat.

The trend in many of these caves now is to attempt to return the cave or areas within them back to pre-settlement times, before commercialization of them took place. This is the case with the Missouri Caverns section of Onondaga Cave. See Figure 1.5 below.

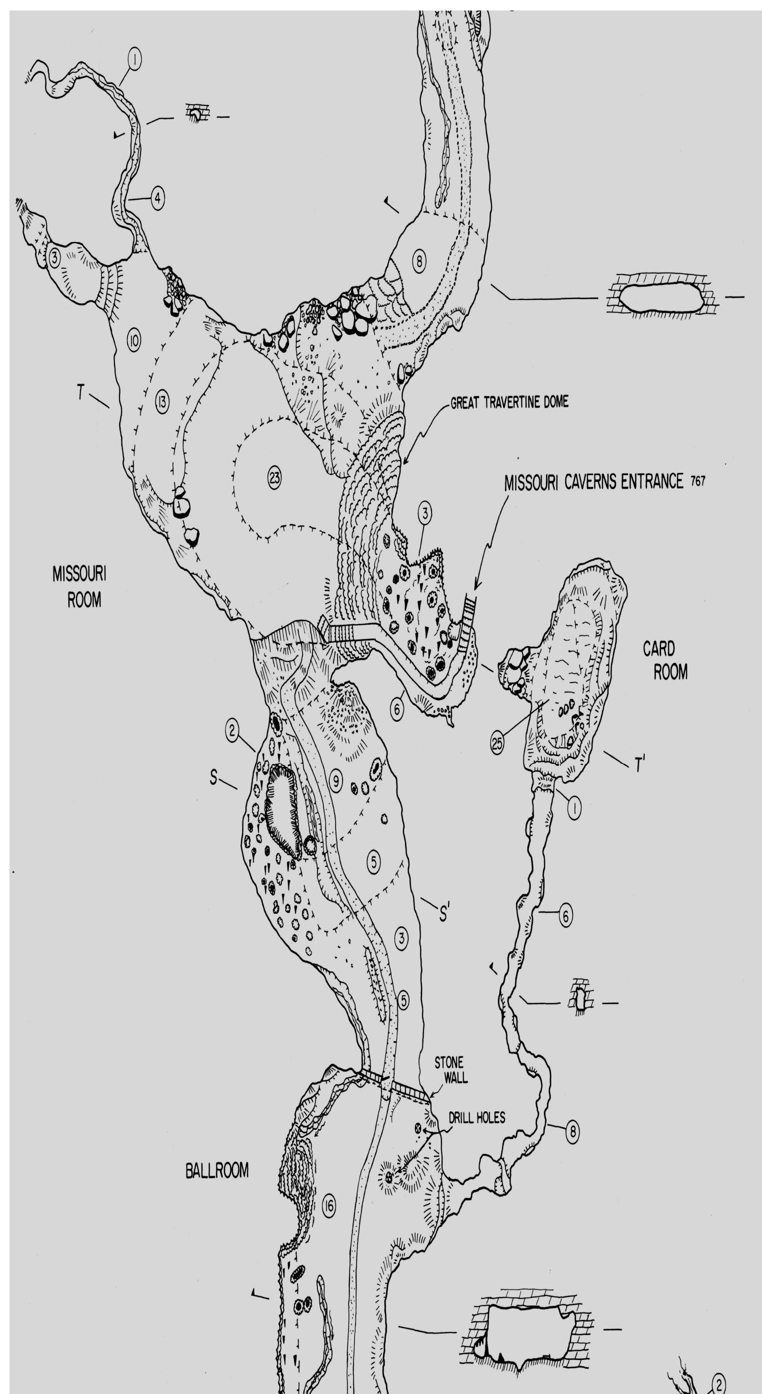


Figure 1.5. Onondaga Cave Map-Missouri Caverns Section

Figure 1.5 shows a detail of the Missouri Caverns area. Traveling from the Onondaga Cave entrance now used for daily tours, it is approximately one-half mile to the point where visitors turn around and approximately one-quarter of a mile further into the passage to reach the sampling area located just inside the door of the old Missouri Caverns entrance. See Figure 1.6 below.

Figure 1.6. Manmade Missouri Caverns Entrance

This is the remains of the cultural icon that housed the Missouri Caverns entrance. Just beyond this door lies the place park employees have deemed "Salamander Heaven" a safe haven for a variety of salamanders, both cave and terrestrial. Currently, the Missouri Caverns entrance is now secured with a steel door that allows for the entry and exit of fauna such as salamanders and bats but requires monumental effort in its removal for human use.

1.2. GOALS AND OBJECTIVES

The primary goal of this study is to focus on the lack of human disturbance in this particular area and relate it to the breeding habits of the salamanders species found within this passage. This study may assist in providing a better understanding of the relationship of human disturbance to speciation and speciation in general throughout the cave system. Additionally, documentation of the species currently residing in the area and identification of potentially interbreeding populations will assist in the development of management plans that address previously altered cave ecosystems and the manifestations thereof.

Objective 1: The purpose of this study is to use salamanders as a model to test for multiple hypotheses. Typically, all species of salamanders react very negatively to a disturbed environment. One in particular, the Cave Salamander is extremely susceptible to change in the ecological balance of a fragile cave ecosystem (Johnson, 2000). "Salamander Heaven" is an area located in the Missouri Caverns section of Onondaga Cave that appears to provide ideal habitat for salamanders. This has not always been the case. The study conducted here will address the theory that removal of a dispersal barrier, in this case human intervention, from this area might have reassigned an ecological balance that was once totally destroyed by daily human activity. And in doing so, it has lead to possible interbreeding habits in this region of the cave. Any results gleaned from this study benefit cave management practices not just for the Department of Natural Resources but other agencies. Examining the salamanders inhabiting this area both morphologically and genetically could provide clues that support the idea of interbreeding or potentially a new species that may

never had existed if humans continued to degrade their habitat. The question looms, has the stability of the last sixty years had such an effect that multiple species within the same genus are willing to interbreed, potentially as a result of a limited habitat and a restricted food source?

Method One: Phenotypic Analysis: Several treks were made to the sampling site in order to gather the morphological data necessary to conduct a phenotypic visual assessment of the populations chosen for this study: *Euryrea lucifuga, Eurycea longicauda* and *Eurycea longicauda longicauda*. Specifically, these visuals were used as a comparison technique to establish the presence of questionable, possibly hybrid species. A series of measurements were taken and each specimen was photographed. Analyses of these data through regression analysis were used to compare the different species, looking specifically for the relatedness of those of questionable origin to those of known origin.

Since phenotype is the overall interactive expression of the genotype of the individual with its given environment, it is possible to see a great deal of phenotypic variation among individuals in some areas. Yet, this variation may fall short in comparison to the amount that would be evident when using a simple method of examining the genotypes of individuals. Conversely, high amounts of phenotypic variation within the population could be the result of environmental effects on gene expression, and may not be accompanied by similar genetic variability (Martin, 2004). In order to assess any genetic variability to support interbreeding, a second method was employed.

Method Two: Genotypic Analysis: A molecular means of assessing variation was used to analyze the genetic information that was gathered; Amplified Fragment Length Polymorphism originally developed by Vos et al (1995). Amplified Fragment Length Polymorphism or AFLP generates banding patterns that can be used to examine variation. It is a recent technique using a Polymerase Chain Reaction or PCR that can be utilized to compare several individual DNA samples in quest of similar or dissimilar repeats in the alleles. The technique has become a popular method of analysis for several reasons. It is less expensive than previous forms of the technique, the data is generated from a sequencer rather than a polyacrylamide gel and that data can be easily incorporated into software packages that generate phylogenetic distance trees to determine the extent of variation. Tissue samples were collected from each of the three species thought to be involved in the interbreeding and samples were collected from four specimens that appeared to be some form of, or a combination of all, of the three known species.

Hypotheses: The three species of *Eurycea* show signs of interbreeding, evidenced by phenotypic and genotypic data. The questionable specimens may be a new distinct species of *Eurycea* and cessation of human interaction (removing a dispersal barrier) in this area played a role in this interbreeding.

2. REVIEW OF LITERATURE

2.1. CAVE MANAGEMENT AND RESTORATION

Caves have always been a great place to find historical artifacts, especially any systems that had once been utilized by humans for shelter. Sadly, the impact later humans had on these finds can never be reversed. Many caves are now protected, though, in an attempt to protect the few artifacts that remain. Once researchers came to the realization that they contained archeological materials, new ways of managing the caves needed to be addressed (Brown, 1986). Human impact in the form of vandalism was occurring. Artifacts of significant historical value were being stolen or destroyed and cave ecosystems are being decimated.

An increased interest in cave restoration has developed over the past several years and people with a genuine interest in preserving these environments became more agitated with vandals breaking off and stealing speleothems from pristine passages in caves. In addition, an awareness to address cave habitats in conjunction with accommodating man's needs was heightened. Restoration of cave habitats in Karst topography generally piques the interest of dedicated cavers. Often times, small towns have been incorporated into areas with caves, sinkholes and losing streams. Dealing with restoration issues such as tourist routes, sewage disposal and effluent discharge, trash removal from sinkholes, boot prints in cave passages and removal of exotic debris are important aspects of their restoration efforts (Lewis, 1993). Sometimes, though, cave restoration efforts can produce a totally different effect than one might have anticipated. Nothing can be assumed in an environment where so little is known. As some individuals at Mammoth Cave found, sometimes dealing

with these issues in the name of restoration produce a totally different affect --habitat that has basically already adapted to conditions introduced by man needn't be altered (Lewis, 1993). It was in the Cathedral Domes section that a determination was made to actually leave wooden debris that would have normally been removed for restoration purposes. After investigation and census surveys, it was discovered that the debris had provided a food source large enough to support a very diverse community of aquatic fauna. Removing it would put this troglobitic community in jeopardy. Unfortunately, miscommunication during the summer months allowed a continued clean up in these areas. In addition to physical damage to the area, the stream and the animals, within months, the amphipod population had decreased significantly. Now disturbed debris blanketed the streambed and provided a nutrient windfall for the microbes in the water. Left undisturbed, the wood most likely would have provided a nutrient source slow to release for the aquatic community.

Attempts to restore these environments to pre-settlement times are directly proportionate to the enthusiasm generated by doing so. Restoring a subterranean environment such as this could provide clues to extant life, recent life forms and previous life forms as it offers a subsurface view of places like no other. Those interested in cave restoration consider a number of factors based on the particular needs of the system. Some caves have old rusty handrails that need to be removed, as in the case of Onondaga Cave, while others have severe algae growth problems as a result of too much artificial lighting. Carlsbad Caverns underwent extensive restoration several years ago and the focus was not only to employ careful cave

restorative techniques but to also ensure that these techniques were incorporated into a thorough cave management plan (Rohde, 1981).

However, all actions have some sort of consequence. Each management action should be carefully administered and monitored as human use of an area, while sometimes destructive, can also be determined beneficial. There can be a delicate balance between use and healing.

In addition to these aspects, it is important to gain a comprehensive awareness of human impact on these pristine environments. Everything we do on the surface of the ground affects what goes on beneath it. Land development, chemical contaminants, sewer systems, and land disturbance can have monumental impacts on cave systems.

Contributing to sedimentation and nutrient loading or loss within a system can have a huge impact on the types of organisms that live there and their population densities. Introduced food sources in a very limited environment can change the way the inhabitants utilize the resource. Many of these ecosystems have endemic species. Alterations could have devastating impacts.

Cave management has become an integral part of research in cave systems throughout the world. As mentioned previously, caves are hidden beneath our feet. The old adage goes without saying, “out of sight out of mind.” Increasing human impact is decreasing the existence of this unspoiled environment. As it stands, there are no real laws governing the use and abuse of caves or the endemic species or microbes that live there. Researchers have recently come to realize that we should be

doing something to identify, quantify and protect the microbes, invertebrates, and other species that could potentially be the key to the future existence of mankind.

Show caves tend to see the most need for good, quality cave management to minimize human impact (Gurnee, 1991). Several aspects of the environment could easily be altered for the sake of commercialization. Inspecting each of these aspects prior to the conversion of a natural cave to a show cave is vital. In addition to understanding the natural aspects, they must also investigate more specific questions as they pertain to commercial use. Is the cave safe and does it provide a quality educational experience? And, site accessibility and location are important factors, too. Implementing a plan to minimize the impact of just one person visiting a show cave or any cave can be important as well (Stitt, 1978). Stitt (1978) took into consideration *several* internal and external factors for impact. A study conducted at Onondaga Cave also addressed the need for limiting public access and to develop a plan supplementing the need to minimize impact during certain times of the season. It also addressed a staffing plan and a general idea of attendance for budgeting purposes (Vale, 1997). The need to utilize professional assistance for trial design and lighting must also be considered, especially if the owner even remotely cares about the ecosystem they are preparing to disturb.

Unfortunately, private ownership sometimes hinders these concepts, as the revenue generated from these caves is someone's livelihood. To admit that humans might have a significant impact on the cave ecosystem equates to committing financial suicide. Privately owned caves sometimes fall prey to this lack of cave management as well. Oftentimes, the property owner either does not know that a cave

is located on their property or they have difficulty enforcing trespass. Amazingly enough, those in quest of cave denigration know where the caves are better than those committed to preserving them. These harmful human activities are threatening the diversity of many of the species within cave ecosystems. And, because of shocking declines in amphibian populations, research is focusing more on salamander, frog and toad populations (Riley et al. 2003).

By employing reasonable and logical cave management practices housed within well written management plans, education, entertainment and research will complement each other and can be maintained all the while still preserving, conserving and protecting this unique underground world.

2.2. HUMAN IMPACT ON CAVES

The last twenty to thirty years have brought with them an urgency to more effectively understand all preservation aspects of cave ecosystems, both wild and show caves alike. Because the cave environment is basically nonrenewable in nature, most actions are irreversible and irretrievable (Stitt, 1978).

Several studies of varying degrees have been conducted to gain useful knowledge for better management of these fragile ecosystems. Scientists have been investigating a myriad of reasons as to how human impact, in particular, has affected these cave environments. The current focus seems to be leaning toward different styles of cave management (Buecher, 1993). More importantly, the thoroughness of managing every aspect of human impact is paramount prior to permitting any impact to occur.

Better cave management brings with it a regulatory aspect crucial to future development. Pre-development studies can provide better focus on several aspects of cave environments such as the geology, biology and hydrology of the system in question. In 1993, Buecher published findings of a pre-development study of Kartchner Caverns that identified several of these aspects. The study surrounded the idea that a cave that had been kept secret for many years was about to be open to the public and every aspect of this cave environment needed to be studied and documented before significant human impact would take place.

For years, cave ecosystems have not been taken seriously. Researchers are beginning to see a shift in the attention paid to this type of environment. With the recent discovery of caves on Mars, for instance, they are redirecting their attention to these pristine and extreme environments (Malik, 2005). Studying the delicate ecosystem, the biodiversity and the microbes that live there can lead to an enhanced understanding of survival in extreme conditions. More specifically, studying the chemoautotrophic microorganisms that exist in these environments could lead to a keen understanding of how other organisms might survive in such unorthodox surroundings.

In the short time that any serious research has been taking place in caves, literally hundreds of new species of subsurface dwellers and invertebrates have been identified. Caves have become a great research ground for the millions of microorganisms yet to be discovered. Developing and following sound cave management plans incorporating regulatory conditions would play a key role in implementing research with proven results.

All aspects of research undoubtedly will provide important insight on not only our current environmental state, but the ecosystems that have been abused and the ones that we must move to protect. The scientific community can ill afford to turn their heads away from the study of caves as an extreme environment at this juncture in our existence.

2.3. REVERSING HUMAN IMPACT

Onondaga Cave was transferred to the State of Missouri nearly 25 years ago. In that time, unfortunately, only minor documented research of the cave system has been done with little comparison to other cave systems. The fortunate aspect, though, is that the natural resource has been well protected during this time period. Just recently, has preliminary research been conducted that will begin to reveal more about this particular cave system. Conducting studies of varying parameters and then using them in comparison research must lead to some new conclusions. Finding a new species could be monumental in the effort to understanding what might have influenced this change over time. Studying the removal of the human factor in these areas and comparing this to areas where people inhabit regularly, will lead to a better understanding of the changes that have occurred and may still be occurring to the ecosystem.

As referenced earlier in Table 1.1, the biota of Onondaga Cave is extraordinarily diversified. Not all caves can boast this fact. There is a definite symbiotic relationship with cave dweller and environment. The more human disturbance that occurs in a cave's fragile ecosystem, less and less cave dwelling species will be found there. Many caves in Missouri have been abused beyond repair.

Any damage that occurred will take hundreds of years to begin the reversal process. Onondaga Cave was well used as a commercial cave for many years, however, many of the passages were considered off limits to regular visitors. By allowing only individuals with a legitimate interest in the cave to enter these areas, the impact was kept to a minimum.

Once the State of Missouri took possession of all the cave systems in Onondaga Cave State Park, access to most became restricted and several are closed to human activity in totality (Miller, 2005). Not only is entering many passages prohibited, all commercial evidence has been removed, returning the passages to a natural state that compared to their original existence. The Missouri Caverns section is a perfect example of a passage being allowed to start the reversal process.

The irony of this human intervention is that it again takes human intervention to preserve a very unusual habitat that may exist nowhere else in the state. Further study could lead to a closer look at other biota so sensitive to a changing environment. A purpose of studies such as this one might potentially convince cave owners with a sincere interest in preservation, to take a second look at the approach taken to protecting these unique and fragile environments.

Recent research conducted throughout these extreme environments has uncovered more than just a few animals inhabiting these well-kept secrets. Bacteria, fungus and algae thriving in a dark, cool and very damp environment are providing clues to researchers that could one day enhance human survival. Protecting and preserving now could literally lead to important scientific discoveries extending man’s inhabitation of the earth (Boston, 2000).

Finding that removing the human barrier is beneficial in the preservation of cave dwellers and advantageous in their adaptation to that change might alter the way the cave system is utilized and how management plans are implemented. Promoting the most pristine environment should, theoretically, promote thriving communities of biota.

2.4. PHENOTYPIC EVIDENCE

A phenotype illustrates an observed quality in organisms. It can refer to the organism's morphology, their development or their behavior, however, in contrast, phenotype does not refer to the genotype- the inherited instructions that the organism carries. This compared and contrasted concept was proposed by Wilhelm Johannsen in 1911 to clearly differentiate between an organism's heredity and what that heredity actually produces (Churchill, 1974).

The phenotype is not just a product of genotype, it is influenced by the environment more or less. Phenotypes are a framework of traits or characteristics. Some are controlled by the individual's genes but others are controlled by genes that are substantially affected by "extragenetic" or environmental factors (Brenner, et al 2002).

As detectable characteristics, variation in observed genotypes could be as a result of silent mutations that, due to some simple change in amino acid base pair frequency without changing the sequence, might provide the organism a selective advantage.

Until fairly recently, visual phenotypic analysis was the primary tool and an adequate method for determining conclusions in scientific studies of hybridization,

speciation and population variation. Researchers simply did not have the necessary molecular tools at their disposal and what they did have was limited. Now, several tried and true molecular genotypic methods have been developed for more conclusive analysis.

2.5. GENOMIC MOLECULAR EVIDENCE

2.5.1. Polymorphic Banding Patterns. There are several different techniques that can be used to determine genetic differences. These techniques produce genetic fingerprints or polymorphic banding patterns. Several variations of this technique currently exist for example, Random Amplified Polymorphic DNA (RAPD), Random Fragment Length Polymorphism (RFLP), and Amplified Fragment Length Polymorphism (AFLP).

Amplified Fragment Length Polymorphism, or AFLP, was developed by Vos et al. (1995), and produces the polymorphic banding pattern similar to that seen in RAPD or RFLP procedures, but with less primer combinations. AFLP has also been successfully used to compare groups at species and population levels in both flora and fauna. One major advantage to using the AFLP fingerprinting technique, in particular, is the large number of polymorphisms that the process can generate. The technique is also capable of differentiating individuals in a population and examining genetic diversity. Maughan et al. (1998) found that AFLP produced more polymorphic loci per primer than either RFLP or RADP in their study of Soybean diversity (*Glycine Max* and *Glycine soja* Leguminosae).

The Amplified Fragment Length Polymorphism technique has been applied to a variety of different plant and animal studies. Law et al. (1998) have utilized the

technique for Plant Variety Registrations. Barker et al. (1999) through investigation of genetic diversity in Salix (Salicacaee) found 645 polymorphic bands with primers using AFLP as opposed to 170 bands using 20 RAPD primers. Rieseberg et al. (1999) looked at introgression between cultivated sunflowers and a sympatric wildflower. Beismann et al. (1997) studied the distribution of two Salix species and their hybrid. The more loci that is available for comparison in an individual analysis the better the accuracy and significance of the results (Gorman and Renzi, 1979; via Martin, 2004).

In reviewing literature, it appears that AFLP has been applied to plant materials more so than animals. However, this also appears to be changing.

In a comparison of techniques study, Robinson et al. (1999) identify other advantages of using the AFLP process to include that no sequencing information is required, the PCR technique is relatively fast and a high multiplex ratio is possible.

Among several reasons AFLP was chosen for this study was the fact that individuals sampled did not have to be destroyed in the process. This meant taking only a minute DNA sample from each specimen. Only a few voucher specimens were taken for the study; those of questionable origin. This allowed the majority of the specimens used in this study, for the most part, to be left intact.

2.5.2. Limitations Associated with Banding Patterns. In contrast to the advantages, Robinson et al. (1999) also indicated potential problems with using the technique. One such problem consists of users having proficient knowledge and skill to utilize the process. While AFLP is not a difficult technique, it is a tedious process with specific steps requiring good pipetting and mixing skills. These specific steps must be followed diligently.

Another issue is that the costs associated with the project tend to be expensive. Also, the enzyme and primer selection can have an impact on the production of reliable banding patterns and the number of polymorphisms detected. Ridout and Donini (1999) found that by changing the enzyme combination from EcoR1/Mse1 to Pst1/Mse1 several more polymorphisms were detected in barley. So, the choice of enzyme and primer combinations can play a major role in the amount and quality of variation revealed.

Robinson et al. also indicated that partial digestion and poor amplification can affect reproducibility. This takes us back to the user and the process. Missing a step, repeating a step or mixing ingredients during a step can have devastating effects on the results. These mistakes can lead to a number of issues for example, too many bands to score, no bands at all, or the number of bands actually amplified. Preliminary screening of primer combinations is strongly suggested by Hartl and Seefelder (1998). After they evaluated 60 primer combinations for their analysis of hop, they found that only eight of the combinations actually provided reliable banding patterns. It is well documented in other literature that testing multiple primer combinations prior to analysis produced only a fraction of combinations that resulted in banding patterns conducive to reliable scoring.

It has been found as well that RAPD analysis requires that a number of random primers also be tested. Commonly, over 50 different primer combinations must be tested in order to produce just 10 to 20 strong primer combinations that provide the large numbers of polymorphism (Kimberling et al. 1996, Evans et al. 1997, and Ritland et al. 2000) needed for consideration of many loci. Again, the

more loci that is available for comparison in an individual analysis, the better the accuracy and significance of the results (Gorman and Renzi, 1979).

AFLP produces more repeatable and reliable results than RAPD analysis requiring fewer primers to produce similar results (Vos et al. 1995). Because there is less time invested in the process overall, it has been found that AFLP has been the favored method of analysis over RAPD in the past few years, and therefore will be the method I used for this study.

The advantages and problems I encountered while using the AFLP technique will be addressed in detail in the section that discusses optimizing the process. Throughout the entire process, when problems were encountered, they had to be immediately corrected prior to moving forward with the next step. Since each step of this process relies on the previous step, any mistakes made within one step will eventuate themselves in each and every subsequent step.

All of the processes mentioned above take time to produce viable results. With each iteration of this fingerprinting procedure, some time is being taken out of the overall equation. As researchers refine the process and document their findings, future uses of the technique become slightly easier. But, for the most part, it takes not only time but also patience and focus to utilize the procedure to your advantage. By first optimizing the process, time can also be saved throughout the project. A large section of this thesis is dedicated to optimizing this process so that hopefully, the next individual using this technique in a similar study might find this to be useful and time saving information.

2.6. SUMMARY

As stated previously, it has only been in recent years that concerned individuals have begun to identify the devastating consequences of human impact to cave ecosystems. Cave management plans and restoration efforts to reverse these deleterious effects are merely in their infancy.

Restoration efforts have mainly focused on removing material items that man has deposited into the cave systems. And, most of the studies that have been done to date surround the human impact on, specifically, bat populations mainly those with roosting maternity colonies. These studies are warranted because evidence indicates that human activity in caves adversely affects bat populations (Mann et al. 2002). Mann et al. specifically studied the effects of cave tours on *Myotis velifer*. They focused on aspects such as light intensity, time of day, size of tour, noisiness of the group and the season itself. Mainly, they were identifying the management implications associated with constant human activity.

My study stands to accomplish a similar objective by identifying a management implication from a different perspective; looking at the removal of human activity in a given area through the use of slightly different methods. They used a number of visual techniques. Other studies have used phenotypic visual techniques. But, because genotypic techniques weren't readily available, many studies that could have used them were limited as a result. In addition, this study will further enhance the use of AFLP analysis in relation to amphibians and the possible implications of interbreeding.

3. MATERIALS AND METHODS

The Missouri Caverns section of Onondaga Cave is located at the Eastern most end of the Onondaga Cave system. Because a feud between landowners occurred, a second entrance was excavated in the 1930's to accommodate visitors to that section of the cave. Refer to Figure 3.1. The section in question exists at an elevation of 725 ft. along the longitude 121° 13' 46.719" W and latitude 38° 3' 35.589" N.

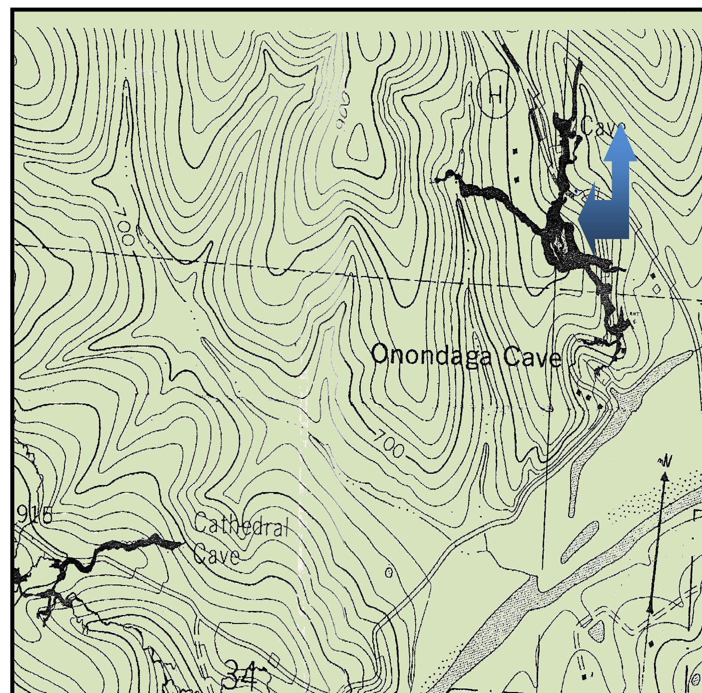


Figure 3.1. Topographic Map of Onondaga Cave

Note in Figure 3.1 of the topographic map that the Missouri Caverns section is the passage extending north and south. It is approximately three-quarters of a mile in length.

3.1. SAMPLING METHODS

3.1.1. Species Selection. Because two species and one subspecies already known to interbreed exist in Onondaga Cave, *Eurycea longicauda longicauda* (Long-tailed Salamander) and *Eurycea l. melanopleura* (Dark-sided Salamander), data collection and subsequent DNA analysis could provide information that will support the theory that one or both of the *E. longicauda* species might be breeding with *Eurycea lucifuga* (Cave Salamander). Visual assessments of the physical characteristics of several of the specimens utilizing the area provide the impression that some sort of interbreeding is occurring among the three species.

Cave Salamanders with unusually long tails, Cave Salamanders that are visibly shorter than their common length, and Cave Salamanders with tail markings of the Dark-sided Salamander are just a few of the odd physical characteristics observed. Table 3.1 references the dates that samples were identified, measured and photographed. Several trips were made to the sampling site where unfortunately, either there were no salamanders at all or species that utilize the cave entrance but were not used in this study. Treks were made at different times of the day, different days of the week and during different weather conditions. Treks were made during different seasons as well. Quite often, there was no rhyme or reason to when they were there and how many would be there. More about this randomness is discussed in the discussion and conclusion sections of this thesis.

Table 3.1. Sampling Dates

Sample Date	Samples taken	Sample Date	Samples taken
09/05/05	8	07/07/07	6
01/07/06	0	08/13/07	3
03/15/06	0	08/20/07	4
05/11/07	16	08/25/07	1
06/18/07	7	08/28/07	2
06/25/07	2	09/07/07	1
07/02/07	1	10/04/07	2

3.1.2. Photographic and Whole Animal Vouchers. Photographs were taken in the natural setting. Mentioned previously, disturbing salamanders too much in their environment can have adverse effects. All photographs were saved to a CD for future reference. A thumbnail print of their head was developed for use in identification during subsequent visits to the sampling site in order to avoid duplication of sampling.

Ideally, for AFLP analysis of potential interbreeding, several samples from each species should be taken and three to five samples from any unusual specimens must be taken for consideration. To allow for statistical evaluation of phenotypic data an attempt to capture 30 samples from each species at the sampling site was made. Ideally, a tail sample was to be taken from at least 10 individuals in each of the thirty sample sets. Refer to Figure 3.2. and 3.3. below.

Figure 3.2. Cave Salamander

Figure 3.3. Unusual Specimen

Only four vouchers were taken from the sampling site. Each of these individuals had some sort of characteristic that defined it as a questionable specimen. Voucher specimens will be deposited in the herpetological collections of the University of Kansas Natural History Museum and Biodiversity Research Center. Vouchers were euthanized in a 1:1000 solution of MS-222, fixed in formalin, and preserved in 70% ethanol.

3.1.3. Tissue Sampling. Procedures outlined in the USGS National Wildlife Health Center "Standard Operating Procedures-Anesthesia of Amphibians in the Field" (2001) were followed. The tip of the tail was removed from some of the specimens examined representing each of the *Eurycea* groups. A new razor blade was used for the tissue removal of each animal to eliminate sample contamination. The tail of each animal was then treated with Bactine© to thwart infection. The tail samples from each animal were placed in a one ml micro-centrifuge tube and

suspended in 0.75 ml of 70% ethanol. Each vial was marked with the specimen number and transferred from the cave sampling site and stored at 4°C for later use in molecular analysis.

3.2. PHENOTYPIC ANALYSIS

A set criterion of data was collected from each of the species listed above. This same set of data was also collected from four salamanders that exhibited any of the unusual characteristics stated above or a combination thereof. All measurements were captured in millimeters. The characteristics chosen for this study included head width, head length, total length, femur length, costal groove count, weight, and on a few specimens, snout to vent length. See Table 3.2 below for a sample from each species.

Table 3.2. Sample Salamander Data

Means Phenotypic measurements for Salamanders studied							
Species	Measurements in mm						
	TL	HL	HW	FL	CG	W	SV
Cave	162.0	17.0	11.4	8.4	11	5	87.2
Dark sided	118.5	9.1	8.1	5.6	10	2	69.8
Long tailed	130.1	12.7	8.9	5.6	14	3	76.6
Odd	142.2	14.0	9.5	6.3	11	5	72.3

3.3. WHOLE GENOMIC EXTRACTION

3.3.1. Extraction of DNA from Whole Tissue. Extraction was done through the use of the REDExtract-N-Amp^{TM} Tissue PCR Kit provided by SIGMA. The protocol provided with the kit suggests that the mixture should be incubated at room temperature for 10 minutes before addition of a final reagent that renders the tissue partially digested but releases a large amount of genomic DNA from the sample. The documentation also states that a more complete digestion can be achieved by incubating the sample at 55°C for 10 minutes instead of at room temperature.

After consultation with Adam Martin, the Missouri S & T cDNA Center's Laboratory Supervisor, I found this suggestion to be useful for all samples in this study, especially those that had been stored at 4°C for a long period of time. Therefore, the samples in this study were incubated at 55°C for 10 minutes (Martin, 2004). It is possible that at room temperature, any aged samples could be more resistant to enzyme activity and may not provide the necessary amounts of genomic DNA for later applications. But at the higher temperatures, ample amounts of DNA could be extracted from the aged samples. A list of complete protocols used in this study is found in Appendix B.

3.3.2. Optimizing the Extraction Method. The AFLP reaction calls for high quality as well as a large quantity of DNA to be digested and analyzed. This could have posed a problem because of the enzyme-rich reaction used to extract the genomic DNA from the tissue sample. To determine which procedures would result

in the best AFLP reaction, two tissue samples were sacrificed in order to find the optimum techniques to satisfy both requirements of the AFLP reaction.

One variable to consider is the concentration of DNA. The amount of tissue available for digestion was limited in order to ensure minimum impact on the organism (i.e., only tail tips were used). This results in a restricted amount of DNA available for extraction. If the procedure as outlined by the extraction kit did not produce DNA of a high enough concentration, a possible change in the protocol might increase the concentrations.

Again, because lab supervisor Adam Martin had already tested this assumption, I was able to proceed under the premise that that the protocol provided with the kit would produce an ample amount of DNA for analysis purposes (Martin, 2004). Large amounts of proteins and other molecules present in the sample can affect the AFLP digestion process (Vos et al. 1996). A second sample was used in the extraction procedure as described in Appendix A, this time testing the quality of the sample. After the reaction was complete, this second sample was in a partially digested state, similar to the first sample. This sample was then subjected to a column binding purification process attempting to determine if the sample would produce an ample amount of purified, higher quality DNA for the AFLP reaction process. It was determined that the purification test did not provide an enhanced or purified DNA sample adequate enough for the study. It was then determined that the procedure outlined in the extraction kit would suffice. Many aspects of the protocol that comes with the kit are very useful and will likely be the one to use.

3.4. AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP)

3.4.1. Summarization of Technique. The AFLP technique consists of four specific steps. The first is the digestion stage, in which whole genomic DNA is cut by two restriction enzymes (6-base restriction site). *Mse1* is the frequent cutter used and *EcoR1* the infrequent, respectively. Table 3.12 details the primers used in this study.

In the second step of the process, an amount of double-stranded DNA, with known sequences and overlap sequences for the restriction sites of the enzymes, is introduced into the mixture. This is sent through a ligation reaction to fuse the known sequences, or adapters, to the unknown fragments created by the restriction reaction. In each step, the samples spend a specified time in the thermo cycler.

In the third step, a diluted sample of the newly ligated mixture is sent through a pre-amplification technique. This is similar to a standard PCR; the general protocol for this reaction is listed in Appendix B. This reaction includes complimentary primers to the known sequence of nucleotides in the adapters, with the addition of a single nucleotide overlapping the unknown sequence of each fragment. This process serves to increase only the fragments that contain the additional nucleotide at the beginning of their unknown sequence on both ends. This effectively cuts the number of fragments to be resolved by a factor of 16. This necessary step is important when dealing with large genomes to ensure effective amplification of the final product during the next step of the reaction. Once this reaction is finished and gone through the thermocycler phase of the step, a portion of the mixture is again diluted and used in the next step of the procedure.

The final stage of the AFLP technique is called selective amplification. This is another standard “step down” PCR reaction that uses primers complimentary to the adapters used during the initial ligation reaction and added nucleotides from the pre-amplification reaction. The difference though, is the addition of two more nucleotides on both primers, again cutting the amplified bands but this time by a factor of 256. This, plus the addition of a florescent label on one of the primers, produces a product that contains a random sample based on the selection of additional nucleotides during the entire initial restriction reaction. Once mixed, this final step is sent through the step down thermocycler process.

The last step includes adding a buffer, usually Formamide and a 600 Liz size standard to a dilution of the final step (whatever the researcher deems necessary). This mixture is lightly vortexed and then loaded into the sequencing machine for an analysis that will produce the unique banding pattern characteristic of AFLP.

3.4.2. Optimization and Adaptation of Procedure. See Appendix A for detailed protocols of the following procedures. The AFLP procedure was carried out through the use of reagents found in the IRDye Fluorescent AFLP^{R} Template Preparation Kit for Large Plant Genome Analysis provided by LI-COR. This kit is actually intended for use in the analysis of plant genomes. However, with some modification, it can easily be adapted to use in other organisms, including salamanders. The Template Preparation Kit does not come with selective amplification primers. Therefore, the researcher must also purchase the AFLP Selective Amplification Kit which includes multiple final primer sets or purchase the

amplification primers separate of the set. Some steps are not necessary if a few changes to the procedure are made, mainly in the form of alterations in the amounts of primers and reagents used during the final stages. These few steps can greatly decrease the overall cost of the technique.

The initial digestion and ligation reaction proceeds as directed by the instructions with the reagents provided with the AFLP kit, with only minor differences. It is indicated that the reaction is to include approximately 100 ng of DNA in a total of 9 µL of water. In order to approach this value, the entire 9 µL should be taken from the extraction solution gained from the tissue sample. With only this minor adjustment, the use of synthesized oligonucleotides and running the annealing reaction independently are not required. Again, because the lab supervisor had already encountered this dilemma, I was able to proceed accordingly. This extra reaction would have been necessary to leave the adapters in a form that would readily ligate to the restriction sites. This simple adjustment cuts the extraction time in half. This would also be necessary if the kit did not come with standard adapters for which the sequences can be found easily online. LI-COR does not publish the sequences themselves.

Also included with the kit is a supply of pre-amplification primers that are already mixed with the other reagents for the reaction. The diluted mixture from the prior ligation reaction is added and run through the thermal cycler as suggested. The use of the primers provided for this step was the easiest approach rather than an attempt to develop pre-amplification primers independently and optimizing reagent amounts for the reaction.

A number of modifications were needed for the final selective amplification stage. The purchase of the fluorescent labeled primers can be expensive and the sequencing service of the providing company can be as well. In combination, they would have made the total cost of this method well out of the range necessary for it to be a viable method for use in cave management studies. An inexpensive alternative is the production of unique oligonucleotides that contain the same sequence as the pre-amplification primers with the addition of the desired nucleotide pairs.

For this study, the lab supervisor was able to provide these pre-produced oligonucleotides due to his involvement with a similar study conducted on the Blanchard's Cricket Frog, *Acris crepitans blanchardi* (Martin, 2004). A description of the optimization is provided below.

Optimization must be done on the reagents involved in the final reaction. LI-COR suggests the use of a duplex primer method involving both their IRDye700 and IRDye800 labeled EcoR1 primers, which come with the suite of di-oxy phosphates already added within the reagent. As mentioned previously, these items were part of the pre-amplification kit that must be purchased separately. The absence of these products requires either developing a new protocol or the use of a manufactured mix. Given the large numbers of samples to be analyzed, the creation of a bulk working mixture for the selective amplification could be developed or the use of Accuprime PFX Supermix, a product premixed by Invitrogen could be used. For the samples associated with this project, the Supermix option was chosen. If the bulk mixture option is chosen, the mixture contains 237 µL of ddH20, 60 µL of 10x reaction buffer specific to the Taq polymerase used, 50 µL of each primer (*EcoR1* and *Mse1*) and 50

μL of an equal mixture of di-oxy phosphates. Once this mixture is completed and the pre-amplification solutions are ready, a final addition of 3 μL of Taq polymerase (5 units per μL) should be pipetted in and vortexed to ensure homogeneity. The total mixture will be sufficient to perform 50 selective amplification reactions, minus any pipetting error. The Supermix provides a premixed solution of most of the ingredients found in the bulk mixture, again saving mixing time and reducing the chance for pipette error. The final reaction took place in 26.5 μL of solution. This included 22.5 μL of the Supermix combined with 2.0 μL of MSE Primer, 1.0μL of 5'-6-FAM iridescent dye and 1.0μL of the diluted DNA solution.

The pre-sequencer tray solution contained a total of 11μL of solution; 9 μL of the Formamide and 1 μL GenScan 600 Liz size standard solution and 1 μL of the diluted pre-amplification solution containing the selection of fragments done in the final step. The instructions suggest that dilution values for each step may need to vary depending on the organism used. The given amounts were found to be effective for this study after running a series of three test solutions.

Refer to the Tables 3.3. through 3.10 listed below for each of the optimization techniques used prior to doing the actual analysis of all of the samples. When working with limited amounts of genomic DNA, it is paramount to conserve that quantity so that there are sufficient amounts for the actual final run. For this particular project, it would have been difficult to retrieve more tail samples at the last minute because the process was short of extracted DNA. It was difficult enough to get the original samples due to the sheer randomness of their availability.

Table 3.3. Maize Control Samples

Reagents in µL	Maize Control Samples			
	Control 1	Control 2	Control 3	Control 4
DNA Sample	1.0	1.0	1.0	1.0
Liz Size Standard	2.0	2.0	2.0	2.0
Formamide	17.0	17.0	17.0	17.0

Maize comes with the kit and is recommended to run the process to ensure that the all kit ingredients were in working order. The Maize was run through the entire AFLP process. The only issue resulted at the sequencer tray step where ingredients are added with the completed DNA sample for analysis. Table 3.3 depicts the process used to test issues with the size standard. I mixed Control sample #1 and #2 myself. Control sample #3 was size standard that had be frozen as opposed to refrigerated and Control sample #4 was mixed by the lab supervisor as he is well versed in lab mixing techniques. I asked for his involvement as I felt that my mixing technique in a previous test might be posing a problem. This was, perhaps, the case as Control sample #4 yielded the best results. Control sample #3, as anticipated, had issues as it is highly recommended that the size standard be refrigerated and never frozen. And, as can be seen from this example, it never hurts to involve a few others in the mixing technique just to ensure that it is being mixed well and properly.

Table 3.4. 600 Liz Size Standard Test

Reagents in µl	DS 1 tail sample testing varying amounts of diluted DNA			
DNA sample	1.0	2.0	3.0	4.0
Liz Size Standard	2.0	2.0	2.0	2.0
Formamide	17.0	16.0	15.0	14.0

Since there were questions as to the viability of the Liz size standard, a test was performed with the Liz size standard on hand to see if it worked and at what intensity of DNA it would work the best to give optimal peak intensity, Table 3.4 above . New product was purchased and the test was repeated on this new shipment of Liz size standard as well. The conclusion was that both ingredients were viable.

Table 3.5. Replacement of Liz Size Standard

Reagents In µl	Replacement of Liz Size Standard with EDTA				
	Control #1	Control #2	Maize	DS Tail	
Liz Standard	0.5	0.5	0.5	0.5	
EDTA	19.5	19.5	18.5	18.5	
DNA Sample	0	0	1.0	1.0	

However, just to be sure, a test was also conducted replacing the Formamide with EDTA theorizing that the Formamide might be degrading the Liz size standard. Table 3.5, identifies the ingredients involved. These samples were run through the sequencer. The results indicated that the Formamide was not degrading the Liz as the size peaks were clearly visible in control sample #2 and both the maize and the tail sample, only absent in control sample #1. This strongly indicates that there was just a mixing or pipetting problem. Working with such small amounts of reagents increases these risk factors. To test the sequencer four controls were used; two with a mix of Liz size standard and Formamide and two with a mix of Liz size standard and EDTA.

Table 3.6. Optimizing Reagents for Sequencer

Samples test to determine accuracy of sequencer												
Reagents In µl	C1	C2	C3	C4	M1	M2	M3	M4	T1	T2	T3	T4
Formamide			9.0	9.0		9.0		9.0		9.0		9.0
EDTA	9.0	9.0			9.0		9.0		9.0		9.0	
Liz	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
DNA AFLP recommended					1.0	0.5			1.0	0.5		
DNA 1:10 Dilution of recommended							1.0	0.5			1.0	0.5

Basically, the test detailed in table 3.6 would determine if the capillaries in the sequencer were working properly as they do occasionally have to be replaced. The DNA was tested at the rate specified in the AFLP step 4 process and it was also diluted to a 1:10 solution and mixed with EDTA/Liz mix as opposed to a Formamide/Liz mix. After running it through the sequencer the results concluded that the capillaries were working fine, again good sizing peaks were reported with all controls. It appeared that diluting the DNA had no affect on the peak intensity and it was decided to stick with using the recommended 1.0µl amount as indicated in the AFLP protocol.

After the AFLP process was done using the Maize controls and the issues with the sizing standard were resolved, one tail sample was randomly selected, in this case labeled DS1 (Dark-sided Salamander sample #1 of 9) and run through the entire AFLP process in order to ascertain any issues that might be associated with the salamander DNA. Subsequently, any samples in the tables above denoted as a tail sample were from this sample after completion of the AFLP analysis. Some control and tail sample tests were combined to cut down on tables that would have ultimately appeared repetitive. Tables 3.7 through 3.9 below describe how the different techniques were optimized prior to the full sample run. It is important to test this as having too much of the dye can over amplify the results and therefore, not necessarily give true primer peaks. Keep in mind though, that the electropherograms produced from the sequencer after analysis are very detailed and provide several pieces of information that may or may not be beneficial on the analysis. Therefore, a *few* over amplified peaks will not impede the overall results.

Table 3.7. Optimizing the Eco primer

Reagents in µl	Maize Control sample and DS4 tail sample subjected to varying amounts of 56-Fam Ecoprimer			
	Maize Control Sample #4		DS 1 Tail Sample	
	Sample #1	Sample #2	Sample #1	Sample #2
Supermix	22.5	22.5	22.5	22.5
Mse1	2.0	2.0	2.0	2.0
56-Fam Ecoprimer	1.0	0.5	1.0	0.5
Pre Amp DNA	1.0	1.0	1.0	1.0

The point of this test depicted in table 3.7 was to decrease the amount of primer in order to increase the signal intensity. After analysis, both the samples with 1.0 µl primer were the most optimal. This is step 4, the selective amplification process. Problems occurred with the randomly selected tail, where initially 2.0 µl of the 5'-6Fam Ecoprimer was added to the mix producing peaks so intense many were off the scale. Therefore, the test was run with 0.5 µl and 1.0µl on both the Maize and the tail which resulted in the 1.0µl mix as the most optimal in both cases. In this case, since several were off scale, it made it impossible to accurately analyze the overall results. Once the amount was decreased and a new sample test was run, many of those off the scale peaks were then within the acceptable range.

Table 3.8. DNA Dilution Factors in AFLP

Reagents in µl	Optimizing AFLP step one using differing dilutions of a DS 1 Tail Sample					
Extracted DNA Sample	.5	1.0	2.0	4.0	6.0	8.0
5X reaction buffer	5.0	5.0	5.0	5.0	5.0	5.0
EcoR1/Mse1 primer	2.0	2.0	2.0	2.0	2.0	2.0
Deionized Water to 25 µl	17.5	17.0	16.0	14.0	12.0	10.0

Once some of the individual reagents were optimized, one of the next steps was to look at the best amount of genomic DNA for the cleanest, strongest signal. Both of the highlighted samples in table 3.8 were optimal. But in order to determine which would be the best overall amount, an experiment was performed on just the highlighted samples changing the amounts of 5’-6-Fam Eco primer. Again, the best peak intensity is the desired result. Also, as mentioned previously, optimizing each step of this process benefits the researcher in the long run. Limited amounts of DNA will likely be available during the actual comparison so test runs are very important to the overall success of the procedure. Some of the reagents purchased for this analysis come in small quantities and can be expensive. Unless, several hundred dollars are allotted to purchase these items, being conservative will save both time and funding.

Table 3.9. Optimizing Genomic DNA

	Optimizing the 6Fam Eco primer dye using DS1 tails at both the 6µl and the 8µl dilution rates			
Reagents	DS1 Tail at 6 µl dilution rate		DA1 Tail at the 8µl dilution rate	
6Fam EcoPrimer	0.5	1.0	0.5	1.0
Supermix	22.5	22.5	22.5	22.5
Mse1	2.0	2.0	2.0	2.0
Preamp dilution	1.0	1.0	1.0	1.0

After generating this test and obtaining the sequencer results, the highlighted column in table 3.9 was the most optimal combination to use for the entire series.

From table 3.8, a test was performed on the tail samples 4.0 µl and the 6.0 µl solutions first at the recommended dilution according to the protocol of 1:40 and then that was also diluted by a factor of 1:10. Both of these were then tested using three different M*se*I primers. Several primer combinations could be used to do this research. Subsequently, a large amount of time and effort could be invested in this process. Referring to Table 3.10 below, it can be seen that a significant amount of effort went into testing just three primers. Luck would have it that two out of the three were optimal, however, having assistance from the lab supervisor and his previous work went along way to saving time initially.

Table 3.10. Optimal M*se* Primer for Study

Comparing Mse primers												
Reagents in µl	Tail sample @ 4.0µl solution						Tail sample @ 6.0µl solution					
	1:40 dilution			1:10 of 1:40			1:40 dilution			1:10 of 1:40		
	1	2	3	1	2	3	1	2	3	1	2	3
Supermix	22.5	22.5	22.5	22.5	22.5	22.5	22.5	22.5	22.5	22.5	22.5	22.5
Ecoprimer	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
DNA	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
M*se* 1	2.0			2.0			2.0			2.0		
M*se* 2		2.0			2.0			2.0			2.0	
M*se* 3			2.0			2.0			2.0			2.0

After analysis, referring to table 3.10, it was determined that the two best primers were M*se*I-1 and M*se*I-3 associated with the 6.0 µl 1:40 dilution overall. They produced highly visible allele peaks as opposed to *Mse* 2. The *Mse* 2 primer produced incredibly average peaks compared to the other two primers in both the 1:40 dilution and the 1:10 dilution of the 1:40 dilution. Not to say that these are the very best two primers overall, but for this test they proved better. Several primer combinations could be tested. Referring back to Section 2.5.3 Limitations of Polymorphic banding, the most optimal primers to produce the best results could be identified and the enzyme and primer selection can have an impact on the production of reliable banding patterns and the number of polymorphisms.

Again, the choice of enzyme and primer combinations can play a major role in the amount and quality of variation revealed.

3.4.3. Visualization of Results. The sequencer produces viable results in just a matter of a few hours. GeneMapper version 3.7(2003), was the software package chosen to analyze the results once the data was run through the sequencer. The initial result identifies the presence of and the amounts of alleles but assigns a wide variation of numbers to them. The software must be calibrated so that the result is an output of the numbers 0 and 1 indicating the presence or absence of allele peaks amongst the samples tested. Note also that the report displays the intensity of the peak as well.

The report initially generated must be converted into a text file and imported into Excel format for use in other analysis programs. See Table 3.11 in the next section. Once this information has been reformatted as a data set for a number of taxa, it will be imported into PAUP 4.0 (Swofford, 2002) a software program used to analyze the information and produce a phlyogenetic distance tree. The trees can be visualized through the use of another software program, Treeviewer (2001). Basically, this analysis will produce a minimum evolution "score" to measure evolutionary change based on the differences or transformation of characters. Essentially, the distance is calculated between the observed sequences based on the observed differences and then these distances become the basis for the criterion in the analysis program that assign a minimum evolution score.

3.5. AMPLIFIED FRAGEMENT LENGTH POLYMORPHISM (AFLP)

Even though the optimization and adaptation tables seem superfluous, it cannot be stressed enough the importance of optimizing the procedures before sacrificing the extracted DNA samples. A limited amount is available and one could easily use most, if not all, of the extracted DNA in a "learn by doing" approach to performing this analysis.

Ideally, the actual reactions were to be performed on 10 tail samples from each of the three species studied and any of the questionable specimens that were collected. In this study, ten samples were collected from the Cave Salamander, nine samples from the Dark-sided Salamander, only three samples from the Long-tailed Salamander and four samples from the four questionable specimens; one from each specimen. The analysis could actually be done with just one sample from each species. However, the more samples that can be collected for analysis, the more loci that can be compared, the more enhanced the results. It was relatively simple to collect all the Cave Salamander specimens necessary for the study. The Cave Salamander prefers to be within the cave dwelling, hence its name. It was slightly more difficult to collect the Dark-sided Salamander specimens as they tend to go back and forth between terrestrial and underground habitats (Lannoo, 2005). The Long-tailed Salamander was particularly difficult to collect specimens for the study. This salamander is actually listed as found in caves in Crawford County (Johnson, 2000). However, Onondaga Cave exists in the Northern most section of the county and the range for the Long-tailed Salamander ends in the southern most part of the county according to Johnson (2000). Over the two year sampling period, only three Long-

tailed specimens were actually captured for measurements. This species prefers the leafy forest floor and old logs, but will occupy caves during the fall and winter months. During all visits to the sampling site few, if any, were observed. But, those that were captured were included in the analysis since the Dark-sided Salamander is a subspecies of the Long-tailed Salamander. It was thought gathering some genomic DNA from the limited Long-tailed specimens might provide some interesting information that could add to the analysis.

AFLP was performed on the entire set of tail samples following the protocols listed in Appendix B. Refer to table 3.11 for a complete list of the primers used in the reactions.

Table 3.11. AFLP Primers for this Project

Step	Primers	Sequences for each primer
4 Pre amp	**EcoR1** **Mse**	**5'-GAC TGC GTA CCA ATT CA-3'** **5'-GAT GAG TCC TGA GTA AC-3'**
5 Selective amp	**EcoR1** **Mse 1** **Mse 2** **Mse 3**	**5'-/6-FAM/ACT GCG TAC CAA TTC AGG -3'** **5'-GAG TCC TGA GTA ACA T-3'** **5'-GAG TCC TGA GTA ACT A-3'** **5'-GAG TCC TGA GTA ACT T-3'**

Note that the M*se*I-2 primer was listed but only tested during optimization. The primer peaks it produced proved pathetic; therefore, it was not used in the study.

Once the reactions were run with a size standard and panel to complete the sequencer step, they were initially analyzed using GeneMapper 3.7(2003). The software converts a series of the tail sample data detailing the presence and/or absence of alleles and to what extent into an electropherogram. This chart shows the peak intensities for the alleles reported. See Appendix C for examples. An allele report is then generated by manipulating the data in the program into a series of 0's and 1's. "1" represents the presence of an allele and "0" represents the absence.

3.6. STATISICAL ANALYSIS

The phenotypic data generated from this study was analyzed using Mystat, (2002) an analysis program designed for students provided by Systat and SigmaPlot. The main focus was clustering the data in scatterplots in an attempt to generate the preliminary conclusion that the questionable specimens were phenotpyically distinctly different.

AFLP sequencer analysis was initially generated in Genemapper 3.7 (2003). Once converted the genetic information was used to generate distance trees.

Distance trees were generated for the AFLP data using the default parameters for distance in PAUP 4.0 (Swofford, 2002) utilizing the Windows format and command system in Windows XP. Analyse-If for Excel (1997) was utilized for the regression analysis of the phenotypic data collected from the specimens. Mystat (2002) was also utilized for some of the scatterplots. Both can be downloaded free from the internet.

4. RESULTS

4.1. PHENOTYPIC ANALYSIS

4.1.1. Overview of Data Collection. For the phenotypic analysis, the set of measurements mentioned in section 3.2 was recorded for each of the specimens. The information was gathered for use in some different statistical analyses to determine if the relationships among morphometric variables were allometric. The analyses would also determine if those relationships were consistent among the specimens examined. This type of analysis is commonly used "in many organisms where the ratio between increments in structures of different size remains roughly constant, yielding a relatively great increase of one variable with respect to another on a linear scale" (Sokal/Rohlf, 1980).

Each salamander was identified by species and specimen number, i.e. CS1 for Cave Salamander sample #1 and its measurements were recorded in an Excel spreadsheet file for comparison analysis.

Regression analysis was run on some of the phenotypic information using Analyze-It (1997) plug in for Microsoft Excel to better understand the allometric relationships. As mentioned previously, these types of analysis software are available as free downloads from the internet. Another good source is the University computer lab or Information Technology graduate students.

4.1.2. Scatterplots. By utilizing scatterplots, all the parameters were taken into consideration to initially determine their usefulness. Several combinations were plugged into a scatterplot graph using Mystat (2002) and the results were reviewed.

When different variables across all samples were plotted against each other, several interesting preliminary results emerged. Total length was compared to all of the other variables. Specimens in this study were closely observed for any sign of tail brakeage before measurements were taken. Since no obvious tail breakage was observed in the specimens measured it is assumed that using this measurement is a true measure of body size. Affects of tail breakage are particularly obvious in that the tail grows back differently, either unusually shaped or distinct in color.

It was determined from the graphs that, since total length was being used, then head length would be of little use since it is actually part of the overall measurement. However, it was pitted against one other measurement, that being head width. Weight was used as a variable with total length even though the outcome may not be useful. The weight was measured in grams and that number was always an even number with no decimal places. As a result, all the species weight fell into one of four measurements. Costal grooves as a variable with total length would likely be ineffectual as well since that count also were even numbers and fell into one of four measurements. Unfortunately, I neglected to get snout to vent measurements for all specimens therefore there was not enough data to utilize it. Refer to scatterplots in Figures 4.1 through 4.4 below. Ideally, we wanted to see if the undiagnosed specimens grouped as a species of their own or if they shared mixed characteristics.

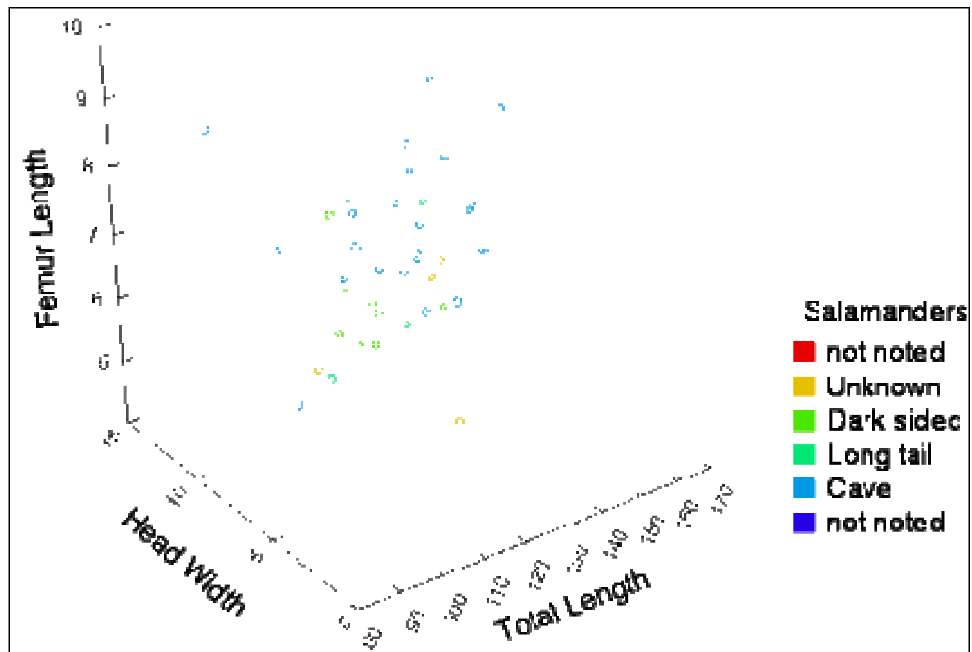


Figure 4.1. Scatterplot—Femur Length vs. Head Width vs. Total Length

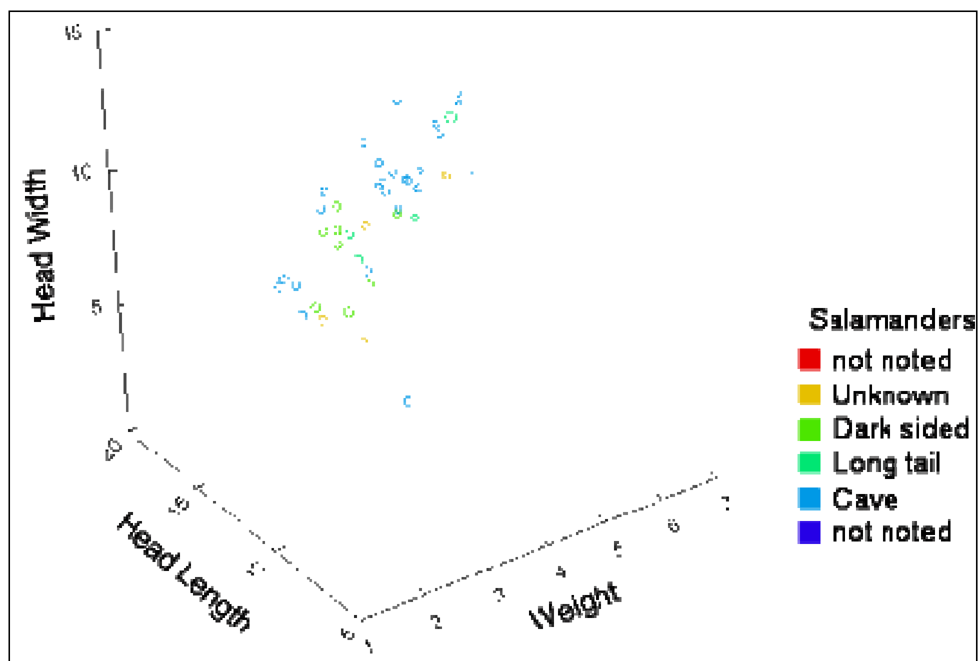


Figure 4.2. Scatterplot—Head Width vs. Head Length vs. Weight

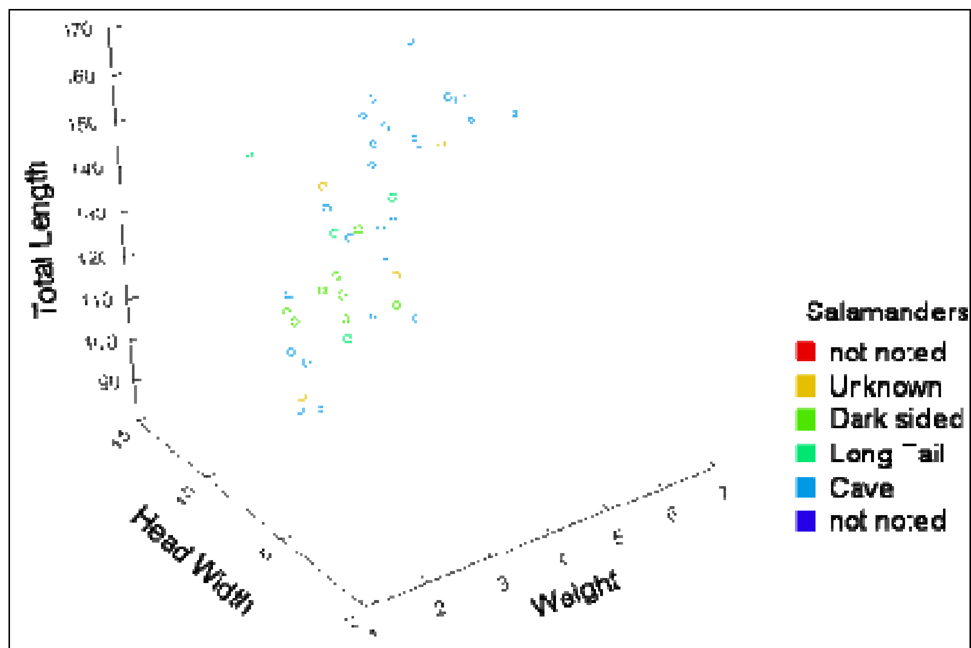


Figure 4.3. Scatterplot—Total Length vs. Head Width vs. Weight

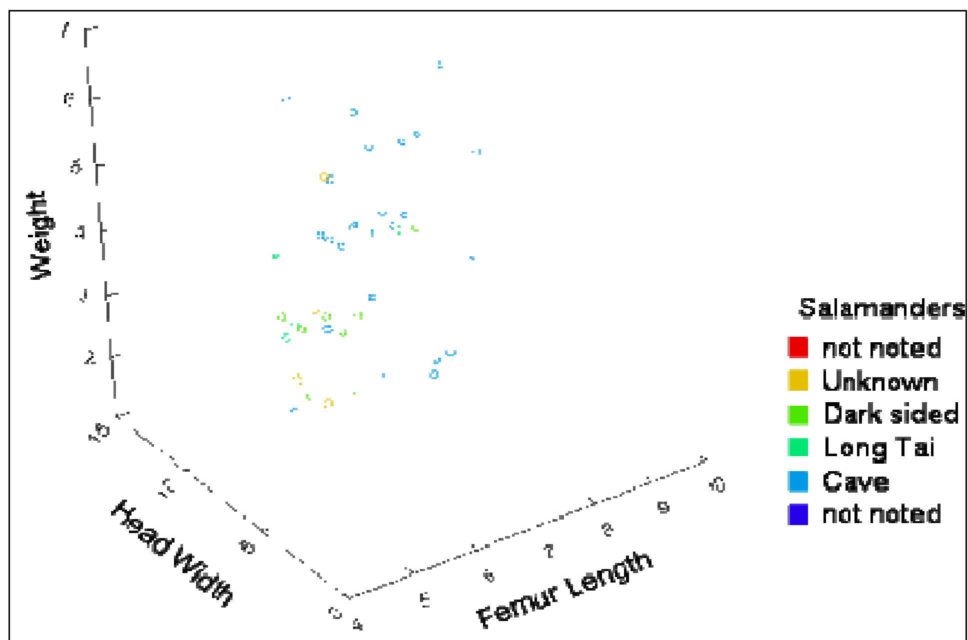


Figure 4.4. Scatterplot—Weight vs. Head Width vs. Femur Length

4.1.3. Regression Analysis. Refer to scatterplots, Figures. 4.1 through 4.4 above. These graphs were initially utilized to visualize the data. In each of the four graphs, note that the unknowns aren't actually phenotypically distinct. They tend to be interspersed with the other species. Yet, they don't actually fall out with any particular group either. Regression analysis was performed for each group using a pair wise comparison.

Regression analysis basically analyzes two variables. It shows a functional relationship between the two variables. The process utilizes the data to predict values for one of the variables when the other or multiple variables have a specified value. According to Sokal et al. data regression estimates the relationship of one variable with another one in terms of a linear function of another; this is also known as allometry.

Regression analysis was applied to following four combinations:
Head Width versus Femur Length, Head Width versus Head Length, Weight versus Total Length and Total Length versus Femur Length. The confidence intervals around the slope were produced for all the groups. This is promising as confidence intervals are good indicator signs of real relationships between the two variables. The first two combinations had good intervals. Table 4.2 illustrates this. The third group showed a slightly larger confidence interval and the fourth group had an overwhelming confidence interval. Regression analysis is a form of hypothesis testing through the use of precise calculations that I will not go into in depth except to say that it involves parameters such as standard deviations, probability, standard error, degrees of freedom, residuals and slope. This information can be easily

referenced and a number of computer programs are now available to quickly provide needed analysis.

Once the regression analysis was done and confidence intervals were established, pairwise comparisons of the slopes for the unknown species versus each of the other species (Cave Salamander, Long-tailed Salamander and the Dark-sided Salamander) using the same set of parameters, was performed.

Because the fourth comparison, Total Length versus Femur Length, had such large confidence intervals, there seemed to be no real relationship between the variables. Based on this information, a pairwise comparison of the slopes was not done on this data as it appears that it would not prove useful. Instead, I chose to compare the Weight versus the Total Length mainly to use a dependent variable other than Head Width against the independent in search of any new results.

Confidence interval graphs were done for each group; Cave, Long-tailed, Dark-sided, and Unknown. Four sets of variables were analyzed in search of positive correlations associated with the allometric relationships (depicted by the slope). Specifically, I was looking for statistical difference with, in this case, 99% confidence.

Table 4.1 below summarizes the t-test values used in comparison of slopes for all of the regression analyses listed previously. Once the slopes were calculated using the regression analysis, the comparison of the slopes of the lines for the questionable or unknowns versus each of the other groups were done. Table 4.2 depicts the statistical information necessary for the calculations.

This information tells us if they are truly statistically different from one another. The results of the analysis will either prove that the unknowns are phenotypically different or prove that they are not.

Because the slope of the lines follow along *t* distributions, a simple *t*-test was performed. This is particularly important for this study because the sampling issues talked about previously will not be such an issue. Ideally, a sample size of thirty specimens for each group was to be collected. In this case, the smaller sample sizes will not affect the standard deviations and calculations for confidence.

In the Head Width versus Femur Length category, the unknowns were statistically different from the other three species with 0.99% confidence. In the Head width versus Head Length analysis the unknowns were not statistically different from the other three groups. Also, in the Head Width versus Total Length analysis the unknowns were not statistically different from the other three groups. In the last analysis, Weight versus Total length the unknowns were not statistically different. All *t*-test results were less than the standard degrees of freedom for this analysis.

It is likely that with other combinations of pair wise comparisons, statistical differences could be found. With the amount of morphological measurements that were taken, over forty different combinations could be examined in order to determine this information. But, because it could be extremely time consuming, some were selected mainly based on their being a dependent or an independent factor to see how they compared to each other. Actually, even more combinations could be generated by changing the dependent and independent axes.

Table 4.1. Summary of *t*-test Values

	Pair wise Comparison of t-test Results			
Salamanders	**HW vs. FL**	**HW vs. HL**	**HW vs. TL**	**W vs. TL**
Cave vs. Unknown **Confidence Interval** **1.69 @ 95%*** **2.46 @ 99%****	3.15**	0.2118	0.035	0.0668
Long vs. Unknown **Confidence Interval** **2.13 @ 95%*** **3.74 @ 99%****	3.7605**	1.719	0.1667	0.3791
Dark vs. Unknown **Confidence Interval** **1.83@ 95%*** **2.82@ 99%****	4.389**	0.6655	0.044	0.424

These values were produced after extensive mathematical calculations; the step of which are available through the use of any good bio-statistical analysis textbook. Keep in mind though, that it is just as important to keep the regression analysis figures organized as it is to keep the molecular data organized. Working with several different numerical values can be confusing and easily transposed.

Table 4.2. Summary of Regression Statistics

Summary Statistics for Regression Lines

Head Width versus Femur Length

Sal'mander	r^2	s	b	intercept	n	CI	p	m ss	r ss	F
Cave	0.21	1.12	0.5555	5.535	29	0.1279 to 0.9831	0.0128	8.89	33.76	7.11
Long	0.15	3.3	1.158	2.913	4	-7.254 to 9.570	0.6137	3.82	21.76	0.35
Dark	0	0.81	-0.0154	8.598	9	-1.0907 to 1.069	0.9822	0	4.62	0
Unknown	0.95	0.56	2.763	-7.85	4	0.797 to 4.729	0.263	11.61	0.63	36.57

Head Width versus Head Length

Sal'mander	r^2	s	b	intercept	n	CI	p	m ss	r ss	F
Cave	0.35	1.01	0.4797	2.567	29	0.2232 to 0.7362	0.007	15.1	27.6	14.72
Long	0.96	0.69	-1.086	22.25	4	-1.733 to -0.438	0.187	24.7	0.95	52.09
Dark	0.2	0.73	0.2066	5.983	9	-0.1604 to 0.5736	0.2249	0.93	3.69	1.77
Unknown	0.19	2.22	0.6113	0.5495	4	-3.1822 to 4.404	0.5598	2.37	9.87	0.48

Head Width versus Total Length

Sal'mander	r^2	s	b	intercept	n	CI	p	m ss	r ss	F
Cave	0.2	1.13	0.02925	5.504	29	0.00599 to 0.0525	0.0157	8.43	34.22	6.65
Long	0.36	2.86	0.1307	-6.512	4	-0.395 to 0.6573	0.3973	9.3	16.31	1.14
Dark	0	0.81	0.00278	8.199	9	-0.0672 to 0.0728	0.9279	0.01	4.61	0.01
Unknown	0.25	2.14	0.05404	1.005	4	0.2307 to 0.3388	0.5	30.6	9.18	0.67

Weight versus Total Length

Sal'mander	r^2	s	b	intercept	n	CI	p	m ss	r ss	F
Cave	0.67	0.87	0.0390	-2.054	29	-1.675 to 4.875	0.105	1.68	19.95	23.76
Long	0.34	0.57	0.6576	1.629	4	0.516 to 1.629	0.516	2.228	0.088	0.266
Dark	0.106	2.81	0.2321	3.684	9	-0.283 to 1.308	0.785	2.094	0.033	0.08
Unknown	0.79	0.29	0.2120	-3.533	4	0.446 to 1.819	0.21	0.669	2.961	3.31

4.2. MOLECULAR ANALYSIS

This part of the analysis was very challenging from start to finish. It was also frustrating at times. While extracting the DNA was favorable in this instance, it can be a difficult part of the process and the finished product may not be viable. It is very difficult to keep the samples separated and keep them accurately marked during the analysis phase, the data conversion phase and PAUP program phase. This was done with only a few minor errors but it required monumental effort and clear and concise thinking throughout the entire process. Overall, the completed batch of samples came out well. Only a few samples had to be removed from the tree analysis as the primer peaks were weak and not of good intensity. Likely, the source of this issue stems from a trip through the thermocycler. The cap on the vial was not tightly closed and the DNA was compromised as a result.

Once the tail data was converted into the 0 and 1 format, it was imported into an Excel tab delimited text file so that it could be utilized in the PAUP program to produce the necessary analysis to generate a tree in Treeviewer. See table 4.1 for an example of the data in text format. Each tail had a varying number of alleles that the program generated. Also, a number of different approaches were taken in comparing the data. They included the following:

1. The total tail data set utilizing the Mse I-1 primer was compared to each other and scored. Each of the four binsets within the Mse I-1 series were compared to each other and scored; Cave Salamander, Dark sided Salamander, Long Tailed Salamander and the Questionable Salamanders.

In addition, each questionable salamander was scored comparing it to the other salamanders from all binsets.

2. The total tail data set utilizing the MseI- 3 primer were compared to each other and scored. Again, all binsets were compared and scored in the Mse I-3 series identical to those described for the MseI-1 series.

Once converted to an Excel tab delimited file, command references are added to develop a Nexus file for complete processing in PAUP. A heuristic search was performed with optimal criterion set to distance, developing a number of rearrangements of the data to construct the best possible tree and assigning branch lengths to calculate a minimum evolution score.

Again, with every aspect of this project, keeping the data organized was paramount. It is extremely easy to mix up the measurements in the phenotypic data as there is not much difference in sizes. It is also easy to mix up samples in the genotypic analysis because of working with such small samples sizes and a multiple specimens from multiple species. The data for the phylogenetic tree analysis is also easy to mix up if the numbers aren't closely monitored. It is important to name the specimens with simple, easy to remember names. And, when dealing with the text files be careful with cutting and pasting. It is very easy to mix up information. It helped considerably to save multiple files with good labeling and delete anything that didn't work the first time. Saving too many files becomes confusing especially when you save them with similar names. Until one gets familiar with this phylogenetic tree program, several test runs should be made in order to get the information in precisely the right order. Refer to a sample data set in Table 4.3. below.

Table 4.3. Data Set from the Q5 Mse3 Binset

LT2	1	1	0	1	1	1	1	1	1
CS1	0	1	0	1	1	0	1	1	0
CS2	0	1	1	1	1	0	1	0	1
CS3	0	1	0	1	1	0	1	1	0
CS5	0	1	0	1	1	0	1	0	1
CS7	1	1	0	1	1	0	1	1	1
CS9	1	1	0	1	1	0	1	0	0
LT1	1	1	0	1	1	0	1	1	0
QS3	1	0	0	1	1	0	0	0	1
QS5	1	1	1	1	1	1	1	1	1
DS5	0	1	1	1	1	0	1	1	1
DS9	0	1	1	1	1	1	1	0	1
CS4	1	1	1	1	1	0	1	0	0
CS8	0	1	1	1	1	0	1	1	0
C10	0	1	0	1	1	1	1	1	1
QS2	1	1	1	1	1	0	1	1	0
QS4	0	1	1	1	1	1	1	0	1
DS2	1	1	1	1	1	0	0	1	0
DS4	1	1	0	1	1	0	1	0	0
CS6	0	1	0	1	1	1	1	0	1
LT3	0	1	1	1	1	1	1	1	1
DS6	0	1	1	1	1	1	1	1	1
DS8	0	1	0	1	1	1	0	1	1

The optimal criterion set to distance generated a completely tree comparing branch length or observed differences in the alleles to determine how closely related the species actually could be to one another. One of the reasons this type of analysis was important in this project particularly involves the questionable specimens. Ideally, the results to be looking for would be a tree that grouped the questionable specimens on their own branch. Unfortunately, this was not the case, questioning the theory that they could possibly be a stand-alone species.

After assessing all of the combinations of binsets, it was determined that, for this analysis, only the information compiled for what was called the total batch for each primer was utilized. Total batch is defined as all of the samples analyzed for each of the primers. Also, both primer batches were combined in order to compare

the primers to each other. As a result, three phlyogenetic trees were generated. All three trees can be referenced in Appendix D-F.

As mentioned previously, four questionable specimens of unknown origin were examined and compared to the known species based on visually observed unusual phenotypic characteristics. Each of the four specimens had a combination of characteristics from each of the other species. The observed characteristics are noted in the following discussion below.

The specimens in question are labeled QS2, QS3, QS4 and QS5 respectively in the phylogenetic trees.

The visually observed phenotypic characteristics for QS2 included that it looked like a Cave Salamander, it was the size of a Dark-sided Salamander and it had tail markings similar to a Dark-sided Salamander. Based on the information provided in the trees it appears that, in the M*se*I-1 tree it is closely related to Dark-sided Salamanders. In the M*se*I-3 tree it appears to be more closely related to the Cave Salamanders. But, in examining the two trees it can be noted that, overall, there does not seem to be a large distinction between the Cave Salamanders and the Dark-sided Salamanders residing in this particular cave when focusing on the clades in which the QS2 is situated in. This observation is made though, based on the two primers used. The possibility exists that a different interpretation could result if other sets of primers were used.

The visually observed phenotypic characteristics for QS3 included that it had Long-tailed markings but the coloration of a Dark-sided Salamander. Based on the information the trees provided here, it appears that, in the M*se*I-1 tree and the M*se*I-3

tree it is very closely related to the Dark-sided Salamanders. In fact, there is very little distance between it and several Dark-sided specimens therefore, it is reasonable to assume that this questionable specimen is a Dark-sided Salamander.

The visually observed phenotypic characteristics for QS4 included that it had the markings of a Long-tailed Salamander, the size of a Dark-sided Salamander and the coloration and patterning of a Cave Salamander. Based on the information the trees provide for this specimen it appears that, in both the M*se*I-1 tree and the M*se*I-3 tree it is closely related to two other salamanders but this time the Cave Salamander and the Long-tailed Salamander.

The visually observed phenotypic characteristics for QS5 included that it had an extra long tail like the Long-tailed salamander, combined markings of the Long-tailed and the Dark-sided Salamanders, and it was the size of a Cave Salamander. As seen with the QS4 sample, it pulls out with Cave Salamanders and Long-tailed Salamanders.

It might be noticed that the tree combining both primer sets was not referenced in the above text. Upon examination of this tree, no new or unpredicted results appeared that would be of use in analyzing the data.

Mentioned previously, and reiterated here, the Dark-sided Salamander is a subspecies of the Long-tailed Salamander. Therefore, we should be seeing them clustering together in the AFLP phylogenetic trees. Yet, in all three trees, the Long-tailed Salamanders are consistently clustering with the Cave Salamanders. Based on this observation, it appears that there is a great deal of genetic distance between the two subspecies the Long-tailed Salamander and the Dark-sided Salamander;

supporting the theory that they should, perhaps, be considered separate species. However, more interestingly, the Long-tailed Salamanders in this cave population seem to have a genetic makeup comparable or similar to the Cave Salamander; supporting the theory that they are interbreeding--backed up by the evidence noted above.

So, in conclusion, the following can be said concerning the results and subsequent discussion about what is taking place here. First, the four specimens were utilized in this analysis based on visually observed phenotypic characteristics. Each had something uniquely unusual about them to make them suspect. The visual assessments parallel the DNA tree analysis in all four cases. The scatterplots identified earlier consistently tell us that the four groups mentioned are not phenotypically distinctly different however, they are not definitively with the others either; supporting the possibility that they could be hybrids. Most of the regressions performed were not significant. However, the visual assessments looked like the unknowns were grouping with the others. In other words, phenotype says that these unknowns are not actually new species but they are not known species either; a strong indication that they may be hybrid.

Provided that somewhat ideal primers were used, applying Amplified Fragment Length Polymorphism in this study had proved useful in determining which species of salamanders might have been breeding together to make the phenotypically distinct morphs. In this case, there seems to be three distinctive occurrences taking place:

1. Cave salamanders and dark sided salamanders might be interbreeding; referring to the results from the QS2 sample.
2. Cave salamanders and long tail salamanders are potentially interbreeding; referring to the results from both the QS4 and the QS5 sample.
3. There are definite dark sided salamanders with distinct characteristics; referring to the results from the QS3 sample.

4.3. COMPARISON OF PHENOTYPIC AND MOLECULAR METHODS

Both methods utilized data related to the salamanders to determine if these questionable samples were distinct species, actually interbreeding or if the characteristics observed were merely variations within each of their own species.

A series of analyses for the phenotypic data was performed that yielded promising results. The regression data was precise and the statistical analysis supported the theories identified.

AFLP analysis also proved to be a promising approach to this analysis. The trees generated yielded pointed toward the prospect that something was definitely taking place among the genetics of the groups studied. When compared to the visual assessments, it could be said with certainty that something was occurring.

Computer software has made considerable strides in recent years and many new programs are available for data analysis. This aspect of the research will likely only continue to improve.

AFLP is becoming a more popular approach to analysis. It has been widely used for plant genome studies in the last several years (Law 1998, Barker 1999, Reisberg 1999, Beismann, 1997) and a few vertebrates for sex identification. It

reduces time constraints and is somewhat more cost effective and the ability to refine the primer choices is paramount. Some genetic studies involving the use of salamanders have been done with RFLP and microsatellites (Weins 2003, Riley 2003). One main reason AFLP was chosen for this study was that there seems to be limited documented literature on the use of AFLP in relation to salamanders. Microsatellites could have been used. The technique combines the use of small alterations within the genetic sequence with a more direct measurement of variability that of genomic DNA. It is most useful in species were the short tandem repeats are already known. If not, a genomic library of genetic sequences has to be developed for the species; requiring several PCR reactions and sequencing before the actual analysis can begin (Martin, 2004). This must be done one at a time and through the use of gel electrophoresis. While they can be used for species identification across a broad spectrum of animals, AFLP can process the information similarly but through the use of a sequencer and a software program that analyzes the data efficiently. Microsatellites also have expensive start up costs and taxonomic limitations (Robinson, 1999).

Overall, both approaches were beneficial in the complete analysis of the salamanders. However, using AFLP as a model for genetic analysis in salamanders was meant to aid in the advancement of this particular approach as it is reasonable to assume that an increasing number of hybrids will be discovered in the future. Salamanders are widely distributed among ecosystems and they are amenable to both molecular and quantitative studies. They should prove to be respectable models for future genetic investigation (Beebee, 2005).

Much time was spent during this investigation optimizing the AFLP process in relation to salamanders under the premise that the knowledge gained will benefit any future studies of salamander populations using the AFLP technique.

Of course, this particular study merely opens the door and broaches the question of interbreeding. In order to truly make some good, solid conclusions a much larger data set needs to be gathered. This would make the AFLP analysis more viable and regression analysis more refined, providing more definitive values.

5. DISCUSSION

5.1. HYBRIDIZATION

Human impact on caves and restoration practices associated with them were discussed extensively in previous sections of this document. Presently, human disturbance has been identified and addressed in a number of ways through the use of varied management practices among concerned speleological organizations. Today, no real evidence points to a link between human disturbance and the possibility that salamanders might be hybridizing in areas where this "human" factor has been removed. Human disturbance of cave ecosystems and lack of concern will always be issues that need to be addressed in some form in the future. Perhaps, one should consider a thought pattern such as this as an ongoing hypothesis for future generations to investigate. In view of that, what other possible factors could be employed in an attempt to understand why there might be inbreeding among the species in this genus? Could other environmental factors unique to cave ecosystems be involved?

The preferred habitat for all of the *Eurycea* species discussed consists of moist areas or water located in woodlands, rock outcroppings, caves, springs or cold creeks (Johnson, 2002). They eat mostly invertebrates that enjoy the same or similar habitat. All of them lay eggs in water or the moist cracks and crevices located in the rock outcroppings. The *Eurycea* species are lungless and lack gills; therefore, they breathe through their skin. These salamanders have limited breeding periods, mostly from November to April. Cave salamanders, in particular, must have *limestone* rock outcroppings for breeding (Johnson, 2002).

All are nocturnal and can be seen after heavy rains. Certain environmental conditions can limit their breeding habits for example, fluctuations in the water levels in pools or ponds that they may use to breed can have a significant effect (Stebbins, 1985). Visual observation at Onondaga Cave shows that the salamanders seem to prefer the natural pools within the cave as opposed to the manmade pools constructed of concrete developed by private owners in the 1970's.

As mentioned previously, the Long-tailed salamander's range does not actually reach Northern Crawford County. However, the subspecies of the Long-tailed salamander, the Dark-sided salamander is found extensively throughout Crawford County. The visual assessments, the statistical analysis and the genotype study of this genus indicate that some sort of interbreeding is taking place. What might be some of the environmental parameters influencing this hybridization? And, why would they actually do this? Typically, inbreeding is detrimental, costs are high and it decreases fitness in a species. A mixture of genes from organisms that are distinct enough to be called separate species don't usually produce healthy, fit offspring that can survive (Stebbins, 1985).

In a recent study on interbreeding between invasive species and native salamander species, Fitzpatrick et al. (2007) found that interbreeding between the California Tiger Salamander, a native, and the Barred Tiger Salamander, an invasive, are producing offspring with an increased ability for survival. They also indicate that this hybrid could potentially replace the parental populations as they are more resistant to disease, better at predator escape and more efficient food gatherers. According to Fitzpatrick et al. (2007), concerns among conservationists swing both

ways. Some feel that this hybridization is beneficial as it is favored by natural selection. Others disagree and feel that it is actually a threat to the native species in the form of genetic impurity.

In a similar study, Veen et al. (2001) identified hybridization and adaptive mate choice in flycatchers. Typically, the offspring of mating between two different species are infertile or have low reproductive rates. In this case, pied and collared flycatchers, two closely related bird species, appeared to be hybridizing. As with the hybrid offspring of the Tiger salamanders, heterosis or hybrid vigor is being exhibited with the hybrid offspring of the pied males and the collared females. More specifically, they are producing more fledglings later in the season than pure collared pairs producing peak performance later on. Also, not all of the offspring are actually hybrids. In addition, Veen et al. (2001) found that this combination also produced more male than female offspring. They hypothesize that this hybridization could enhance species divergence. For instance, any negative outcome of hybridization might put pressure on the two species to evolve better ways to distinguish between each other.

Why would the salamanders in Onondaga Cave be interbreeding then? What factors could be influencing their mate choice? Firstly, let's consider the species versus subspecies. The Dark-sided Salamander is a subspecies of the Long-tailed Salamander. But what actually constitutes a subspecies? According to Stebbins 1985, the characteristics of many species differ in varying parts of their range. In the Ozark region, the geographic range distribution for the Dark-sided Salamander occurs throughout southern and eastern Missouri. The Long-tailed Salamander is restricted

to the southeastern portion of Missouri. There is about a two county wide region where the two species overlap (Johnson, 2002). This would be the point where the characteristics of one change gradually into those of the other. This zone of change is identified as intergradation (Stebbins, 1985). The subspecies that results are also known as geographic variants or geographic races.

As mentioned previously, separate species don't usually produce healthy and fit offspring. According to Stebbins (1985), the subspecies category has been applied in the absence of adequate information and with "considerable subjectivity." Previously, taxonomy based identification was the mode for distinguishing a subspecies from a species. So, as biochemical techniques have been developed, applied and analyzed actual degrees of genetic differences have been assigned to groups of organisms. Stebbins (1985) indicates these techniques have shown that some closely related populations are actually full species as opposed to a subspecies.

Now, in the case of the Long-tailed Salamander, even though its range is in the southern most part of Crawford County, a few specimens were found in Onondaga Cave. What could be the reason for this? Is this a new locality of occurrence? What about the unusual individuals or hybrids? Perhaps, they are a part of the hybridization that has been identified. Actually, there is also the possibility that they were transported outside of its original range, perhaps by humans.

Another perspective to investigate why this hybridization is taking place is to take a closer look at the cave microclimate. The cave ecosystem is fragile and has a unique biological and ecological structure. Caves are actually made up of three zones of cave life; the entrance zone, the twilight zone and the zone of total darkness

(Weaver, 1992). These zones support different aspects of the limited food chain that exists in cave. The entrance zone has limited light and the temperature and humidity can vary. Most of the animals found at or near the entrance are surface dwellers and don't normally go very far beyond the entrance due to the limited food source. The twilight zone begins just inside the entrance and typically ends where total darkness begins. The temperature and humidity are more stabilized in this area but still can minimally fluctuate. This is the zone were various species of insects, frogs, salamanders, cave crickets, and bats can be found. The zone of total darkness supports only troglobitic species, those that are blind and have no pigment to their skin. Temperature of the water, air and rock are constant and the humidity is constant as well. In other words, the temperature is ambient and remains at about 56° year round. Could this aspect of total darkness play a role in the *Eurycea* interbreeding? Since salamanders are brightly colored and this color likely plays a role in the breeding rituals of the salamander, would it be a factor in mate choice if the female could not actually see it due to the lack of light in the cave? Would this then increase the possibility that they would mate with the individual most available to them?

Onondaga Cave has nine total entrances; two are manmade and seven are natural. The area studied, the Missouri Caverns section, is a manmade entrance. All of the entrances, manmade or natural, lie within the entrance zone of the cave where the temperature and humidity fluctuate and the light is very limited and generally non- existent as the twilight zone is close. Salamanders prefer a wet, cool environment generally under leaf litter in shade habitats such as woodlands and forests. These areas are always wet and cool. The cave microclimate mimics these

conditions. It is wet and cool inside the cave in the summer and winter (warmer inside than out; but still cool and not freezing). There are a number of reasons why they might utilize this microclimate and why using it might be a factor in their interbreeding. Food is probably not a significant reason why the salamanders studied are using this section of the cave. Small invertebrates are washed into the area under the door during rain events and they can find their own way in as well since they live in the leaf litter just outside the door. Unlike the troglobites, food would not be a limiting factor as there doesn't seem to be competition for the space. Troglobites, such as the Grotto salamander, are generally limited in number and distribution based on the limited food source (Weaver, 1992). Remember, the troglobites are dependent on the food that other creatures bring in with them and/or leave behind. These areas of total darkness don't see much in the way of large animal or human traffic. Humans actually leave a food source behind in the form of skin cells and hair, lint off of their clothes and bacteria and fungus brought in on their shoes.

The fluctuation in the humidity could be another factor. Salamanders use pheromones to attract their mate. In a fanning motion they attempt to distribute the pheromone throughout the area in which they reside. High humidity could actually assist in this distribution helping to carry the scent (Rossi, 1995). The scent could have a lingering effect since it is suspended in the thick air. This effect is witnessed on occasion when tours are taken into the cave and someone has a particular odor to them, for example the smell of a cigarette. That scent is enhanced in the highly humid conditions and the wafting smell can be identified from a great distance.

Different species of salamanders have been known to utilize habitats that are not necessarily their first choice. Wilson (2003) observed along with others the habits of the Green salamander in the Kentucky, Tennessee, and Virginia area. The Green salamander is primarily a rock crevice dweller. But he and others report seeing this salamander in woody and even arboreal habitats. Obviously, these are not related habitats. There must have been some reason why this salamander chose to inhabit a different area. Apparently, this species of salamander once used this woody, arboreal habitat when the American Chestnut tree was prevalent. But because of a decline in the tree population as a result of the chestnut blight and the loss of the subsequent woody debris, the salamander began using the crevices of rock outcrops instead.

What about factors outside the cave microclimate that could actually have an effect on the microclimate itself? Changing weather conditions outside the cave could dictate what the salamanders consider doing inside the cave. Barometric pressure changes, temperature changes, precipitation and freezing would affect the entrance zone and the twilight zone to some degree, in turn affecting when or if the salamanders use the areas. Wilson (2003), reports that they observed the green salamander becoming more active during periods of high humidity, during misting conditions and during light and moderate rain. But, in the summer they were not seen as often during periods of heavy or prolonged rain; possibly making it too wet for mating rituals. Stebbins (1985) pointed out that conditions of differing temperatures and rainfall were the best guide for field observations. He also indicated that the knowledge of the patterns of daily activity in the salamanders was very useful in studying them. Studying the daily habits of salamanders and the conditions

associated with the cave microclimate would be beneficial information that could be incorporated into a cave management plan. Thorough recording of this type of data may be of help in answering some of these questions.

The cave microclimate must also serve as some sort of protection from predation. One could not say for certain that this would have any direct impact on a salamander's mate choice, however. There are usually factors within a species geographic range that keep them mating with each other to continue the fitness of their species. In addition to those discussed, changes in their geographic range, their moving into a different latitude, changes in the color of their surroundings, changes in behavior and changes in the amount of light they are exposed to could all play a role in their willingness to inbreed (Stebbins, 1985).

Let's not forget good biodiversity and ecosystem integrity. During treks to the sampling site it was surprising that if no other species was seen at the sampling site, an abundance of *Plethodon alabgula* or the Western Slimy salamander was always there; apparently, for a good reason. Welsh et al. (2001) identify the *Plethodontidae* family as a species of salamander that have unique attributes making them excellent indicators of biodiversity and ecosystem integrity. The Slimy salamander is normally a terrestrial, forest floor dweller that occurs in high densities when the habitat exhibits signs of good biodiversity. However, in the heat of the summer they are known to go underground and have been found in Missouri caves (Johnson, 2002). Their presence in large numbers in the Missouri Caverns entrance might provide a connection that correlates biodiversity and ecosystem integrity with *Eurycea* interbreeding.

5.2. APPLICATION OF RESULTS

The questions raised and those that were answered could be beneficial and serve as a model for future discoveries of hybrids. The information could provide constructive background that might be noteworthy in developing many parts of a well defined cave management plan.

The finished project proved to have a threefold result. First, to take a closer look at how cave management, restoration efforts and human disturbance could positively or negatively affect the cave ecosystem. And, what measures could be taken in the development of these plans to include important scientific research as a supplement to the effectiveness of those management plans.

Caves play an important part in the quality of our drinking water. As a result, continued and heightened awareness of this unique underground environment is paramount. As development on the surface above cave ecosystems increases and changes in water quality occur, these actions will have to be addressed.

Second, to apply the molecular technique AFLP, or Amplified Fragment Length Polymorphism as a genetic test to determine if hybridization was truly occurring within the genus *Eurycea*. This technique was chose because it was inexpensive, relatively easy to use, and could be utilized for endangered species or species of concern since it requires only small amounts of genomic DNA. And thirdly, to look at how the information gathered could be applied to assist in answering the question—why do the species in this genus appear to be interbreeding? The intent was to address a variety of environmental issues associated with the cave ecosystem that might provide some insight as to their willingness to interbreed.

For this study, two species of salamanders and one subspecies were observed in a section of the cave that is now highly protected and where minimal human interaction takes place. Sixty years ago, multiple tours of individuals were guided through the area on a daily basis. The three groups studied were *Eurycea lucifuga*, the Cave Salamander, *Eurycea longicauda longicauda* or the Dark-sided Salamander and *Eurycea longicauda*, the Long-tailed Salamander. Particular attention was focused on the Cave and Dark-sided Salamanders as the sampling site is on the northern most boundary of the know habitat the Long tail Salamander; although a few specimens were identified and sampled. The AFLP technique was applied in attempt to identify polymorphisms in relation to each species.

These applications no doubt have improved the assessment of scientific theory on many different levels. Prior to these advancements, species identification was probably done by phenotype alone. And, most of the statistical approaches to analyzing this data only a few years ago might be considered archaic now. Many of the genetic analysis capabilities available today are not concepts that are generations old. Imagine if these genotypic capabilities had been available to researchers, when many of the species we actually have to day were named. The exponential growth in species identification and discovery that is seen on a regular basis today would have started many years sooner as a result.

More research employing the technique in a wide range of applications should be done. Because it can be applied to small sampling sizes, it provides an additional way for researchers to study species of concern. One of the more interesting aspects of AFLP is the software used in analysis. It can analyze small

samples sizes from several groups simultaneously and cross-compare the results. These features will likely draw the attention of progressive analysts as it becomes better documented and more popular.

5.3. CONCLUSIONS

The results of this project were surprisingly interesting and exciting. It raises yet more questions about future studies of cave ecosystems. There are a number of approaches to gaining more knowledge to support this aspect of the hypothesis. First, impact studies need to continue and become more refined. A number of studies were cited in previous portions of this thesis. Environmental impact studies provide the scientific community with much of its basis for developing policy and managing resources.

Next, to enhance a project such as the one covered in this document, habit studies of the salamanders themselves might prove beneficial in helping to narrow down specific sampling times of the day and seasons of the year. One of the issues dealt with was the randomness of the samples. There appeared to be no rhyme or reason to when each type of salamander, if any, would be present in this section of the cave. The unit was visited a number of ways; going in at different times on the same day even late at night and extremely early in the morning. Going in on different days and going in during different weather events, i.e. particularly warm days or particularly cold days. Visits were made during the different seasons. It appeared blatantly obvious that salamanders can be very elusive. Reiterating Stebbins (2005),

knowledge of the daily activity patterns of salamanders was very useful in studying them. A habits study, while potentially a long term event, could prove useful.

Experiencing this randomness raised another question for me. A recent National Geographic television program discussed cave ecosystems where multiple species of bats had evolved to utilize the cave at different times of the day and night to decrease competition for space. Could the salamanders be doing something similar? Or, are they random because they can be? No human activity is occurring in the area that inhibits their movement. In other parts of Onondaga Cave, all employees have observed salamanders utilizing different areas of the cave at different times of the year and it appears to be based on human activity at the time. During the busiest times of the season, we do not see any of the species that normally utilize Onondaga Cave. However, we regularly see different species during the early and late portions of the season went traffic flow is minimal or nonexistent. A habits study might address this theory as well. Another approach to refining the randomness would be a salamander specific pheromone study where pheromones exclusive to a species would be used to "draw out" or attract the particular species of salamander to be sampled.

Because the results of this project seemed promising, much discussion has taken place with colleagues in the caving community. It is of special interest to me to note that several have reported visually observed phenotypic assessments in some of the caves they are mapping in Missouri. Of particular interest, though, is that the anomaly is reported to exist in caves they have mapped in the Ozark region of Missouri. One individual stated that he had seen this in only four other caves out of

about one thousand that he has been in nationwide *and* all four were located in the Ozark Plateau. One of the four, Crystal Cave located in Berry County, Missouri was a former commercial show cave for almost 65 years (Beard, 1999). In 1994, it was closed to the public and is now leased by the Missouri Cave and Karst Conservancy. They have some photo documentation of unusually looking salamanders.

In closing, I mentioned previously that one of the reasons for a salamander being outside its range was as a result of human intervention; someone actually relocating the animal. This may seem farfetched but consider the mindset of the typical commercial cave owner in the 1970's. First of all, there weren't many of them and they all likely knew each other. At that time, this was a big industry and some cave owners went above and beyond to promote and market their "product." People were probably just as scared of bats then as they are today. But, a salamander is not alarming to the general public and, for the most part, is considered pretty. What are the chances that the cave owner here at the time "transported" a few salamanders to Crystal Cave or visa-versa and strategically placed them throughout the cave so the visiting public could see one on their tour?

APPENDIX A

STANDARD OPERATING PROCEDURE

APPENDIX A
Extraction of DNA from Whole Tissue Samples

Materials and Instrumentation:

REDExtract-N-AmpTM Tissue PCR Kit (SIGMA)
0.5 clear micro-centrifuge tubes for specimens
Thermal Cycler
70% Ethanol solution
ddH_20
50 μL pipette man
200 μL pipette man
200 μL pipette tips
Tissue Samples
Forceps

Protocol:

Instructions adapted from kit.

1. Wash forceps in between each sample to keep remove contamination.
2. Remove tissue samples from 70% ethanol solution and place in clean tube.
3. Wash sample three times with ddH_20, discarding water between each wash.
4. Add 100 μL of Exaction Solution to tissue sample.
5. Add 25 μL of Tissue Preparation Solution to sample.
6. Mix lightly or vortex. Ensure Sample is completely immersed in solution.
7. Program a thermal cycler for one cycle of 55°C for 10 min, and one at 95°C for 3 min.
8. Run samples through thermal cycler.
9. Remove and add 100 μL of Neutralization Solution B from Kit and mix thoroughly.
10. It is normal that the tissue may not be completely digested at this point.
11. Solution is now ready for the Amplified Fragment Length Polymorphism reaction.

Samples can be stored overnight at 4°C. For long-term storage remove solution from tissue remnants and place in a new tube. Store at -20°C for up to 6 months.

Standard Procedure for DNA Column Binding Purification Process

Materials and Instrumentation:

1.1. QIAquick Min-elute Reaction Cleanup Kit Product #28204

DNA Extraction Solution
PB Buffer
PE Buffer
2mL tubes
2mL filter tubes
10 µL pipette man
200 µL pipette man
Tabletop Microcentrifuge

Protocol:

To bind DNA, add the following to a 2mL tube:

1. Extracted DNA Solution	100 µL
2. PB Buffer	500 µL
	600 µL

Let mixture sit at room temperature for 5 minutes.

Centrifuge mixture at anything over 10,000 rpm for 1 minute.

To wash, add the following to the each tube from the previous reaction:

1. PE Buffer	740 µL

Centrifuge at anything over 10,000 rpm for 1 minute. Discard the flow through liquid.

Centrifuge again for 5 minutes discarding any liquid and retaining the center of the tube (column) containing 30 µL of filtered and purified DNA.

Place the column in a clean 2mL tube.

To elute DNA, add 30 µL of Buffer EB in the center of the filter column.

Let sit for 5 minutes. Spin 1 minute. Keep the contents of tube.

APPENDIX B

AFLP OPERATING PROCEDURE

APPENDIX B
Amplified Fragment Length Polymorphism
(AFLP)

Materials and Instrumentation:

AFLP Template Preparation Kit (LI-COR)
DNA Extraction Solutions
Selective Amplification Primers
Taq Polymerase and Buffer
0.2 mL micro-centrifuge tubes
0.5 mL micro-centrifuge tubes
1 mL micro-centrifuge tubes
Labels
Thermal Cycler
ddH_20
10 μL pipette man
50 μL pipette man
200 μL pipette man
1 mL pipette man
200 μL pipette tips
1 mL pipette tips

Protocol:

Preparation of Thermal Cycler:
Prepare the following four (4) programs. All 4^oC Soak steps are to allow the researcher time to return and remove reaction. It is not encouraged to allow the full 24 hour duration to expire.

1. AFLP-1
   a. 37^oC for 2 hours
   b. 70^oC for 15 minutes
   c. 4^oC soak for 24 hours
2. AFLP-2
   a. 20^oC for 2 hours
   b. 4^oC soak for 24 hours
3. AFLP-3
   a. 94^oC for 2 minutes
   b. 94^oC for 30 seconds
   c. 56^oC for 1 minute
   d. 72^oC for 1 minute
   e. Repeat steps (b) through (d) 20 times
   f. 4^oC soak for 24 hours

4. AFLP-4
   a. 1 cycle of
      i. 94°C for 30 seconds
      ii. 65°C for 30 seconds
      iii. 72°C for 1 minute
   b. 12 cycles lowering step (4.a.ii) above by .7°C each cycle (STEPDOWN)
   c. 23 cycles of
      i. 94°C for 30 seconds
      ii. 56°C for 30 seconds
      iii. 72°C for 1 minute

Protocol for Digestion:

Add the following to a .2 micro-centrifuge PCR tube on ice:

1.	5X reaction buffer from kit	5.0 µL
2.	EcoR1/Mse1 enzyme mix from kit	2.0 µL
3.	Sample Extraction mixture	6.0 µL
	Volume:	13.0 µL

Mix Gently, Centrifuge at 2000 rpm for 30 seconds.
Place in thermal cycler and run AFLP-1 program.

Protocol for Ligation:

Add the following to each tube from previous reaction (on ice):

1.	Adapter mix from kit	24.0 µL
2.	T4 DNA ligase from kit	1.0 µL
	Final Volume:	25.0 µL

Mix gently, Centrifuge at 2000 rpm for 30 seconds.
Place in thermal cycler and run AFLP-2 program.

Dilute by transferring 10 µL of mixture to a new tube and adding 90µL TE buffer.
Label remaining solution and store at -20°C.

Protocol for Pre-Amplification:

Add the following to a .2 mL tube (on ice):

1.	Diluted ligation mixture	2.5 µL
2.	AFLP pre-amp primer mix (from kit)	20.0 µL
3.	PCR reaction buffer (10X)	2.5 µL
4.	Taq DNA polymerase (5 units/µL)	0.5 µL
	Final Volume:	25.5 µL

Mix gently, Centrifuge at 2000 rpm for 30 seconds.
Place in thermal cycler and run AFLP-3 program.

Once complete, dilute Pre-Amp mix with a 1:40 mix (5 µL of Pre-Amp plus 195 µL of ddH_20) in a new tube.

Store the concentrated portion of Pre-Amp mixture at -20°C for future dilutions. Store diluted portion at 4°C overnight or at -20°C long term.

Protocol for Selective Amplification Working Mix: If chosen.

Add the following to a .5 mL tube (on ice):

1. ddH20	237 µL
2. 10x Taq buffer	60 µL
3. Labeled primer	50 µL
4. Unlabeled primer	50 µL
5. dNTP mixture	50 µL

Store on ice until preparations for final stage of AFLP are done.

6. Add Taq DNA polymerase (5 units/µL)	3 µL

Mix gently or vortex to ensure homogenous mixture. Sufficient to perform 50 selective amplification reactions, barring pipette error (usually around 45-47).

Protocol for Selective Amplification:

Add the following to a .2 mL tube (on ice):

1. Working mix	9.0 µL
2. Diluted Pre-Amp Mixture	2.0 µL
	11.0 µL

Protocol for using Supermix in place of Working Mix for Selective Amplification:

Add the following to a .2 mL tube (on ice):

1. Supermix	22.5 µL
2. Unlabeled Primer	2.0 µL
3. Labeled Primer	2.0 µL
4. Diluted Pre-Amp mixture	1.0 µL
Final Volume:	11 µL

Mix gently and centrifuge at 2000 rpm for 15 seconds.
Place in thermal cycler and run AFLP-4 program.

Dilute to a 1:10 mixture by transferring 2 µL of the solution from AFLP-4 to a new tube and adding 18 µL of Formamide.

Final Samples are ready to be loaded into the sequencer tray.

In a separate tube, mix 550 µL of Formamide with 30 µL 600 Liz size Standard

Mix gently or vortex to ensure homogenous mixture. Sufficient to perform 50 selective amplification reactions, barring pipette error (usually around 45-47).

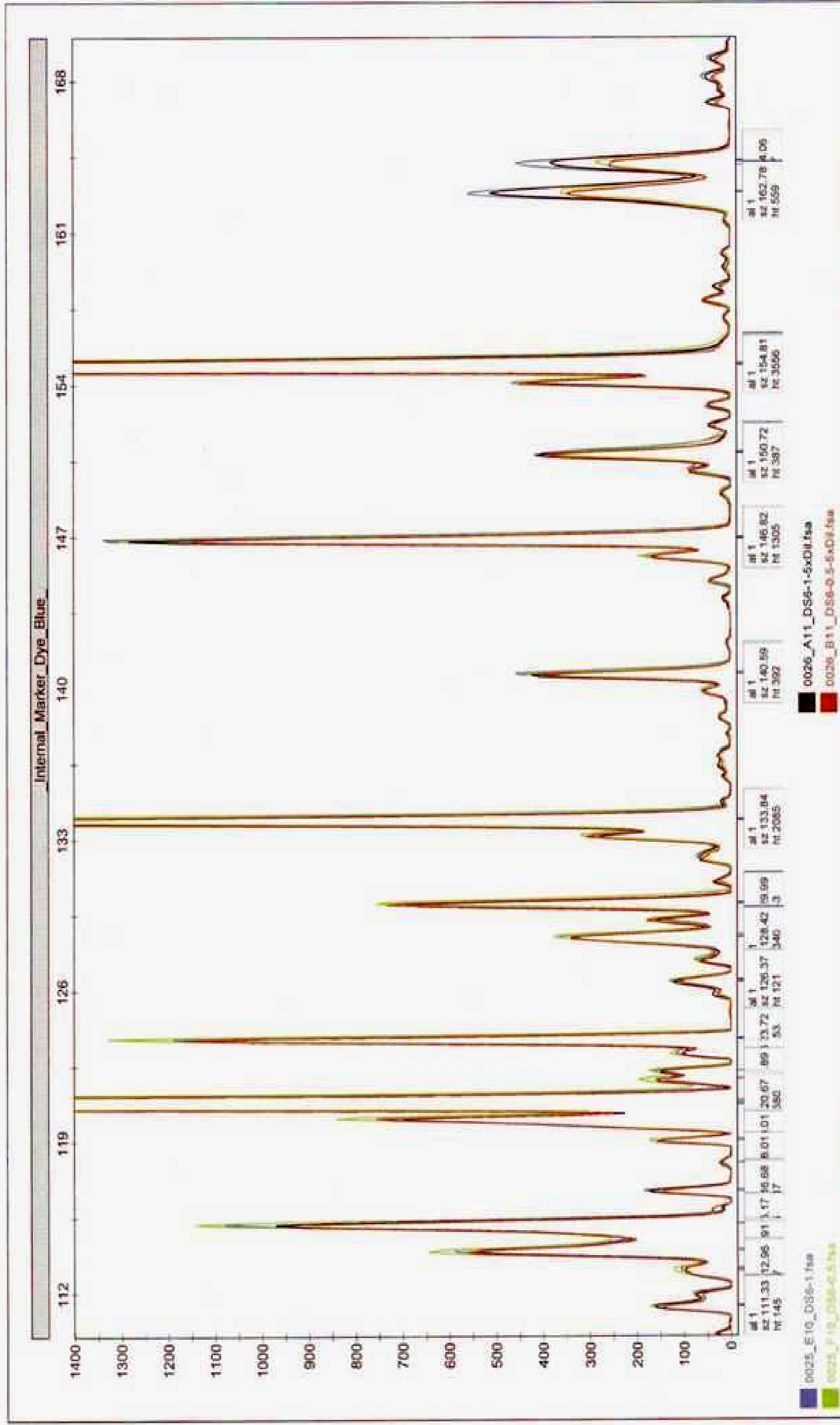
Load the following into each labeled well of the sequencer tray:

1.	Diluted Selective Amplification	1 µL
2.	Size Standard Mixture	10 µL
		11 µL

APPENDIX C

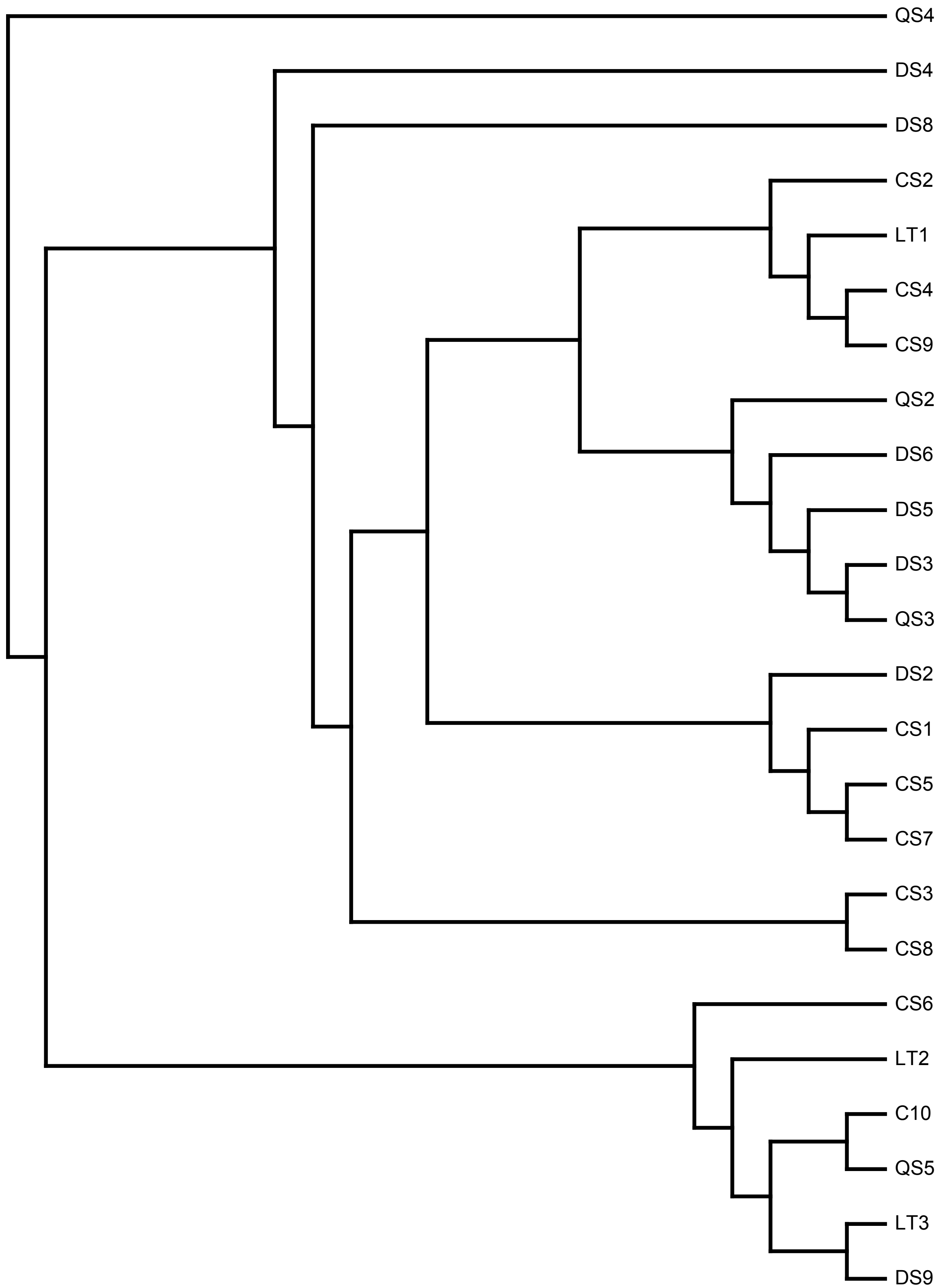
AFLP ELECTROPHEROGRAMS

APPENDIX C
AFLP Electropherograms


Applied Biosystems
GeneMapper v3.7
Maria_NewForm_6ul8ul_test
Internal_Marker_Dye_Blue
0026_A11_DS6-1-5xDil.fsa
0026_B11_DS6-0.5-5xDil.fsa
0025_E10_DS6-1.fsa
Printed by: gm
Fri Oct 19,2007 12:13PM, CDT
Page 1 of 1

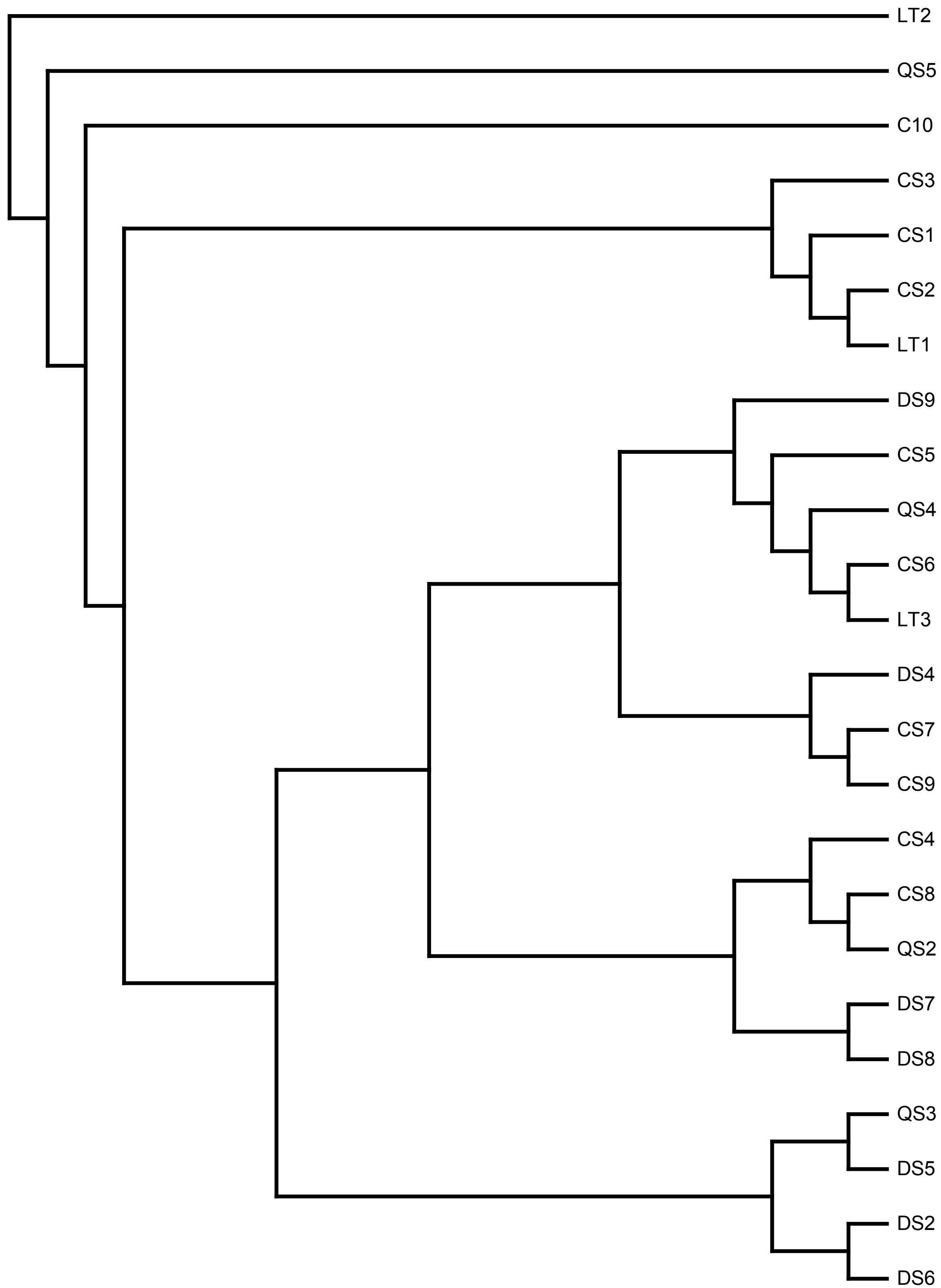
APPENDIX D

PHYLOGENETIC DISTANCE TREE-1


QS4
DS4
DS8
CS2
LT1
CS4
CS9
QS2
DS6
DS5
DS3
QS3
DS2
CS1
CS5
CS7
CS3
CS8
CS6
LT2
C10
QS5
LT3
DS9

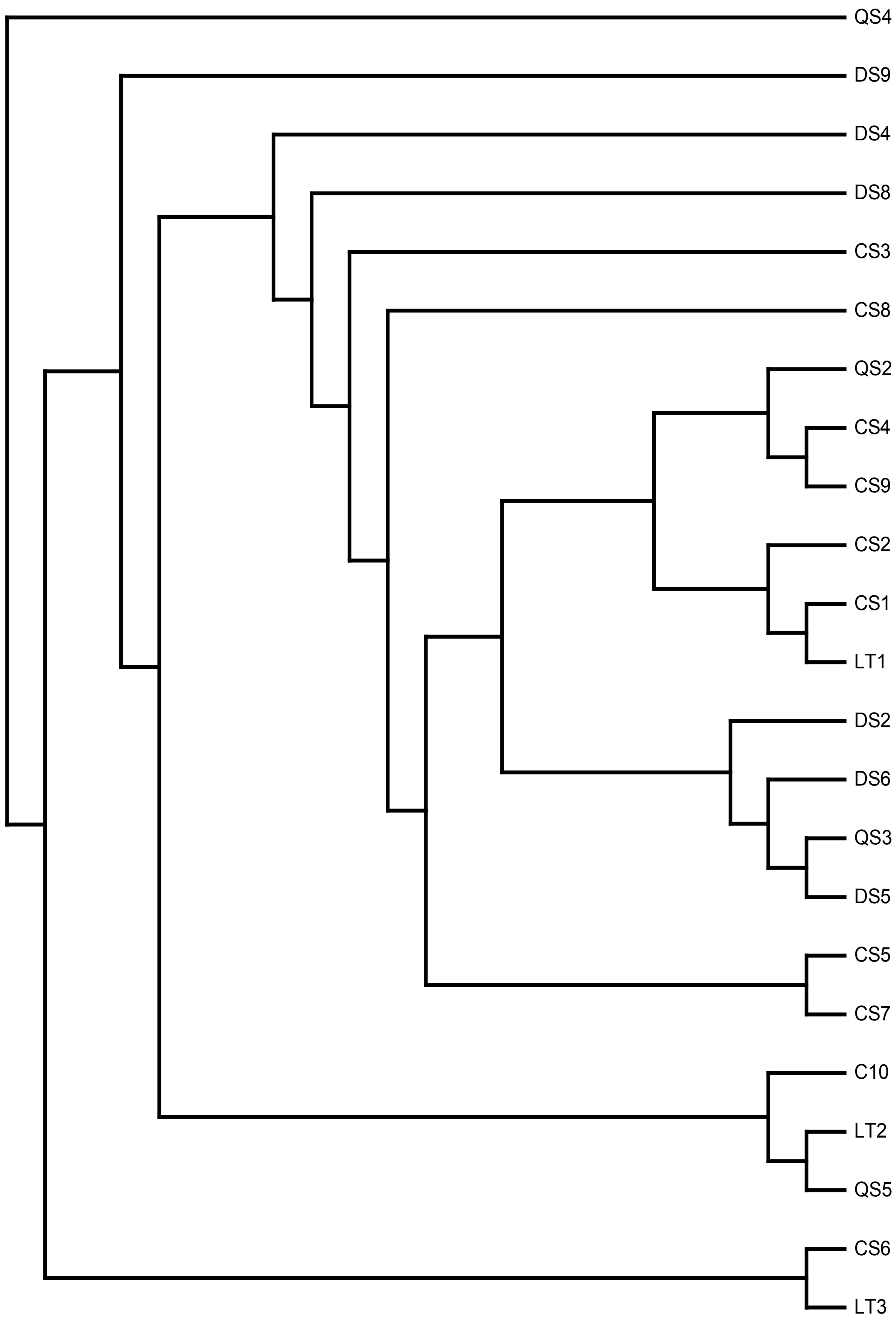
APPENDIX E

PHYLOGENETIC DISTANCE TREE-2


LT2
QS5
C10
CS3
CS1
CS2
LT1
DS9
CS5
QS4
CS6
LT3
DS4
CS7
CS9
CS4
CS8
QS2
DS7
DS8
QS3
DS5
DS2
DS6

APPENDIX F

PHYLOGENETIC DISTANCE TREE-3


QS4
DS9
DS4
DS8
CS3
CS8
QS2
CS4
CS9
CS2
CS1
LT1
DS2
DS6
QS3
DS5
CS5
CS7
C10
LT2
QS5
CS6
LT3

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VITA

Maria Louise Potter was born in St. Louis Missouri on May 22, 1965. Hers was an easy and uneventful birth as her mother was employed at St. Louis University Hospital as an Ophthalmology Technician. It was literally a flight of stairs between the womb and the world. Up until age four, she lived in a subdivision in the outer fringes of North St. Louis. From that point on, she grew up on a ten acre parcel of land in rural Crawford County, Missouri. She attended Bourbon High School where she graduated in the top 10 percent of a class of 64. After high school graduation in 1983, she was accepted to East Central College in Union, Missouri on scholarship where she earned an Associate of Arts Degree with a double major in Biology and Commercial Art in 1986. At that point, she was accepted into St. Louis University on scholarship double majoring again in Biology and Studio Art. Her initial intent was to apply to Veterinary School but later opted to take a more ecological approach to biology. She graduated in1988 with a Bachelor's of Science in Biology and Art. Upon graduation from St. Louis University, she obtained employment with the Missouri Department of Natural Resources working in the resource management and interpretation fields at various state parks. Currently, she is a natural resource manager or park superintendent at Onondaga Cave State Park. It was during this tenure that she chose to pursue a Master's of Science degree and applied to then University of Missouri-Rolla. After receiving her MS in Applied and Environmental Sciences in May 2008, she plans to apply what she has learned to various research projects where she is employed.