2005

Analysis of genotypic and phenotypic differences in Desmognathus quadramaculatus across the Southern Appalachians

Deborah Susan Merritt

Analysis of genotypic and phenotypic differences in *Desmognathus quadramaculatus* across the Southern Appalachians

Thesis submitted to
The Graduate School of
Marshall University

In partial fulfillment of the
Requirements for the Degree of
Master of Science
Biology

By

Deborah Susan Merritt

Thomas K. Pauley, Committee Chair
Charles Somerville, Committee Member
Daniel K. Evans, Committee Member

April 6, 2005

Abstract

DEBORAH S. MERRITT. Department of Biological Sciences, Marshall University, 1 John Marshall Dr., Huntington, WV 25755

Populations of *Desmognathus quadramaculatus* were compared for genotypic and phenotypic differences across 5 states in the Southern Appalachians. Series consisting of 10 salamanders each were collected from West Virginia, Virginia, Tennessee, North Carolina and Georgia. Salamanders were assessed for genotypic differences by using allozyme (proteins with polymorphic loci) electrophoresis staining for 13 loci of the muscle, liver and stomach tissue. Nei's genetic distance was used to score genetic variation between populations. Phenotypic differences were assessed by comparing 10 external morphological measurements between populations. Mahalanobis' distance was used to score morphological differences between populations. Both genetic and morphological differences were correlated with linear geographic distance (km) between populations (r^2= 0.85 and 0.65, respectively). Results of these studies indicate that morphologically cryptic species exist in the *D. quadramaculatus* complex. However, conflicting results of the morphological and allozyme testing indicate that further research, including DNA sequencing is necessary to resolve the taxonomic status of this species complex.

Acknowledgements

Foremost, I gratefully acknowledge Dr. Thomas K. Pauley for all of his guidance and support throughout the completion of my thesis, and without which I would probably still be trying to tell the difference between the head and tail end of a salamander. Many thanks to Dr Steven Tilley, for his whirl-wind tour of the mountains of North Carolina as well as his generous donation of time, supplies and laboratory space. I would also like to thank Drs. Dan Evans and Chuck Somerville for their helpful suggestions to the manuscript. Field assistants Zac and Kathy Loughman, Christopher Barry, Jaime Sias, Vanessa Dozeman and Elizabeth Fet all deserve thanks for their many hours standing knee deep in icy streams (even when we didn't catch a thing). Laboratory assistants, Zac and Kathy Loughman and Christopher Barry, aided in salamander dissection and euthanization. Conceptual advice was provided by Jessica Wooten and Dr. Michael Siedel. Funding was provided by the West Virginia Department of Natural Resources and Marshall University Department of Biological Sciences. This research was conducted under IUCAC permit number 286.

Table of Contents

Title Page i

Abstract ii

Acknowledgments iii

Table of Contents iv

List of Figures vi

List of Tables viii

List of Appendices ix

Chapter 1: Literature Review 1

Introduction 1

Species Concepts 1

Evolution of Desmognathine Salamanders 3

Species Description 6

Study Objectives 7

Chapter 2: Methods 11

Collection 11

Morphometrics 11

Allozymes 12

Tissue Preparation 12

Gel Preparation and Running 13

Chapter 3: Results 19

Morphometrics 19

Allozymes 20

Chapter 4: Discussion 35

Morphometrics 35

Allozymes 38

Chapter 5: Conclusions 40

Literature Cited 43

Appendices..... 47

Curriculum vitae 53

List of Figures

Figure 1: The traditional view of evolution of the subfamily Desmognathinae. Adapted from Titus and Larson, 2003.....8

Figure 2: Phylogeny adapted from Rissler and Taylor (2003) based on mitochondrial RNA analysis of Desmognathine salamanders.....9

Figure 3:
a. One theory on the origin of direct development in the family Plethodontidae. Adapted from Titus and Larson, 2003.....10
b. An alternative reconstruction of the evolution of direct development in the family Plethodontidae. Adapted from Titus and Larson, 2003. 10

Figure 4: Map showing the collection sites, indicated by black dots and county each series were collected in. 15

Figure 5: Measurements taken for morphometric analysis. Figure adapted from Powel *et al,* 1998. 16

Figure 6: Sample gels, MDH stained on TC8 gel January 5 and 7 2005.....18

Figure 7 a, b. Principle component analysis for all samples assessed individually.
a. Principle Components 1 versus 2.....23
b**:** Principle Component 2 versus 3.....24

Figure 8 a,b : Principle components analysis for pooled samples, West Virginia and Virginia combined compared with Tennessee and North Carolina
a: Principle component 1 versus 2 25
b: Principle component 2 versus 3.....26

Figure 9 a, b: Principle components analysis comparing size corrected data for pooled group West Virginia, Virginia, and Tennessee compared with North Carolina.
a: Principle components 1 versus 2.....27
b**:** Principle Components 2 versus 3 27

Figure 10: Size corrected head shape measurements (DV thick and cranial width) for the combined data set, West Virginia, Virginia and Tennessee compared with North Carolina
a. Cranial Width compared between the pooled group and NC 28

b**.** DV Thick compared between the pooled group and North Carolina 28

Figure 11: Principle components analysis comparing size corrected data for individually assessed populations.
a. Principle component 1 versus 2 29

b. Principle component 2 versus 3 30

Figure 12: Phenotypic and Linear geographic distance for five populations of *D. quadramaculatus* (r^2 = 0.65). 31

Figure 13: Nei's genetic distance and Linear geographic distance for six populations of *D. quadramaculatus* (r^2= 0.85). 32

Figure 14: Phylogram of genetic distances where abbreviations represent the state of collection. WVR were collected from Rock, West Virginia, and WVG collected from the Gauley River drainage. 33

Figure 15: Phylogram of Mahalanobis' distances where abbreviations represent the state of collection. WVR were collected from Rock, West Virginia, and WVG collected from the Gauley River drainage. 34

Figure 16: Comparison of diets between North Carolina and all other sampled populations with the follow abbreviations: Co= Coleoptera, Pl= Plecoptera, Ephem = Ephemeroptera, Try= Trycoptera, hym= Hymenoptera, dip= Diptera, Ara= Arachnida, aca= Acari, gas= Gastrapoda, oli= Oligocheta, and iso= Isopoda. 42

List of Tables

Table 1: Significant t-test results Comparing Morphological Characters between Populations, p< 0.05 21

Table 2: Significant t-test results comparing size-corrected morphological characters between populations, p< 0.05 21

Table 3: Linear geographic distances between populations (miles) 22

Table 4: Matrix of Mahalanobis' Distance between populations. 22

Table 5: Matrix of Mahalanobis' Distance P- values 22

Table 6: Distance Matrix of calculated Nei's genetic Distance (*D*) 23

List of Appendices

Appendix 1: Series photographs of collected salamanders 48

Appendix 2: Gel and Stain Recipes; provided by Steven Tilley. 50

Chapter 1: Literature Review

Introduction

Until the advent of biochemical techniques for the study of genetic differentiation of species, it was difficult to determine the status of species that are morphologically similar but genetically diverse (Camp *et al.*, 2002). Recently, studies have been conducted to determine the species status of what were once considered disjunct populations of a widely distributed single species (eg, Highton, 1979; Camp *et al.*, 2002, etc). Many of these genetically distinguishable sub-units have since been recognized taxonomically as distinct species (Highton, 1979, 1989, 1997; Highton and Peabody, 2000; Highton and Webster, 1976; Tilley, 1981; Tilley and Mahoney, 1996). The Black-bellied Salamander of West Virginia, currently *Desmognathus quadramaculatus* is potentially one of these morphologically cryptic species.

Species Concepts

Three major species concepts exist today: the Biological Species Concept, the Phylogenetic Species Concept, and the Evolutionary Species Concept (Frost and Mills, 1990; Smith, 1990). Each concept of a species has its own merits.

Biological Species are considered groups of interbreeding populations that are reproductively isolated from other such groups (Frost and Mills, 1990). While this concept of a species is the most common, and has even been suggested as a Universal Species Concept (Smith, 1990) it is generally difficult to test, and even more difficult to prove in cases of long- lived animals. Allopatric populations of salamanders can result in genetic differentiation, while morphological characters remain constant (Titus and

Larson, 1996). This can result in new taxonomic subunits described as species. Tilley and Mahoney (1996) tested allozyme differentiation at fixed loci in salamanders of the *Desmognathus ochrophaeus* complex and described several new species. In breeding experiments conducted with salamanders of this complex all "species" were found to breed freely together with no significant barrier to reproduction (Tilley, unpublished data). However, these salamanders would never reproduce naturally in the wild due to the vast geographic distance that separates their populations. It is possible that reproductive compatibility in these salamander species could be considered a primitive character (Cole, 1990). Highton (1990) argues that reproductive compatibility should not be considered a single characteristic when determining the species status of animals.

The Phylogenetic Species Concept states that the smallest detectible samples or populations with unique character sets should be considered species (Frost and Mills, 1990). This also refers to geographic populations with one or more synapamorphies (Cole, 1990). In this concept, subspecies do not exist because species are considered the smallest measurable group. Allozyme data can also be utilized in this species concept, and may be expected to increase the number of phylogenetic species, especially when considering disjunct populations (Echelle, 1990).

The Evolutionary Species Concept states that each lineage evolves separately, having a different evolutionary fate or trajectory. The current evolutionary trajectory of a species can be measured by allozyme frequencies at fixed loci (Tilley and Mahoney, 1996). This can provide evidence of current gene exchange and common ancestry through the calculation of Nei's genetic distance *D* (Nei, 1975). Large genetic distances and changes in allele frequencies could indicate evolutionary independence, and should

be considered (Tilley and Mahoney, 1996). The evolutionary species concept will be applied here.

Evolution of Desmognathine Salamanders

Taxonomic work on the Desmognathine salamanders has been abundant in recent years. This subfamily represents the most diverse group of Plethodontid salamanders, with a unique body plan and feeding mechanism (Tilley, 1980).

Many theories have been raised concerning the phylogenetic relationships of what was once considered their own family, the Desmognathidae (Wilder and Dunn, 1920). Until recently, the most commonly accepted theory was that these salamanders evolved from an ancestral aquatic form such as the extant *D. quadramaculatus*; to a more derived, terrestrial form such as the extant *D. wrighti* (Hairston, 1949) (Figure 1). Desmognathine salamanders follow a size gradient from large to small which corresponds perfectly to the species' degree of terrestriality (Hairston, 1949, Bruce 1996). Community structure of Desmognathine salamanders was thought to be a result of competition and predation by larger salamanders (Bruce, 1996; Tilley and Bernardo, 1993). By this logic, the ancestors of *D. wrighti, D. aeneus,* and *D. ochrophaeus* evolved to a more terrestrial state to avoid predation by ancestral states similar to *D. quadramaculatus* and *D. marmoratus*. However, these theories were devised prior to molecular techniques, when scientists were forced to infer phylogenetic relationships from morphological traits (Highton, 1990; Larson and Dimmick, 1993).

The use of mitochondrial RNA to construct phylogenies of Desmognathine salamanders has resulted in conflict with those constructed on the basis of morphological

characters (Rissler and Taylor, 2003). Phylogenetic trees can be used to explain changes in discrete characters, such as reproductive modes or body size (Canatella and Hillis, 1993), which probably represent ancient speciation events (Tilley and Bernardo, 1993).

While evolutionary trends in salamanders of the genus *Desmognathus* were considered to be a phylogenetic reduction in body size and the evolution of direct development as a derived character (Beachy and Bruce, 2003), the opposite appears to be true when mitochondrial rRNA are analyzed to produce the most parsimonious tree (Titus and Larson, 1996; Rissler and Taylor 2003) (Figure 2). This suggests that terrestriality and all of its adaptations (*i.e.* direct development) are basal characteristics in *Desmognathine* salamanders (Titus and Larson, 1996; Rissler and Taylor 2003). Rissler and Taylor (2003) theorize that *D. quadramaculatus* diverged as an early lineage, although their research showed weak support for order of divergence in these salamanders which indicates rapid diversification of these salamanders, followed closely by a reinvasion of the aquatic habitat.

Two theories exist on the evolution of direct development in Plethodontid salamanders. It either evolved twice in the family Plethodontidae: once in the genus *Desmognathus* and once in the branch directly ancestral to Plethodontini and Bolitoglossini; or once in the common ancestor to Plethodontidae (Titus and Larson, 1996) (Figure 3a,b). *Desmognathus* is one of the few genera in the family Plethodontidae that have free living larvae. It follows that it is more likely that direct development evolved in the common ancestor to the family Plethodontidae, inferring that *D. quadramaculatus* is a derived form of Desmognathine salamanders. The evolution of direct development could have occurred by shortening the larval stage and retaining it

entirely within the egg. If this is the case then the reversal (free living larvae) would be feasible as a derived state in *D. quadramaculatus* (Titus and Larson, 1996).

The size of adult *D. quadramaculatus* is also thought to be a derived characteristic compared with basal taxa for this clade (Titus and Larson, 1996; Rissler and Taylor 2003; Camp *et al.*, 2000). Differences in adult body size between populations of *D. quadramaculatus* have been observed throughout the Southern Appalachians (Wooten, 2001). Differences in body size between populations can occur for numerous reasons, including biotic and abiotic factors. Elevation, rainfall and time and size at metamorphosis have all been considered for differences in adult body size.

Time and size at metamorphosis are thought to be the two factors which contribute most to maximum adult size (Beachy, 1995; Beachy and Bruce, 1993; Bruce 1996; Titus and Larson, 1996). Controlling the timing of metamorphosis allows individuals to maximize the probability of growth to a larger size and increasing their chance of survival (Beachy, 1995). The size that is achieved at metamorphosis is controlled by the timing, as well as the individual hatching size and larval growth, which is ultimately dependent upon the resources available (Bruce, 1996; Camp *et al.*, 2000). Camp, *et al.* (2000) observed that salamanders inhabiting wetter climates have an increased size at first reproduction, indicating a larger size at metamorphosis, concluding that adult size may be related to rainfall.

Size at metamorphosis has been found to be indicative of the size at maturation (Beachy, 1995; Beachy and Bruce, 1993; Bruce 1996; Titus and Larson, 1996). After maturation, further growth can occur in males but tends to be very slow, while no further growth tends to occur in females (Beachy and Bruce, 1993).

Community structure of Desmognathine salamanders has been thought to be a contributing factor to the evolution of adult body size (Tilley 1980; Bruce 1996; Hairston 1949). In theory, *D. ochrophaeus*, *D. wrighti*, and *D. aeneus* evolved from their large, ancestral, aquatic form into smaller terrestrial forms to escape predation pressure from these larger salamanders. In this model, predation and competition of juveniles in the aquatic habitat would select for earlier maturation and metamorphosis resulting in a smaller adult size (Titus and Larson, 1996). However, given current cladistic data, it is likely that the large adult body size evolved as a mechanism to utilize large prey items, including smaller salamanders, instead of miniaturization evolving as a mechanism to escape predation.

Species Description

Desmognathus quadramaculatus are large salamanders that measure 10-20 cm in length. They have a characteristically black ventral surface and sharply keeled tail (Green and Pauley, 1987). They belong to the family Plethodontidae, which is made up entirely of lungless salamanders. Lunglessness in Plethodontid salamanders is advantageous for aquatic species such as *D. quadramaculatus*. The presence of lungs would not allow these animals to be efficient swimmers due to their buoyancy, and in their absence, the salamanders are able to sink to the bottom of streambeds and swim against the current (Wilder and Dunn, 1920).

In adult *D. quadramaculatus*, the dorsal surface is brown to black, but in juveniles or sub- adults may have greenish or light brown dorsal blotches (Mills, 1996). Larval stages are also robust, but lack the characteristic black venter, and instead are

marked with a double row of dots starting behind the head and extending above the hind legs (Green and Pauley, 1987). At the time of metamorphosis, the venter turns black.

Desmognathus quadramaculatus is only found in steep, mountain streams with a closed canopy of mixed deciduous trees in West Virginia (Organ 1961). The current range of *D. quadramaculatus* is the mountainous region of Southern West Virginia and south of the Tennessee Valley Divide in the Allegheny Mountains of Virginia southward into Northern Georgia (Green and Pauley 1987; Conant and Collins, 1998; Petranka, 1998). In West Virginia, the distribution of *D. quadramaculatus* is directly associated with the Bluestone River, New River, Gauley River, and Potts Creek Valley (Green, 1967; Pauley 1992; Turner, 1997).

Objectives

The objectives of this study are to (1) determine any differences in the protein frequencies at specific loci using allozymes (genotypic differences), and (2) determine if there are any morphological differences between populations including size and non- size related characters between the sample salamander populations (phenotypic differences).

Figure 1: The traditional view of evolution of the subfamily Desmognathinae. Adapted from Titus and Larson, 2003.

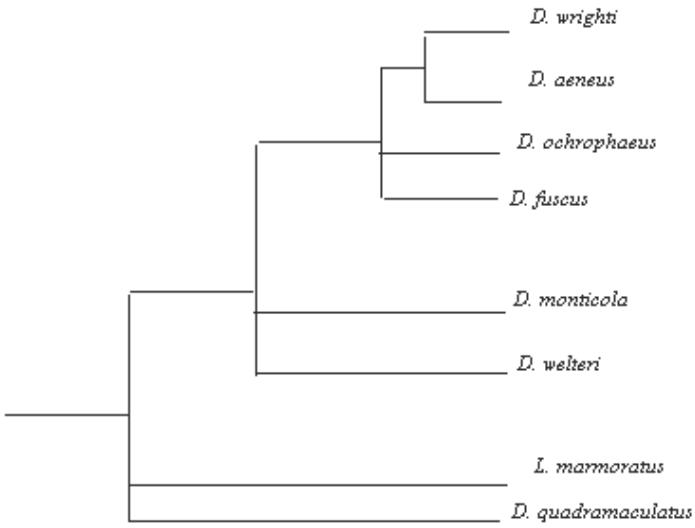


Figure 2: Phylogeny adapted from Rissler and Taylor (2003) based on mitochondrial RNA analysis of Desmognathine salamanders

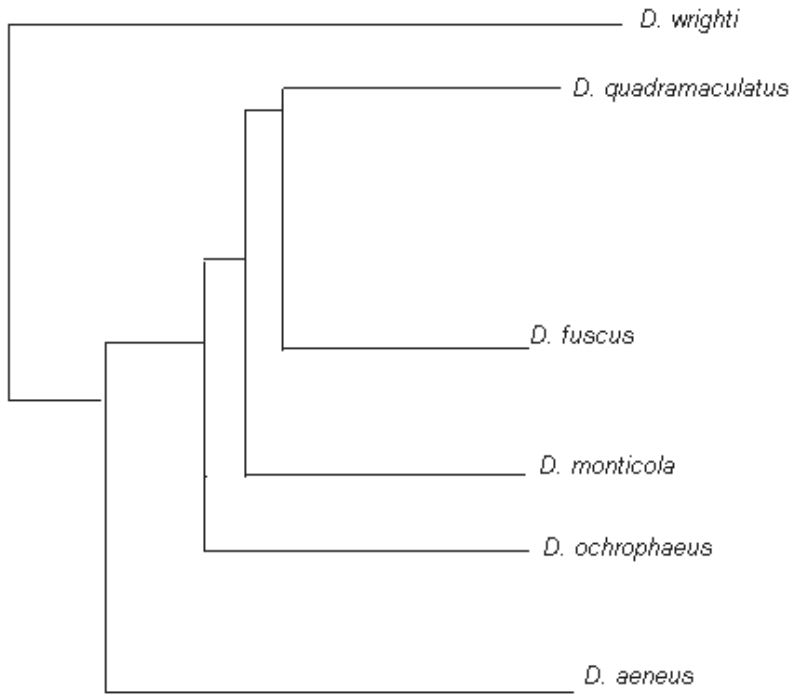
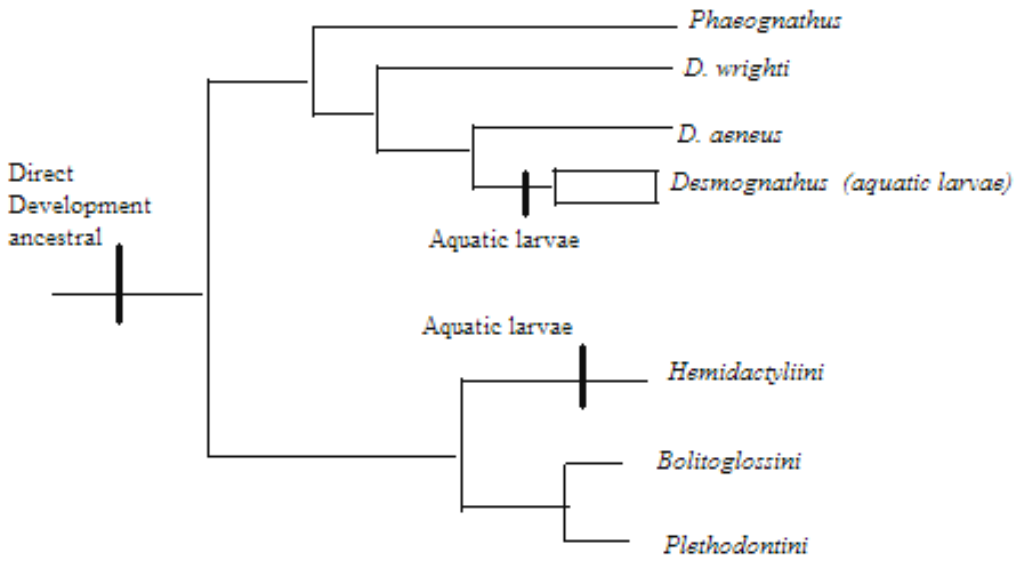
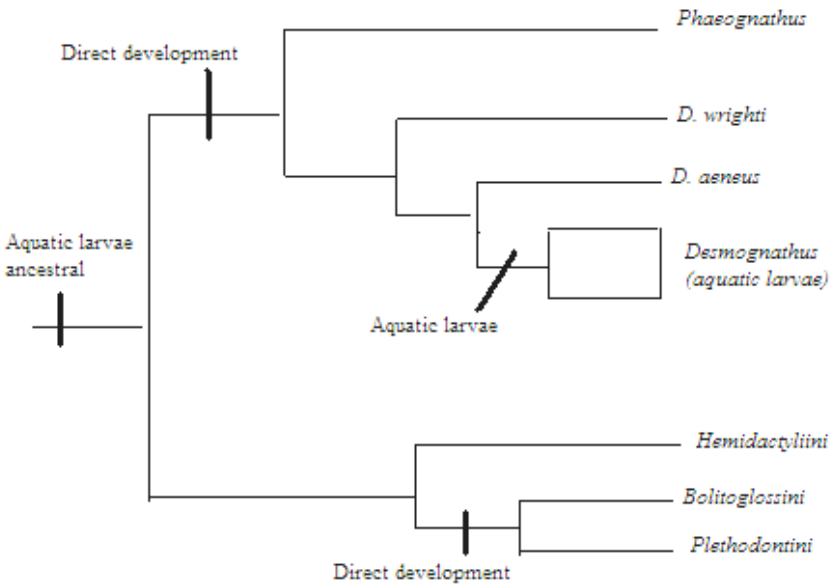


Figure 3: a. One theory on the origin of direct development in the family Plethodontidae. Adapted from Titus and Larson, 2003.



b. An alternative reconstruction of the evolution of direct development in the family Plethodontidae. Adapted from Titus and Larson, 2003.



Chapter 2: Methods

Collection

Desmognathus quadramaculatus were collected in series of 10 animals from West Virginia, Virginia, Tennessee, and North Carolina (Figure 4). Two series of animals were collected from 2 streams in West Virginia (n=20), while one series was collected from each of the other locations (Appendix 1). Salamanders were collected by hand and with dip nets, placed in ziplock bags, and brought back to the lab in a refrigerated cooler. In lab, salamanders were euthanized in 10% chlorotone. The stomach, liver, and a portion of visceral tissue was removed from each animal, placed in distilled water and stored at –70° C until allozyme analysis. Ten *D. quadramaculatus* from Fannin County, Georgia were included in the allozyme analysis. Tissue samples from these animals were provided by Dr. S.G. Tilley from previous work.

The following abbreviations will be used to represent the sample populations: WVR= collected from Mercer County, West Virginia; WVG= collected from Nicholas County, West Virginia; TN= collected from Sullivan County, Tennessee; NC= collected from Carter County, North Carolina; VA= collected from Giles County, Virginia; GA= collected from Fannin County, Georgia.

Morphometrics

Each salamander was measured for snout- vent length at the posterior of the vent (SVL), head width (HW), head thickness dorsal-ventrally at the thickest portion (DV thick), head thickness at the thinnest portion (DV thin), body length (BL), head length (HL), femur length (F), tibia/ fibula length (TB), eye depth (ED), snout length (SL) and nostril distance (ND) (Figure 5). All measurements were taken externally using poly dial

calipers (SPI 2000). Statistical analysis for variation in morphometrics between and within populations was completed using Principle Components analysis (the SAS System version 8.0). Data were pooled based on the results of the allozyme study and analyzed again for differences in morphological characters using the appropriate non-parametric test (SIGMASTAT). Normalization of measurements were made in the data by dividing all characters by the SVL of that animal, and the data were analyzed using the same systems. Mahalanobis' distance was calculated to score the distance between populations based on morphological characters using non-size corrected as well as size corrected data (The SAS System version 8.0). Mahalanobis' distance was compared with Nei's genetic distance as well as with the linear geographic distance in miles between sites.

Allozyme analysis

Methods for allozyme analysis follow those outlined in Tilley and Mahoney (1996) and Camp *et al.* (2002).

Tissue preparation

The samples containing liver, stomach and visceral tissue were thawed at room temperature in a tap water bath. An equal portion tissue and distilled water was placed in a glass test tube so that 1/3 of the total test tube volume was filled (approximately 1.16 mL). Forceps and scissors were rinsed with distilled water between each sample transfer. The tissue was then sonicated using a "Vibra- Cell" instrument (Sonics and Materials Inc, Danbury Ct) for 5 minutes at 50% duty cycle. The disrupted tissue and water was transferred to 1.5 mL centrifuge tubes and spun for 5 minutes at setting 14. The tubes were then aspirated to remove the lipid layer from the surface. Tissues were stored at – 70° C while not in use.

Gel Preparation and Running

The full starch gel and buffer recipes can be found in appendix 2. One gel was made for each buffer system utilized, Tris-citrate, pH 8.0 (TC8), Tris-EDTA-borate, pH 9.1 (TBE), Tris-citrate, pH 6.7 (TC6.7) and Poulik (P). Potato starch (Starch Art Corporation) and the buffer were premixed in volumetric flasks. Aliquot of buffer was boiled on a hot plate in large flasks. The starch and hot buffer were mixed over a hot flame and the temperature was monitored with a thermometer to lot allow the solution to rise above 45° C. The thermometer was rinsed between each buffer system. After NAD (1%) was added to the gels, and vacuum was applied to degas the gel for 15 seconds. The gel was then poured into a mold, allowed to cool until the color was opaque, and wrapped in at least three layers of cling-film to prevent dehydration. The gels were stored at 4° C overnight (from approximately 5 pm to 9 am).

The following morning, the 24 samples that would be run that day were thawed in a tap water bath, and then stored in an ice bath. The four gels were removed from the refrigerator, unwrapped, and 24 wells were placed in each gel. Lane markers were used to keep the lanes straight, and a running dye was placed on the right side of TBE, TC8 and TC6.7. The Poulik gel has a brown band appears as the gel runs, so no dye was necessary. Pre-cut thin layer chromatography papers (~ 0.5 cm^2) were dipped in each sample in placed in individual wells. Forceps were used to handle the wicks, and were wiped with chem-wipes between each sample. The gels were then placed into their appropriate buffer reservoirs and the plastic wrap placed over the top surface. A large ziplock bag filled with ice and water was laid on top of the plastic wrap to prevent the gel from overheating during running. The entire assembly was placed in the refrigerator (4°

C) and electrode leads were attached. The power supply was then set such that a current of 95 mA was run through the TC8, TC6.7, and TBE gels. The Poulik gel runs at 75 mA. The current levels were checked every 10 minutes to prevent a spike in the current from "cooking" the gels and subsequently denaturing all proteins. Gel running times were as follows; TC8: 3.6 hours, TBE: 4.25 hours, P: 3.3 hours, and TC6.7 3.1 hours.

After the gels had run, they were prepared for staining. One to two inches was removed from either side of the gel to facilitate removing the gel from the mold. The top left corner of each gel was notched to mark the gel orientation. The gels were then cut into 3-4 1mm layers using a wire cutter. Each layer was mounted in a separate staining tray and stained. The staining trays were stored in a dark drawer at room temperature because the stains are light sensitive. The stains developed in 10-15 minutes and images were taken of each gel layer with a Nikon DIX camera. The gels were immediately scored for the presence/absence of bands using both the gel and the gel image. A sample gel can be viewed in Figure 6. Migration distances were recorded as follows; "-" no band observed, "X" band present and all bands are in the same position on the gel, "F" band present indicating Fast allozyme (i.e. further along the gel), "S" band present indicating slow allozyme, "FS" both fast and slow bands are present indicating a heterozygote.

All procedures for preparing and running gels were repeated each day for 3 days until all 64 samples had been run. An additional running day was utilized to determine any unclear loci.

The computer program "Neighbor" in the Phylip Package (http://evolution.genetics.washington.edu/phylip.html) was used to calculate the genetic distance (Nei, 1987). "Treeview" (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) was utilized to visualize a phylogram based on genetic distance output from the Kitch program.

Figure 4: Map showing the collection sites, indicated by black dots and county each series were collected in.

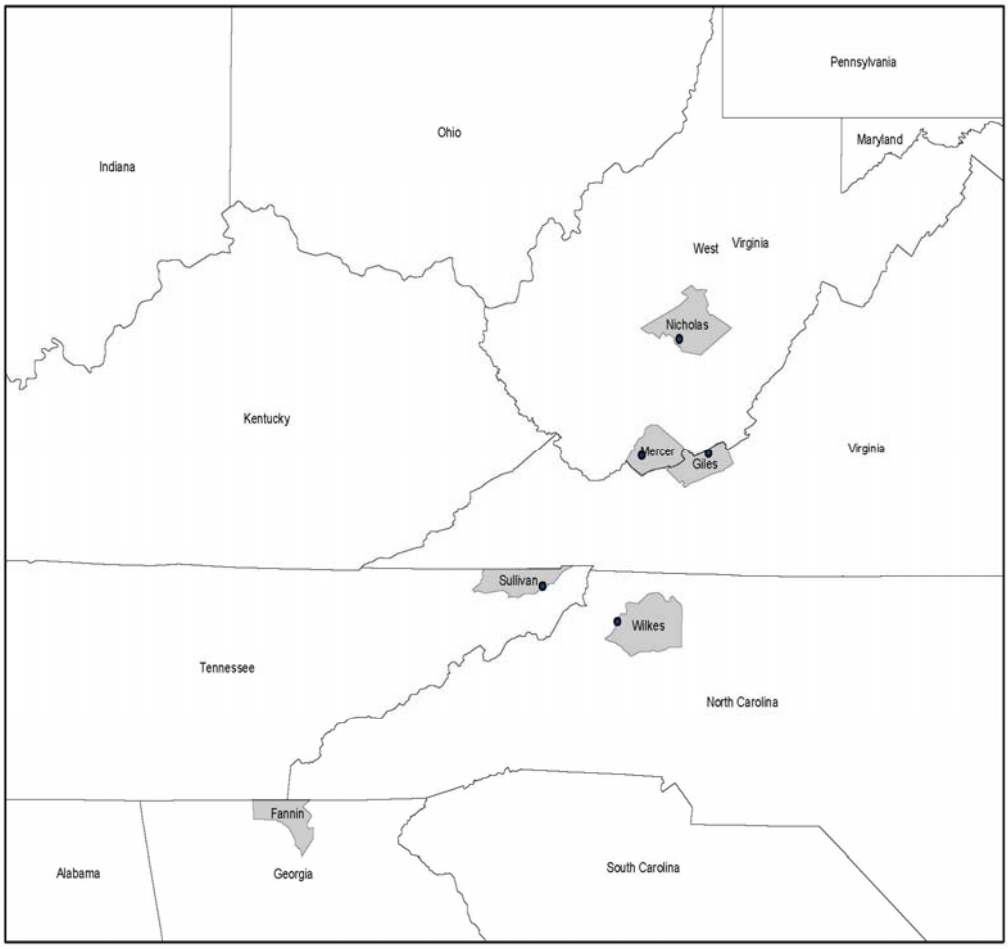
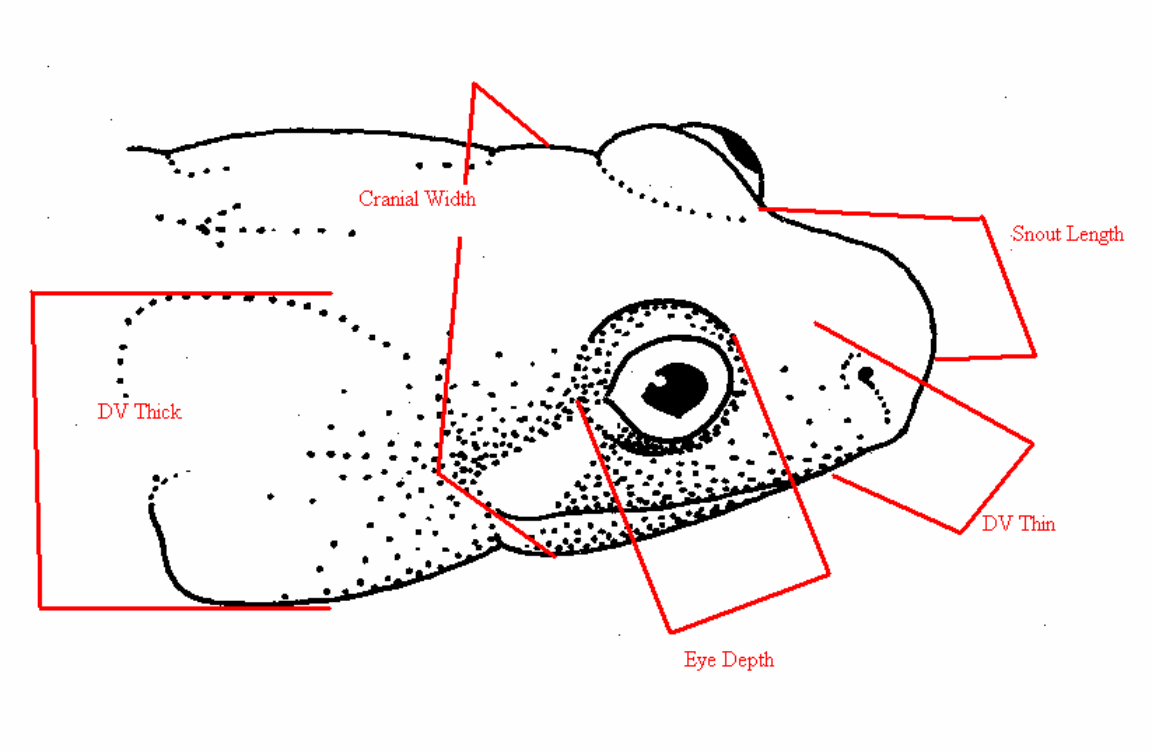


Figure 5: Measurements taken for morphometric analysis. Figure adapted from Powel *et al*, 1998.



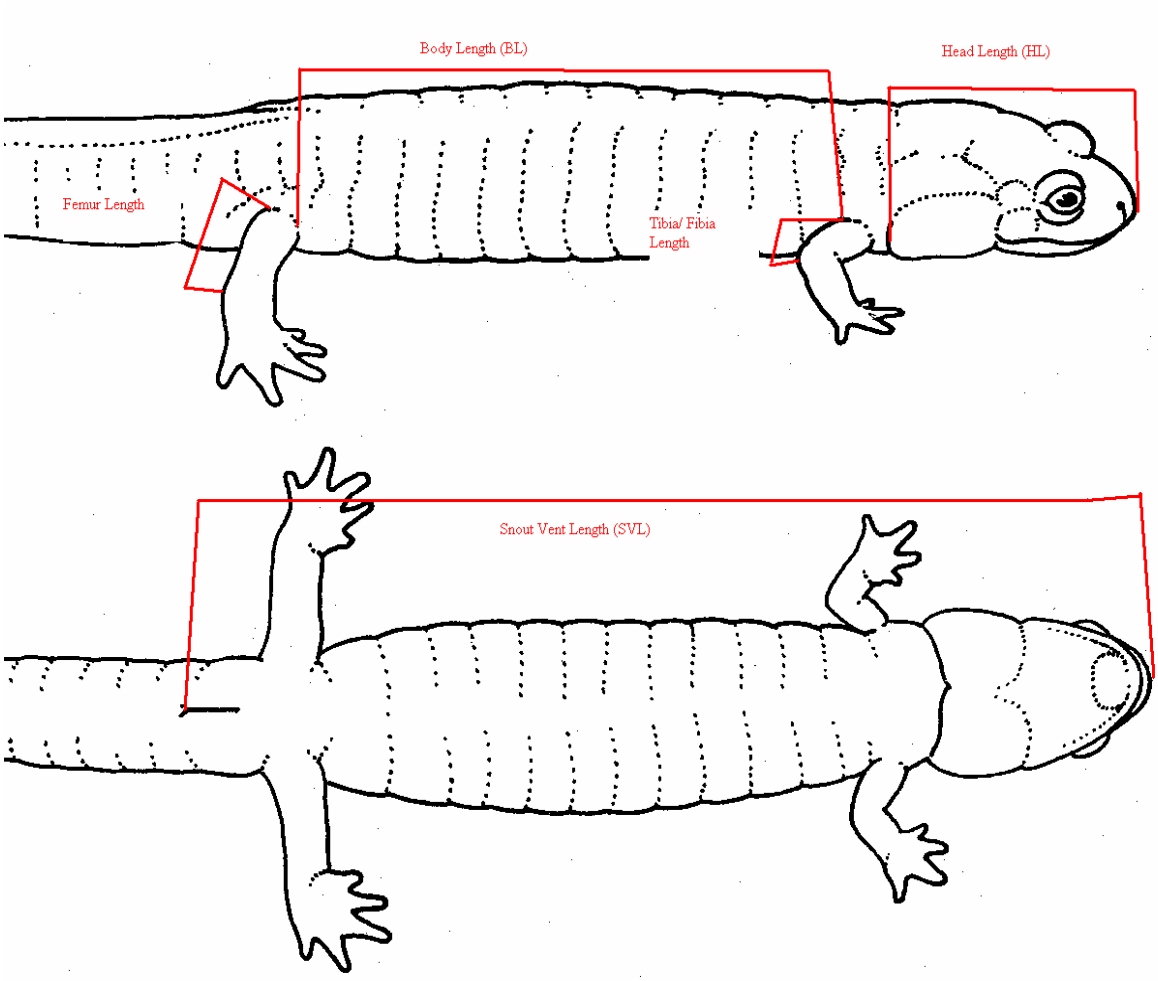
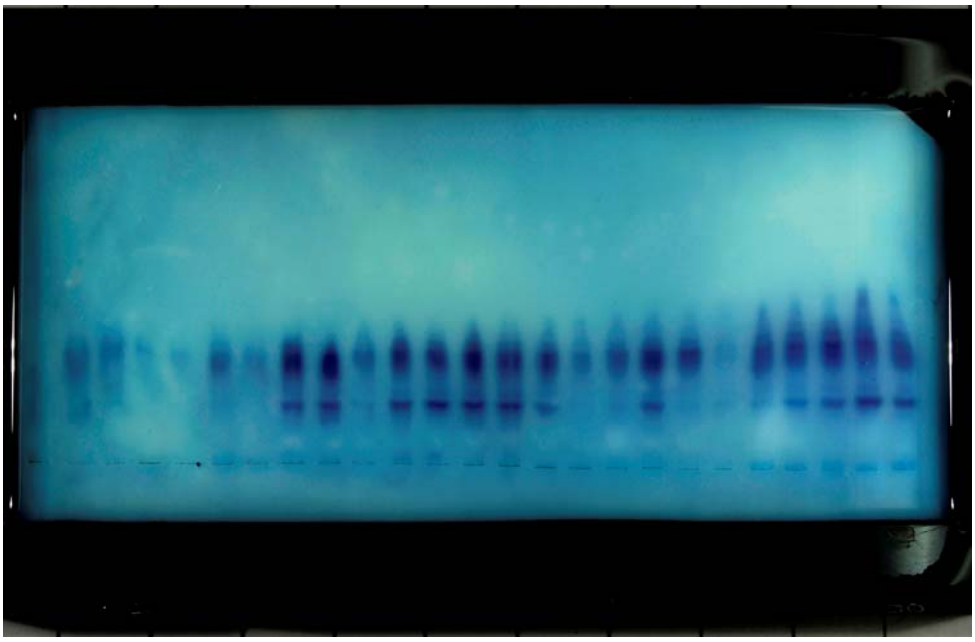
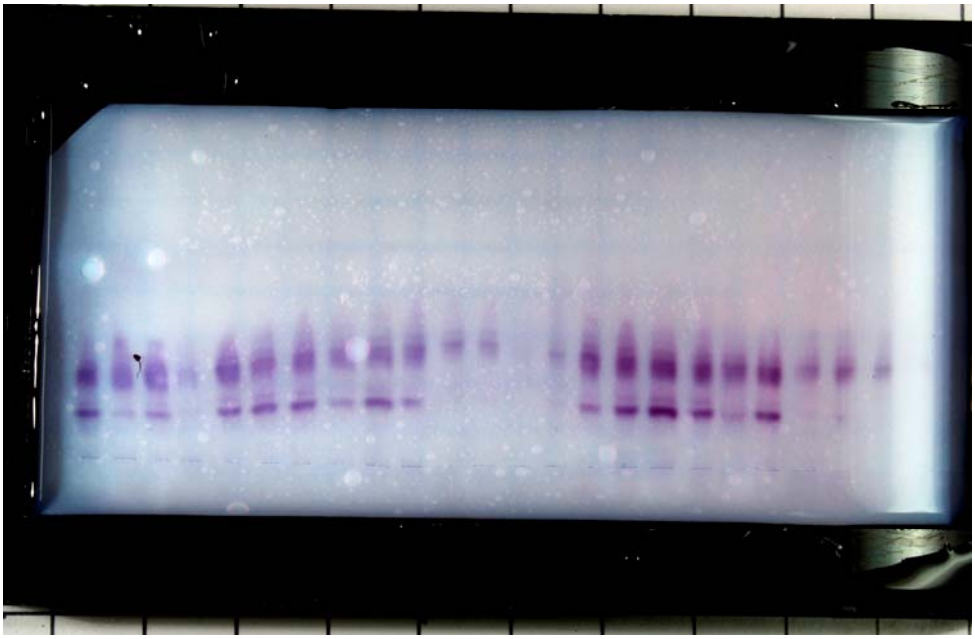

Body Length (BL)
Head Length (HL)
Femur Length
Tibia/ Fibia Length
Snout Vent Length (SVL)

Figure 6: Sample gels, MDH stained on TC8 gel January 5 and 7 2005.





Chapter 3: Results

Morphometrics

Data show that there are no general morphological differences based on the characters measured using Principle Components analysis for each separate population (Figure 7 a, b). Results of a T-test indicate that there are no differences in SVL between populations ($p > 0.05$). Differences were observed for the character DV Thick between WVG and TN, WVR, and VA; for the character Head Length between WVG and WVR and between TN and WVR; for the character Tib/Fib between WVG and NC, WVR ($p < 0.05$, Table 1). When the data were combined based on the results of the allozyme study, no general differences could be detected between the groups (WV, VA, and TN plotted against NC) (Figure 8 a, b).

Size correction of characters not did allow any further separation among the populations between the pooled group and NC (Figure 9 a, b). Significant differences in head shape (Cranial Width and DV Thick) between the combined group (WV, VA, and TN) and the NC specimen were observed using the size corrected data ($p < 0.05$). All other size-corrected characters showed no difference between the combined group and the NC specimen ($p > 0.05$) (Figure 10). Size correction of the morphological characters did allow further separation between the populations when they were assessed individually. (Figure 11 a, b). T-tests revealed significant differences between many of the populations for the following characters using size corrected data and assessing the populations separately: Cranial Width, DV Thick, DV Thin, Head Length, Femur and Tib/fib ($p<0.05$, Table 2).

Linear geographic distance between sites is presented in Table 4. Mahalanobis' distances are presented in Table 5. Mahalanobis' Distance indicate that the Tennessee, Virginia and North Carolina populations are not significantly different from each other ($p>0.05$, Table 5, Figure 15). The two West Virginia populations studied were found to be significantly different from each other, as well as from all other populations ($p< 0.05$, Table 5, Figure 15). There is a weak correlation between linear geographic distance and Mahanalobis' Distance ($r^2 = 0.65$) (Figure 12). There is a strong correlation between Nei's Genetic Distance and linear geographic distance ($r^2= 0.85$) (Figure 13).

Allozymes

Numerous problems were observed while the gels were stained. Many loci did not stain, and were therefore not scored for their allelic frequencies. I speculate that the samples became too warm at some point during the processing or shipping, which caused the proteins to become denatured, and therefore not stain according to protocol.

Table 3 provides the distance matrix calculated by the Phylip program. The data indicate that the Georgia population is the most genetically distinct from all other populations studied. Figure 13 provides a phylogram based on the distance matrix, highlighting the close genetic relationship among West Virginia, Virginia, and Tennessee populations of *D. quadramaculatus*. The North Carolina population of *D. quadramaculatus* was distinctly separated from the pooled group of West Virginia, Virginia, and Tennesee with a distance of D= 0.405465.

Table 1: Significant t-test results Comparing Morphological Characters between Populations, $p < 0.05$

Compared Populations	Character	
WVG and TN	DV Thick	
WVG and WVR	DV Thick	
WVG and VA	DV Thick	
WVG and WVR	Head Length	
TN and WVR	Head Length	
WVG and NC	Tib/Fib	
WVG and WVR	Tib/Fib	

Table 2: Significant t-test results comparing size-corrected morphological characters between populations, $p < 0.05$

Compared Populations	Character	
WVG and NC	Cranial Width	
WVG and WVR	DV Thick	
WVG and TN	DV Thick	
WVG and NC	DV Thick	
TN and VA	DV Thick	
VA and NC	DV Thick	
TN and WVR	DV Thick	
WVR and NC	DV Thick	
WVR and TN	DV Thick	
NC and VA	DV Thick	
VA and TN	DV Thin	
WVG and WVR	Head Length	
TN and WVR	Head Length	
VA and WVR	Head Length	
NC and WVR	Head Length	
WVG and NC	Femur	
WVG and WVR	Femur	
VA and WVR	Femur	
NC and WVG	Femur	
WVR and TN	Femur	
WVG and TN	Tib/Fib	
WVG and VA	Tib/Fib	
WVG and WVR	Tib/Fib	
NC and WVR	Tib/Fib	
TN and WVG	Tib/Fib	
VA and WVG	Tib/Fib	

Table 3: Linear geographic distances between populations (miles)

Site	Distance in Kilometers					
WVR	0					
WVG	95.36	0				
TN	125.93	216.93	0			
VA	51.77	91.09	169.43	0		
NC	128.73	222.47	65.72	194.37	0	
GA	397.15	483.05	272.19	447.29	308.58	0

Table 4: Matrix of Mahalanobis' Distance between populations.

Site	**Mahalanobis' Distance (non-corrected data)**				
WVR	0				
WVG	14.16493	0			
TN	10.65828	11.29603	0		
VA	7.27367	13.1238	2.4216	0	
NC	6.65302	6.00291	3.56494	4.60598	0
Site	**Mahalanobis' Distance (size corrected data)**				
WVR	0				
WVG	14.88343	0			
TN	11.76581	11.69769	0		
VA	7.59556	5.9532	3.36066	0	
NC	9.09053	12.5066	2.03077	4.51016	0

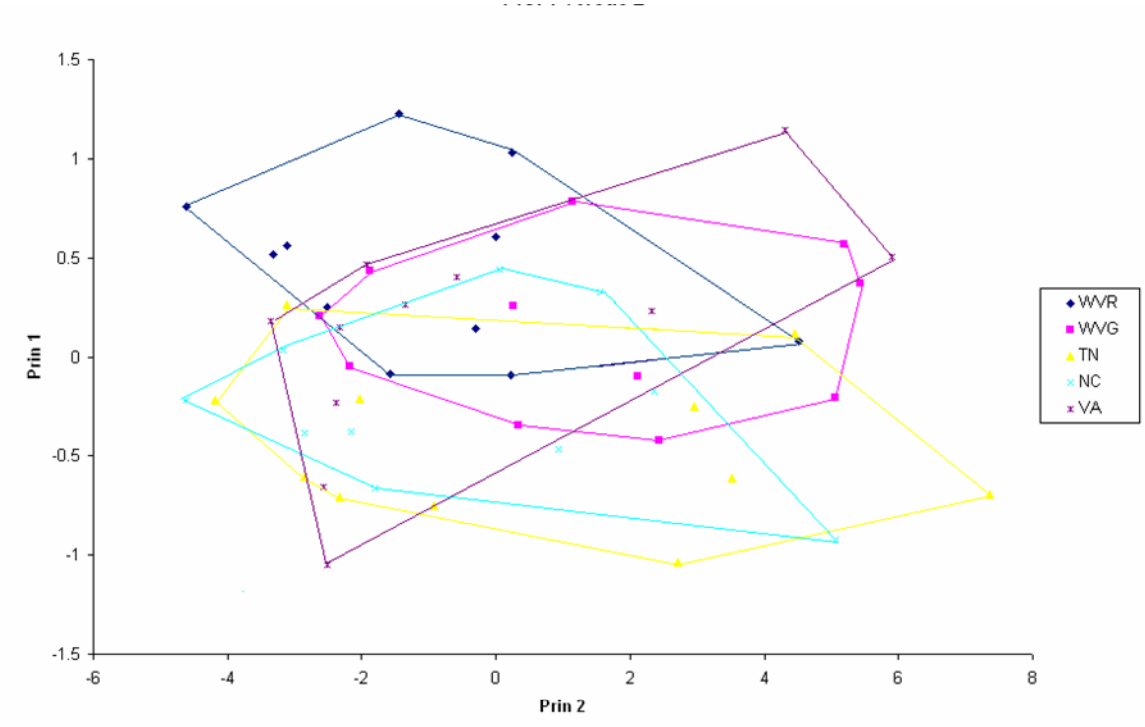
Table 5: Matrix of Mahalanobis' Distance P- values

Site	**Mahalanobis' Distance p- values (non-size corrected)**				
WVR	1				
WVG	<0.001	1			
TN	0.0004	0.0002	1		
VA	0.0096	<.0001	0.5332	1	
NC	0.0124	0.0225	0.2035	0.099	1
Site	**Mahalanobis' Distance p- values (size corrected data)**				
WVR	1				
WVG	<0.0001	1			
TN	<0.0001	<0.0001	1		
VA	0.0029	0.0133	0.1721	1	
NC	0.001	<0.0001	0.5691	0.0695	1

Table 6: Distance Matrix of calculated Nei's genetic Distance (D)

Site	Nei's Genetic Distance (D)					
WVR	0					
WVG	0	0				
TN	0	0	0			
VA	0	0	0	0		
NC	0.404565	0.405465	0.405465	0.405465	0	
GA	1	1	1	1	1	0

Figure 7 a, b. Principle component analysis for all samples assessed individually. WVR= collected from Rock, West Virginia; WVG= collected from Nicholas County, West Virginia; TN= collected from Sullivan County, Tennessee; NC= collected from Carter County, North Carolina; VA= collected from Giles County, Virginia.
a. Principle Components 1 versus 2.



b: Principle Component 2 versus 3

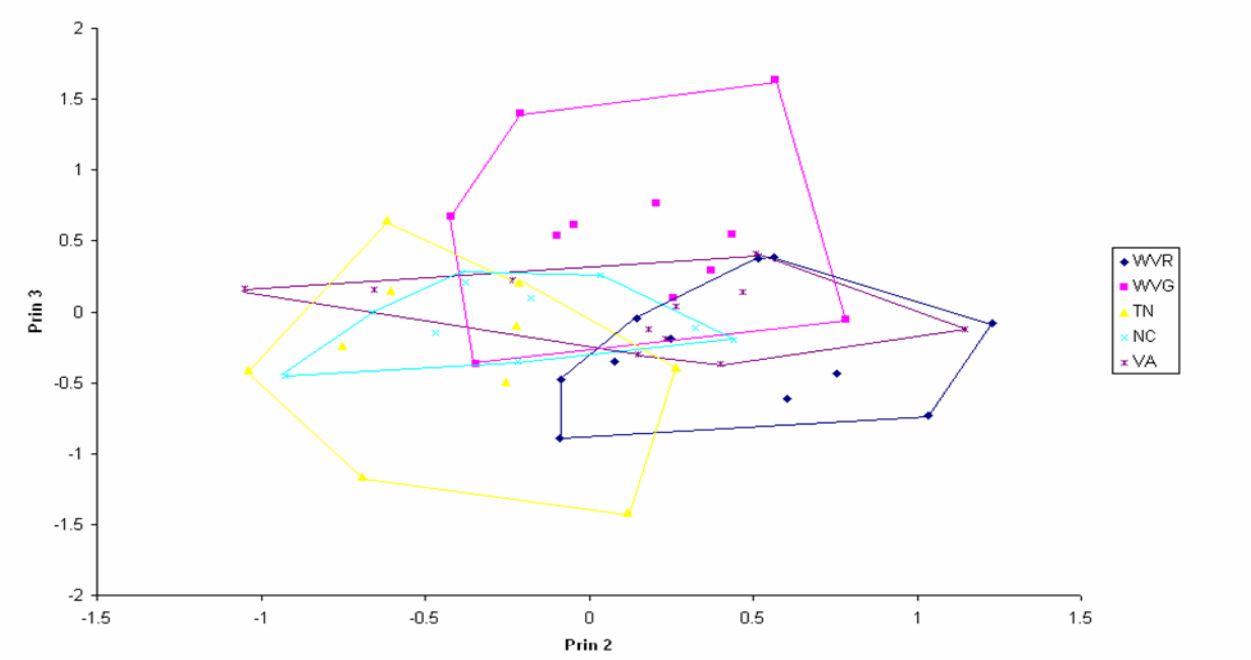
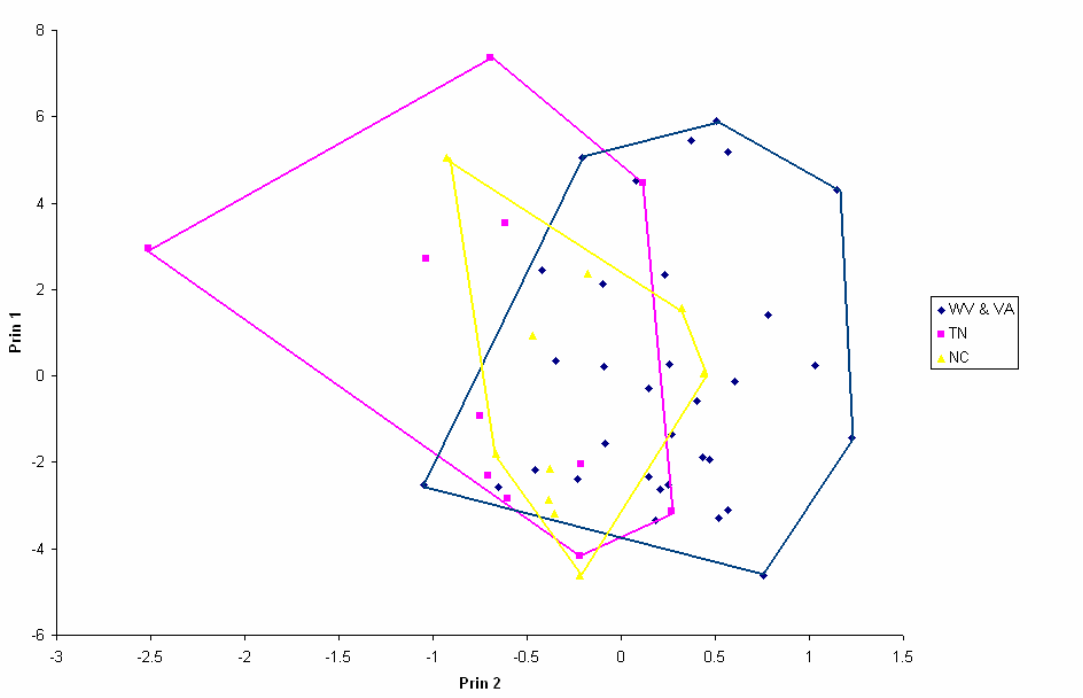


Figure 8 a,b : Principle components analysis for pooled samples, West Virginia and Virginia combined compared with Tennessee and North Carolina
a: Principle component 1 versus 2



b: Principle component 2 versus 3

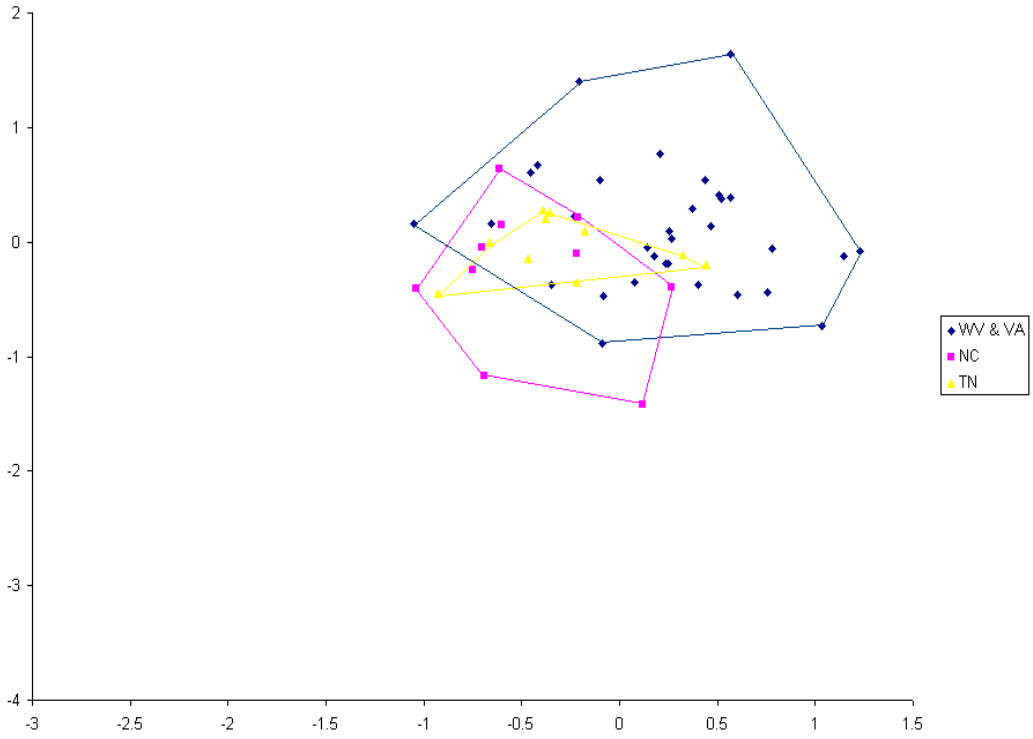
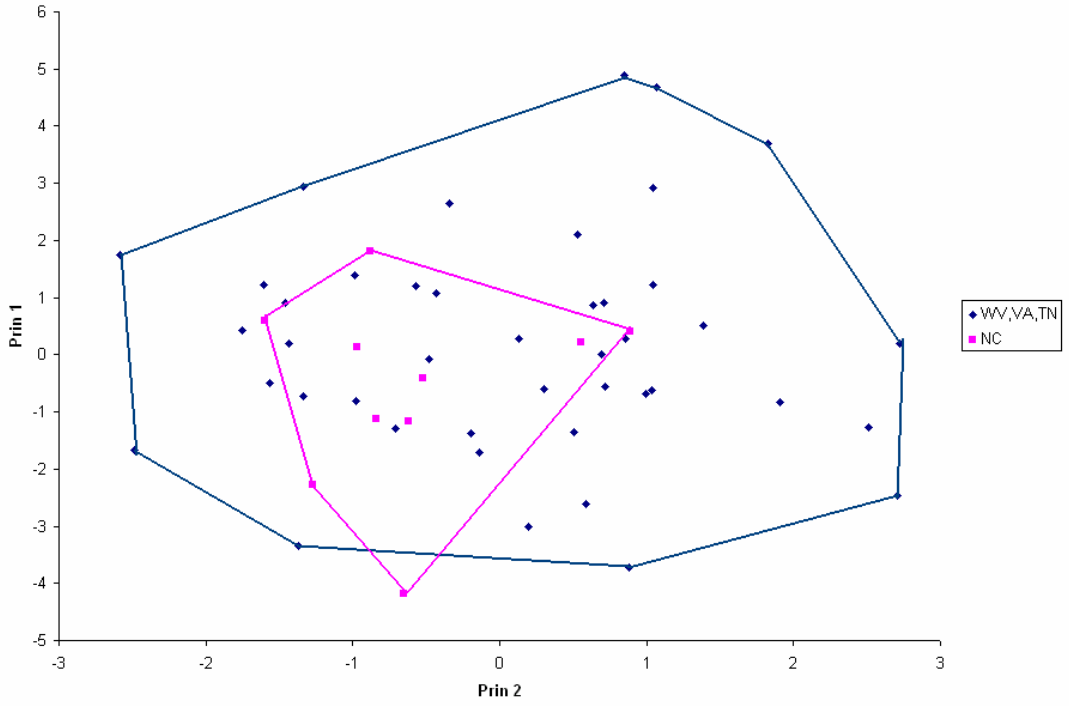


Figure 9 a, b: Principle components analysis comparing size corrected data for pooled group West Virginia, Virginia, and Tennessee compared with North Carolina.
a: Principle components 1 versus 2



b: Principle Components 2 versus 3

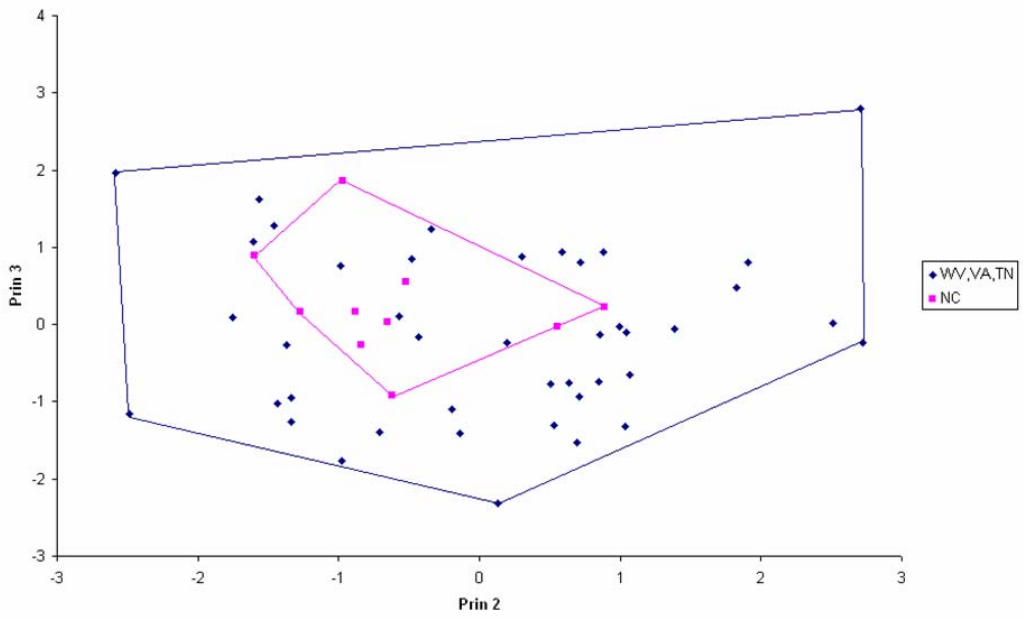
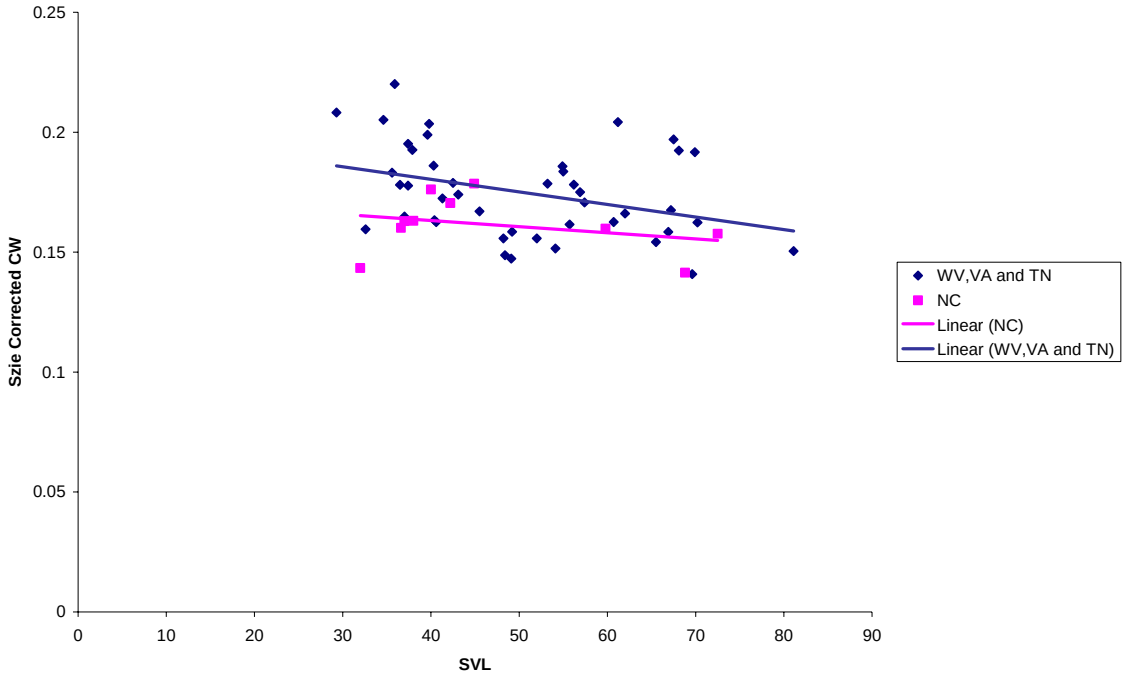


Figure 10: Size corrected head shape measurements (DV thick and cranial width) for the combined data set, West Virginia, Virginia and Tennessee compared with North Carolina **a**. Cranial Width compared between the pooled group and NC



b. DV Thick compared between the pooled group and North Carolina

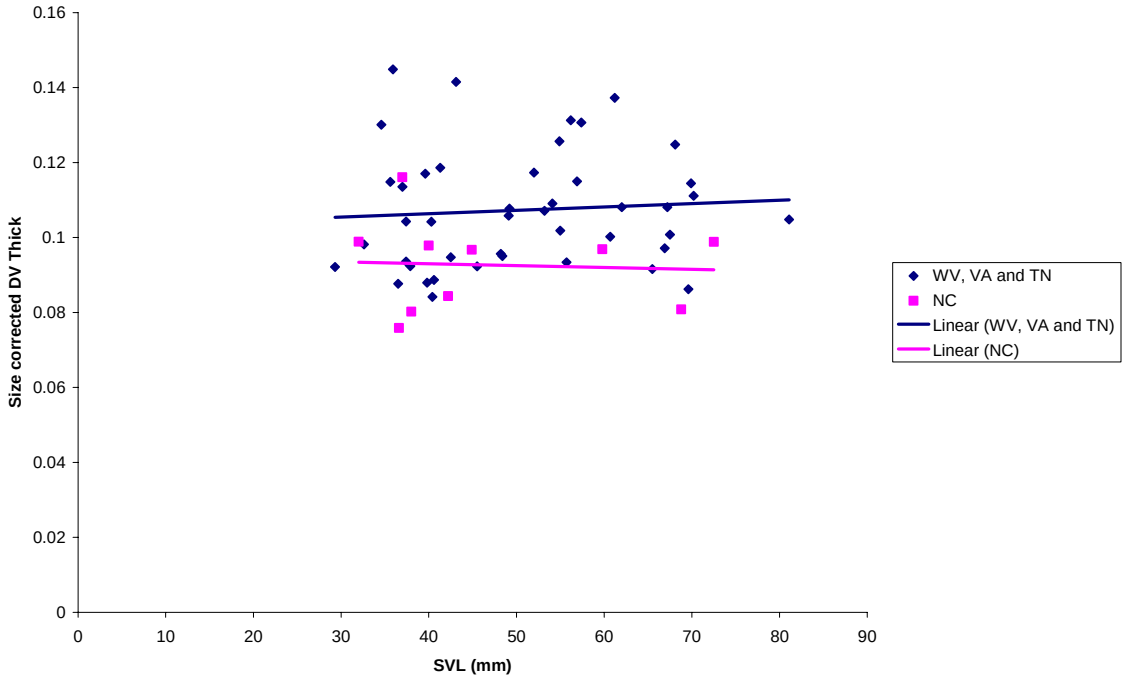
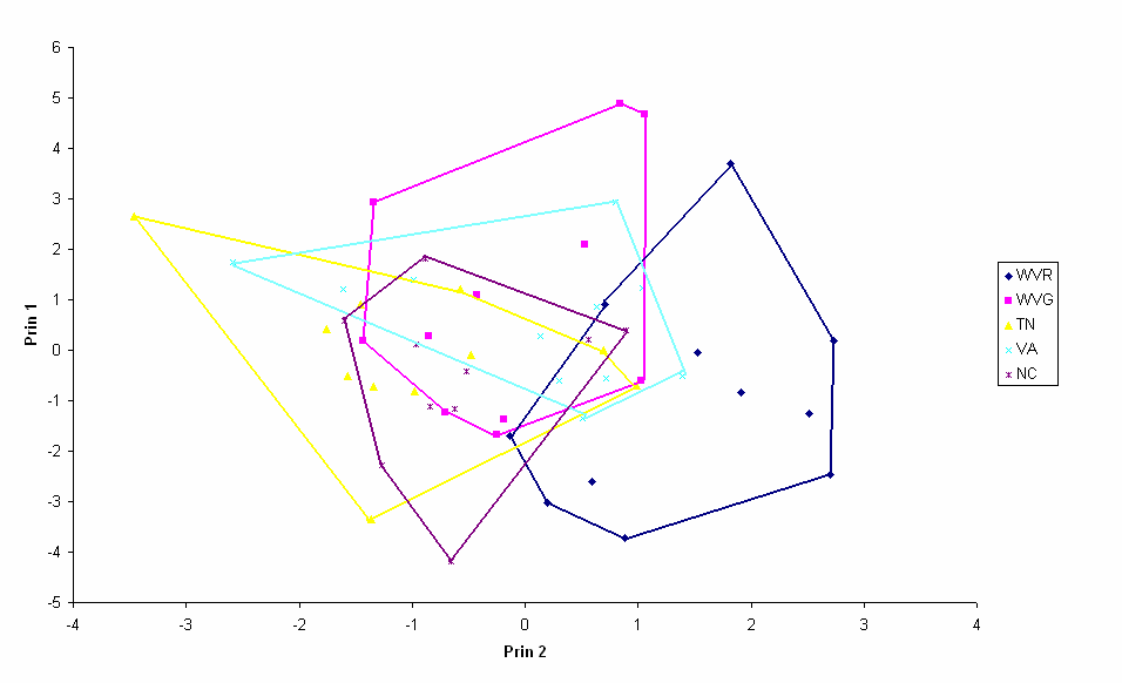


Figure 11: Principle components analysis comparing size corrected data for individually assessed populations.

a. Principle component 1 versus 2



b. Principle component 2 versus 3

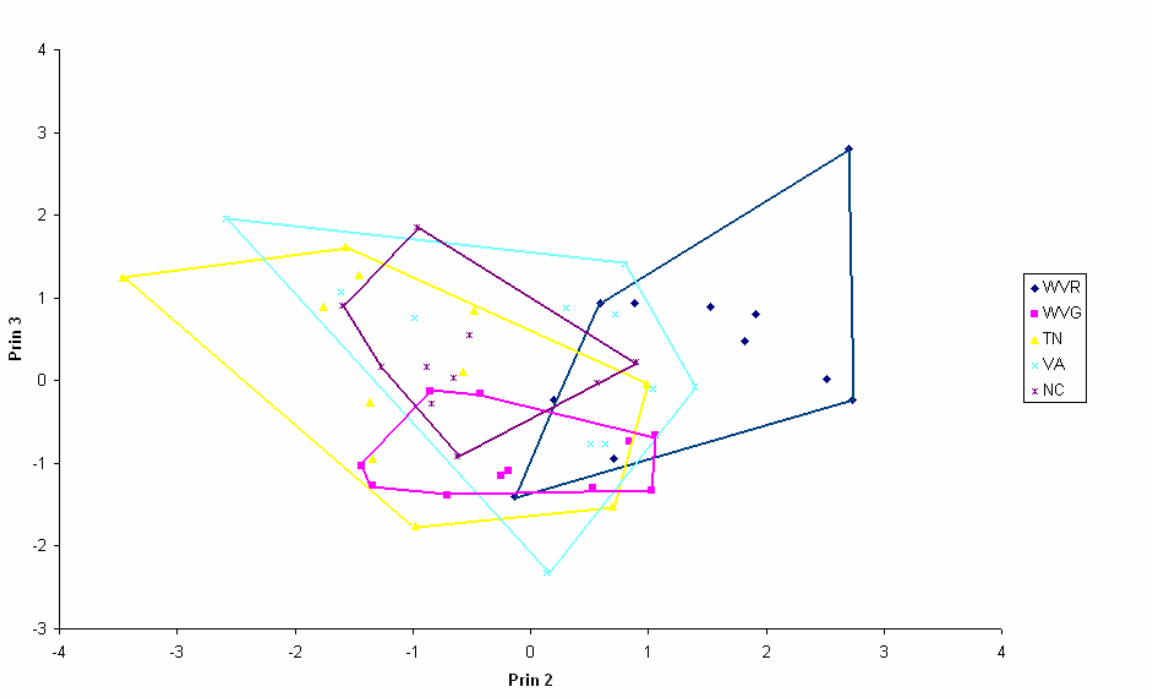


Figure 12: Phenotypic and Linear geographic distance for five populations of *D. quadramaculatus* ($r^2 = 0.65$).

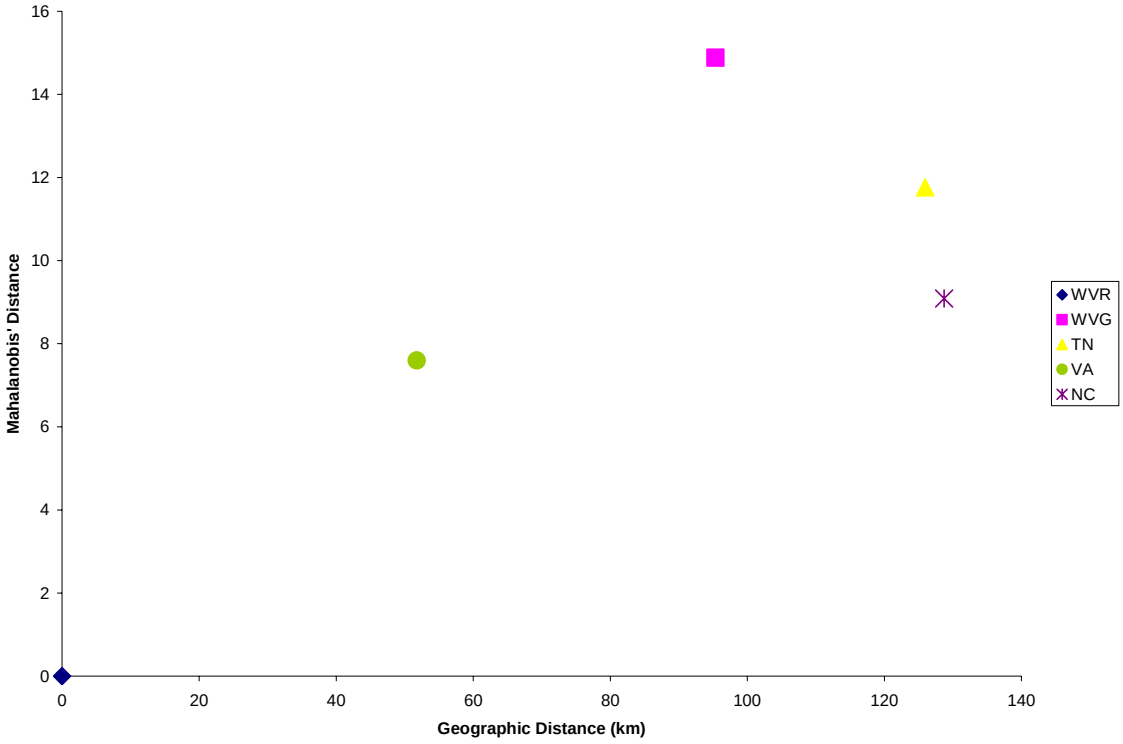


Figure 13: Nei's genetic distance and Linear geographic distance for six populations of *D. quadramaculatus* (r^2= 0.85).

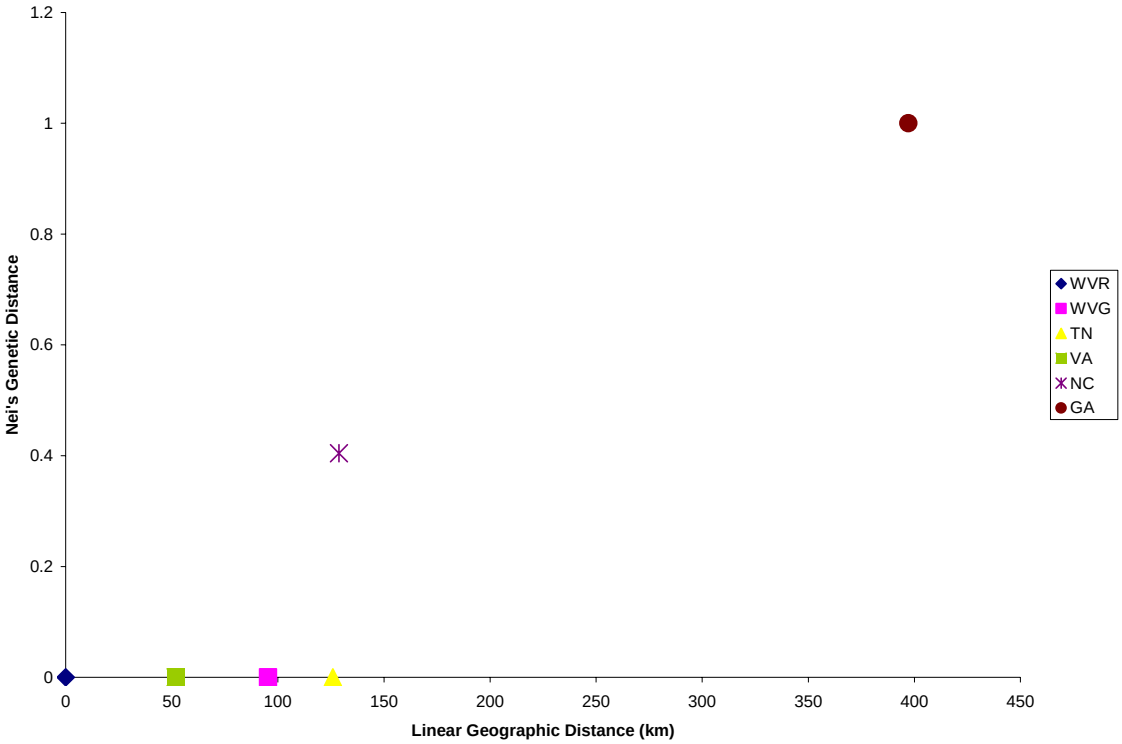


Figure 14: Phylogram of genetic distances where abbreviations represent the state of collection. WVR were collected from Rock, West Virginia, and WVG collected from the Gauley River drainage.

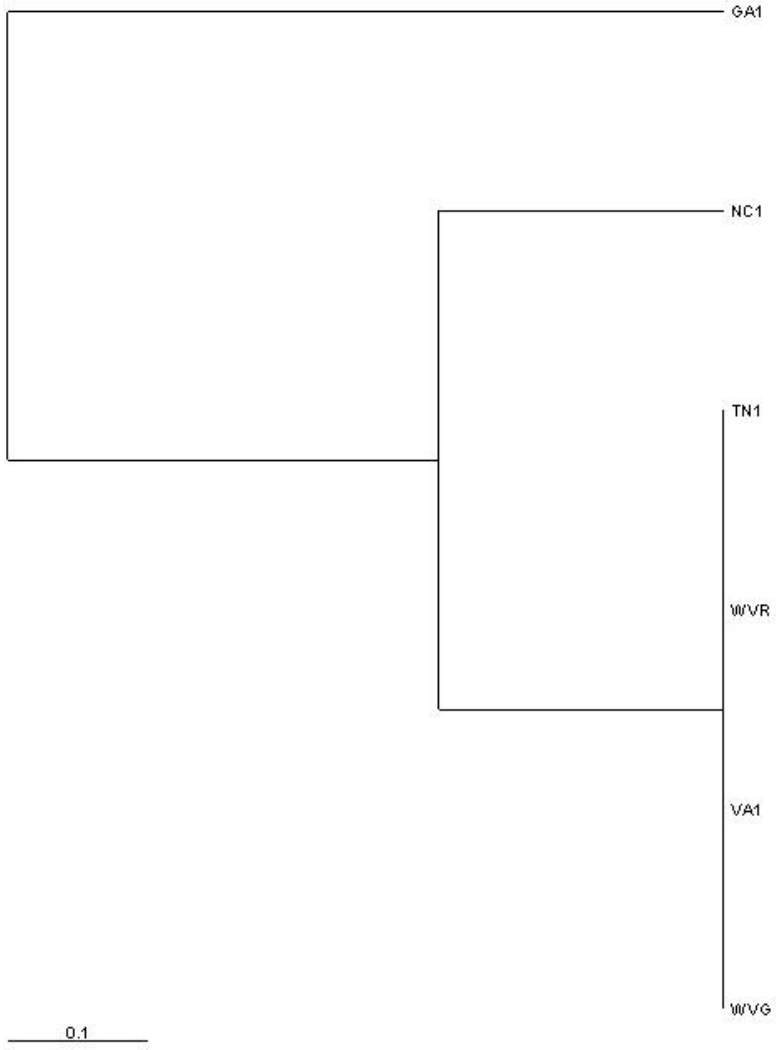
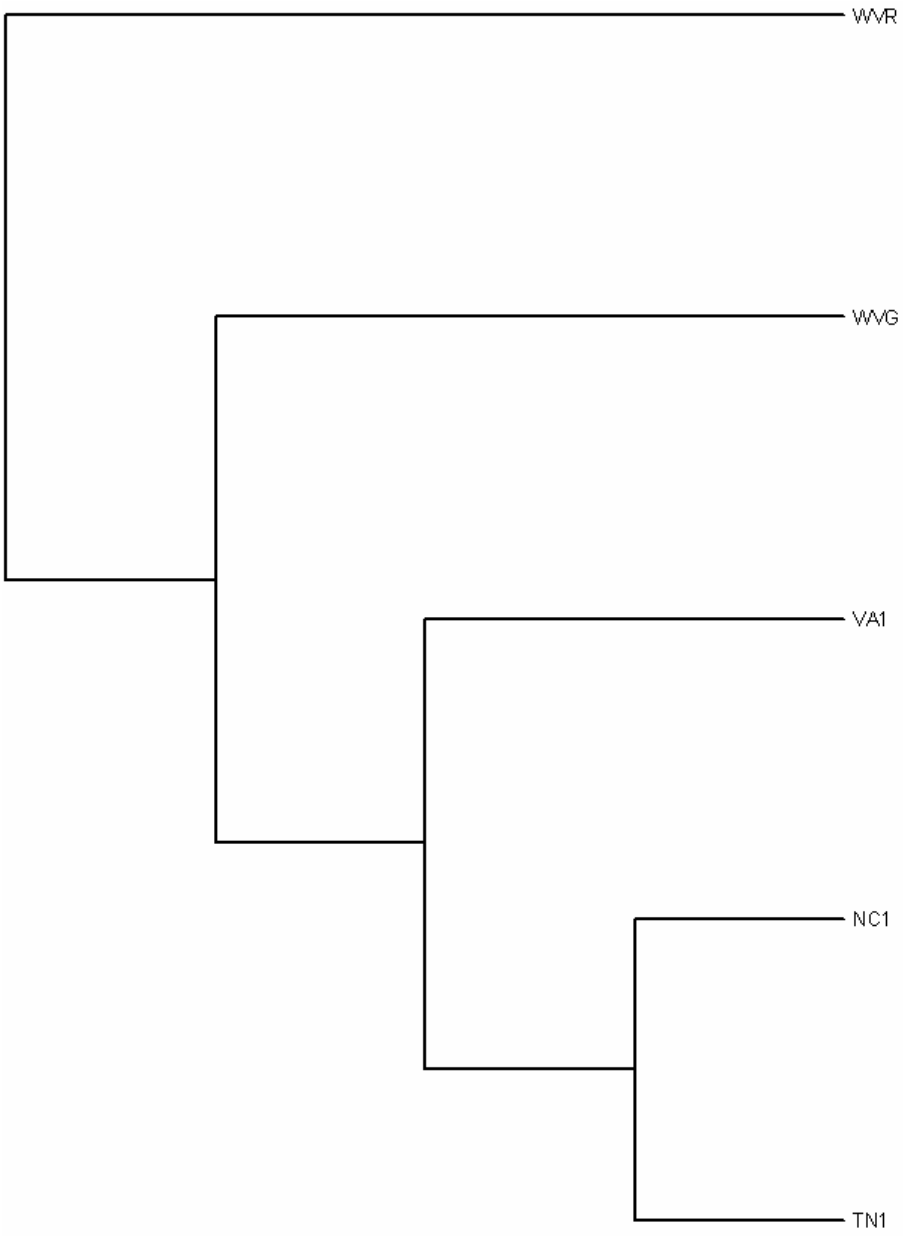


Figure 15: Phylogram of Mahalanobis' Distances where abbreviations represent the state of collection. WVR were collected from Rock, West Virginia, and WVG collected from the Gauley River drainage.



Chapter 4: Discussion

The results of this study show that *D. quadramaculatus* in West Virginia is not a genetically distinct species. The relationships observed were not the expected outcome, however, do provide valuable information on the evolution of this species.

Morphometrics

Whooten (2001) provided observable size differences between West Virginia specimens and those in surrounding states, stating that adult *D. quadramaculatus* from West Virginia are 40% smaller than those in other states (TN, VA, NC, GA). There are no distinct size differences observed between the populations studied (Figure 8). There were few observed differences in morphological characters that are not affected by the overall size of the animal (Table 1). One reason that there are not observable size differences between populations is the sample size. Only 10 specimens were collected from each sample site, and these were not necessarily all adult specimen as in the Whooten (2001) study. A population that had more sub- adults than adults could have biased the results.

Size can be heavily affected by environmental characters such as climate and elevation (Tilley, 1980; Bervin *et al*, 1979). Tilley (1980) found while studying two populations of *D. ochrophaeus* at high and low elevation that the higher elevation populations were larger throughout their life cycles. Colder temperatures at higher elevations affect the maturation rates, resulting in larger size at maturation (Bervin, *et al.*, 1979). As stated earlier, size at maturation dictates adult body size in these salamanders

Beachy and Bruce, 1993). Prey availability can also affect the maximum size a salamander can achieve (Bernardo and Agosta, 2003).

Differences were observed in head shape between salamanders from WV, VA, and TN compared with those from NC (Figure 10). Desmognathine salamanders have distinct head shapes when compared with other salamanders in the family Plethodontidae. The head shape of salamanders in the genus *Desmognathus* is dictated by the feeding mechanism of these animals. These salamanders utilize a "head-tuck" method of feeding, and use large muscles on the sides of their "necks" to pull their food back towards them after they have bitten it. This provides a forceful bite, allowing these salamanders to eat larger prey, including other salamanders. The North Carolina specimen had significantly narrower cranial widths and thinner heads at the thickest portion (DV Thick) ($p < 0.05$, Figure 10); both are related to the size of the feeding muscles. Differences in head shape could be a result of the diet of these salamanders. Walls (1996) reported that larval salamanders of the same species are limited in the prey they consume by the size of their heads. In a study of the effects of size specific predatory interactions of *Gyrinophilus porphyriticus*, Gustafson (1994) found that gape width is the limiting factor determining prey type and size.

In a study of the diets of 5 populations of *D. quadramaculatus* across the southern Appalachians, Loughman *et al.* (2005) describe the stomach contents of the salamanders that were used in this study. Significant differences were observed between the diet consumed by the North Carolina series when compared with those in the other sites combined ($p = 0.025$, Figure 16). The differences in diet consumed could be attributed to differences in the invertebrate fauna of the streams surveyed. Samples were not taken in

the streams for comparison. Differences between diets of these two groups of salamanders can also be attributed to niche separation at different life stages of the animals. The majority of salamanders utilized in the present study were post-metamorphic, however a direct relationship exists between the SVL of the salamander and the size of prey it consumes (Burton, 1976). The size classes of the salamanders in each population would therefore affect the type of prey taken by each animal. Since the same salamanders that were used in the present study were utilized for the diet analysis, a biased sampling may have occurred. Salamanders (limited to 11 per population) were captured based solely on availability in the stream. This effort was not correlated with weather activity, which has been proven to affect the foraging habits of Plethodontid salamanders (Maerz, 2000), and therefore may not have shown a representative sample of the dietary intake of these salamanders.

Plethodontid salamanders are generally considered to be morphologically conservative, with speciation events occurring without any morphological changes (Wake, *et al* 1983). The results of this study show that *D. quadramaculatus* does not follow this pattern of morphological conservatism. Body size (SVL) seemed to mask many of the differences (Figure 7). When size corrected characters were assessed, many morphological differences were observable (Figure 10, Table 3). These differences were not strong enough to indicate distinct separation between the groups analyzed (Figures 7, 8, 9, 11).

The shape distinctions are present in the highest frequency between the northern most population (WVG) and the southern most population (NC) (Table 2, 3; Figure 12). This is not surprising considering the large geographic distance between these

populations. Low gene flow between these populations is one reason why vast amounts of phenotypic variation occurs (Larson, 1984). Large rivers or areas lacking mountainous head-water streams could act as barriers to gene flow between populations of *D. quadramaculatus* because of the habitat that they are typically found.

The separation of these populations based on Mahalanobis' Distance indicates significant but unexpected differences between some populations (Tables 4, 5; Figure 15). The West Virginia populations were found to be significantly different from each other, as well as from all other populations (Tables 5; Figure 15). Due to the geographic proximity of these populations, it was unexpected that they would be separated based on morphological differences (Figure 12).

Morphological differences between the populations studied are probably the result of the combined effects of genetics and the environment. Isolating the cause of these differences is difficult without further study of the climate in each locality, prey availability and gene sequencing.

Allozymes:

Highton (1989) explored biochemical variation within the *Plethodon glutinosus* complex in the eastern United States, ultimately describing several new species based on their *Nei's* genetic distance being greater than 0.15, indicating more than 15% genetic differentiation. Thorpe (1982) found the same amount of differentiation when he surveyed published genetic distances between non-avian vertebrate species. This genetic distance has been used several times as a "bench-mark" for determining the species status

of morphologically cryptic species (Camp *et al.*, 2002; Tilley and Mahoney, 1996; Highton and Peabody, 2000).

Some controversy has risen from the use of this genetic distance to describe new species of amphibians. Highton (1990) argues that this genetic distance was determined because 15% genetic divergence "represented the only level that unified geographically contiguous morphologically and genetically similar groups". He also suggests that this amount of genetic differentiation also is equivalent to the amount of divergence in the genes responsible for reproductive isolation. While this may be possible, it is difficult to test. Salamanders are long- lived, with many species not reaching sexual maturity until after their fourth year (Castenet *et al.*, 1996). Reproductive compatibility was determined for different members of the *D. ochrophaeus* complex that have been distinguished as distinct species (Tilley and Mahoney, 1996) were tested for their reproductive compatibility. It was found that these species would interbreed freely (Tilley, 2004 unpublished data), by looking for the presence of sperm caps in the cloacae of the females. Although these species would not breed freely in the wild due to geographical constraints, there were not any behavioral or morphological distinctions to prevent them from breeding in the lab. These breeding events were not followed through the lifetime of the young to determine whether or not the F_1 generation was viable.

Considering the opportunity for errors in biochemical studies, as occurred in the completion of the present study, it is difficult to rely solely on biochemical techniques for determining species divergence. Genetic distances in this study are reported as high as 1 (Table 1). When considering Highton's arguments, this would be grounds for describing several distinct species in a *Desmognathus quadramaculatus* complex. However, the

lack of data present on all loci could bias the data. The data here provides good basis for future research, however, due to the problem encountered with protein denaturizing, it would be premature to suggest any taxonomic changes at the present time.

This data does suggest some interesting things about the relationships between the geographically separated members of this species. Geographically speaking, Georgia is the most distant population of those sampled; it was also the most genetically distinct (Figure 4, 13, Table 3). It is not surprising that this occurred, however it is interesting to note the correlation (r^2= 0.85). The North Carolina *D. quadramaculatus* also appear to be different from the West Virginia, Virginia, and Tennessee populations which formed a distinct group (Figure15). The populations sampled from West Virginia and Virginia are along the New River Watershed. This, in concert with their geographic proximity (Figure 4, 14, table 3), contributes to their genetic similarity. There is probably opportunity for gene flow between these populations considering stream drift and larval dispersal, although this remains untested at the present time. Geographic barriers to these populations interbreeding should be assessed in future research.

Chapter 5: Conclusions

Comparing Phenotype and Genotype

Variation exists between the results of the genotype and phenotype analyses. Genotypic expression (i.e. protein loci) may not necessarily affect phenotypic variation within a species. Phenotypic variation, at the same time, may not indicate any evolutionary status or genetic differentiation of a species. A change in taxonomic status,

therefore, should be considered as a result of a combination of studies to provide clear distinctions between morphologically similar species.

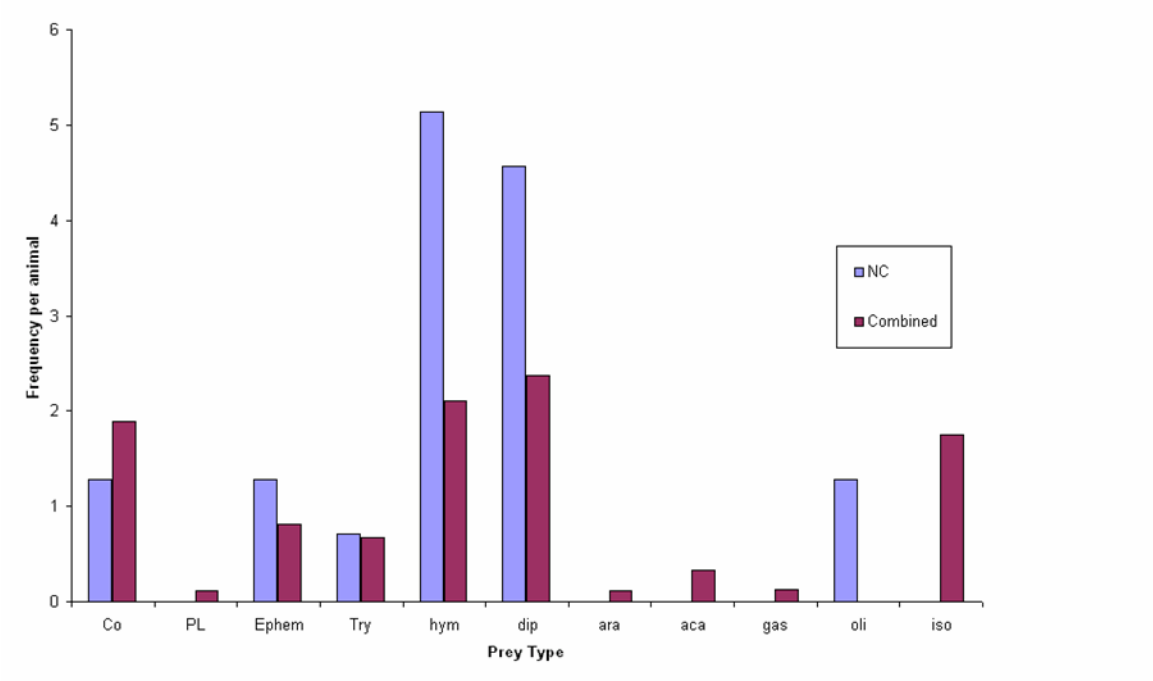
If morphometrics and allozyme frequency were considered separately in this case, two completely different pictures are painted of the relationships between these populations. The results of the allozyme study suggest that the relationships between the groups of *D. quadramaculatus* are closely related to linear geographic distance (Figure 14). The results of the morphology assessment contradicts the grouping of these animals this way, indicating two distinct groups present in West Virginia (Figure 15). Morphometrically, Tennesse, Virginia, and North Carolina form a distinct group with two other groups formed in West Virginia based on Mahalanobis' Distance (Figure 15). Considering Principle Components Analysis, however, there is not strong separation between any of these populations (Figures 8, 9, 10, 12). Based on the allozyme frequencies, West Virginia, Virginia and Tennessee form a distinct group from North Carolina (Figure 14).

Larson (1984) noted that speciation in Plethodontid salamanders rarely includes morphological changes. While the West Virginia populations are most likely not distinct species, they exhibit a large amount of phenotypic size variation (Table 4). It is possible that environmental differences between the populations studied could account for shifts in the life histories of these animals. However, the life histories of these populations were not assessed in the present study. Life history variation between intra- specific populations may account for or even lead to genetic differences between populations (Tilley and Bernardo, 1993). Traditionally, speciation events have been assumed to be

accompanied by life history shifts, however, the available data suggests that in actuality life history shift actually dictate the speciation events (Tilley and Bernardo, 1993).

In order to resolve the differences observed between protein frequency and morphometrics, it is recommended that DNA sequence data be assessed. This could provide valuable insight for resolving the phylogeny of *D. quadramaculatus* across the Southern Appalachians.

Figure 16: Comparison of diets between North Carolina and all other sampled populations with the follow abbreviations: Co= Coleoptera, Pl= Plecoptera, Ephem = Ephemeroptera, Try= Trichoptera, hym= Hymenoptera, dip= Diptera, Ara= Arachnida, aca= Acari, gas= Gastrapoda, oli= Oligocheta, and iso= Isopoda.



Literature Cited:

Beachy, CK. 1995. Effects of larval growth history on metamorphosis in a stream-dwelling salamander (*Desmognathus ochrophaeus*). *Journal of Herpetology* 29(3): 375-382

Beachy, Ck and RC Bruce.2003. Life History of a small form of the Plethodontid salamander *Desmognathus quadramaculatus*. *Amphibia-Reptilia* 24:13-26

Bernardo, J. and SJ Agosta. 2003. Determinants of clinal variation in life history of dusky salamanders (*Desmognathus ocoee*): prey abundance and ecological limits on foraging time restrict opportunities for larval growth. *Journal of the Zoological Society of London,* 259: 411-421

Bervin, KA., DE Gill, SJ Smith-Gill. 1979. Counter-gradient selection in the green frog, *R. clamitans*. *Evolution,* 33:609-623

Bervin KA., and DE Gill. 1983. Interpreting geographic differences in life history traits. *American Zoology*, 23: 85-97

Bruce, RC. 1996. Life history perspective of adaptive radiation in Desmognathine salamanders. *Copeia* 4: 783-790

Burton, TM. 1976. An analysis of the feeding ecology of the salamanders of the Hubbard Brook Experimental Forest, New Hampshire. *Journal of Herpetology*, 10(3): 187-204

Camp, CD, JL Marshall, RA Austin Jr. 2000. The evolution of adult body size in black-bellied salamanders (*Desmognathus quadramaculatus* complex). *Canadian Journal of Zoology*. 78: 1712-1722

Camp, CD, SG Tilley, RM Austin, and JL Marshall. 2002. A New Species of Black-bellied salamander (Genus: *Desmognathus*) from the Appalachian Mountains of Northern Georgia. *Herpetologica,* 58(1): 471-481

Cannatella, DC and DM Hillis. 1993. Amphibian relationships: phylogenetic analysis of morphology and molecules. *Herpetological Monographs*, 7: 1-7

Castenet, J., H Francilion- Viellot, and RC Bruce. 1996. Age estimation in Desmognathine salamanders assessed by skeletochronology. *Herpetologica,* 52(2): 160-171

Cole, CJ. 1990. When is an individual not a species? *Herpetologica*, 46(1):104-108

Conant, R. and JT Collins. 1998. A field guide to reptiles and amphibians of Eastern and Central North America. 3rd ed, expanded. Houghton Mifflin Company, New York, NY.

Echelle, AA. 1990. In defense of the phylogenetic species concept and the ontological status of hybridogenetic taxa. *Herpetologica*, 46 (1):109-113

Frost, DR and DM Hillis. 1990. Species in concept and practice: herpetological applications. *Herpetologica*, 46:87-104

Green, NB and TK Pauley. 1987. Amphibians and reptiles in West Virginia. Pittsburg: University of Pittsburg Press.

Gustafson, MP. 1994. Size-specific interactions among larvae of the Plethodontid salamanders *Gyrinophilus porphyriticus* and *Eurycea cirrigera*. *Journal of Herpetology,* 28 (4): 470-476

Hairston, NG., 1949. The local distribution and ecology of the Plethodontid salamanders of the Southern Appalachians. *Ecological Monographs,* 19 (1): 49-73

Highton, R. 1979. A new cryptic species of salamander fo the genus *Plethodon* from the Southeastern United States (Amphibia: Plethodontidae). *Brimleyana,* 1: 31-36
---- 1983. A new species of woodland salamander of the *Plethodon glutinosus* group form the southern Appalachian Mountains. *Brimleyana,* 9: 1-20
----1989. Biochemical evolution in the slimy salamanders of the *Plethodon glutinosus* complex in the eastern US. Part I. Geographic protein variation. *Illinois Biological Monographs*, 57: 1-78
----1990 Taxonomic treatment of genetically differentiated populations. *Herpetologica*, 46(2):114-121
----1997. Geographic protein variation and speciation in the *Plethodon dorsalis* complex. *Herpetologica,* 53: 345-356

Highton R, and RB Peabody. 2000. Geographic protein variation and speciation in salamanders in the *Plethodon glutinosus* and *Plethodon jordani* complexes in the Southern Appalachian Mountains with the description of four new species. Pp 31-75. *In* RC Bruce, RG Jaeger, and LD Houck (Eds.) The Biology of Plethodontid Salamanders. Klwer Academic/ Plenum Publisher, New York. New York, USA

Highton R, and TP Webster. 1976. Geographic protein variation and divergence in populations of the salamander *Plethodon cinereus*. *Evolution*, 30: 33-45.

Larson, A (1984) Neontological inferences of evolutionary pattern and process in the salamander family Plethodontidae. *Evolutionary Biology,* 17: 119-217

Larson, A and WW Dimmick. 1993. Phylogenetic relationships of the salamander families: an analysis of congruency among morphological and molecular characters. *Herpetological Monographs* 7:77-93

Loughman, Z., DS Merritt, KR Loughman and TK Pauley. (2005) Black Bellied Salamander (*Desmognathus quadramaculatus*) Feeding Ecology Across the Southern Appalachians. *Association of Southeastern Biologists*; Florence, Alabama

Maerz, JCM. 2000 Microgeographic foraging patterns if the Red-backed Salamander. *25th GSA Ecology and Evolutionary Biology Symposium*; Cornell University, Ithaca, NY.
Nei, M. 1975. Molecular population genetics and evolution. North Holland, Amsterdam.

Mills, G. 1996. A study on the life history and seasonal foraging habits of the salamander *Desmognathus quadramaculatus* (Holbrook) in West Virginia. Master's Thesis. Marshall University, Huntington, West Virginia.

Organ, JA. 1961. Studies of the local distribution, life history, and population dynamics of the salamander genus *Desmognathus* in Virginia. *Ecological Monographs.* 31(2):189-219

Pauley, TK. 1992. Report of upland vertebrates in the New River Gorge National River. A report for the National Parks Service. Vol. 3. 1145 pp.

Powell, R. JT Collins, and ED Hooper, Jr. 1998. A Key to Amphibians and Reptiles of the Continental United States and Canada. University of Kansas Press, Lawrence, Kansas

Rissler, LJ and DR Taylor. 2002. The phylogenetics of *Desmognathine* salamander populations across the southern Appalachians. *Molecular Phylogenetics and Evolution,* 27: 197-211.

Smith, HM. 1990. The Universal species concept. *Herptologica*, 46(1):122-124

Stiven, AE and RC Bruce. 1988. Ecological genetics of the salamander *Desmognathus quadramaculatus* from disturbed watersheds in the Southern Appalachian Biosphere reserve cluster. *Conservation Biology*, 2(2):194-205

Thorpe, JP (1982). The molecular clock hypothesis: Biochemical evolution, genetic differentiation and systematics. *Annual Review of Ecological Systematics,* 13: 139-168

Tilley, SG. (1980) Life histories and comparative demography of two salamander populations. *Copeia*, 4: 806-821

Tilley, SG. 1981. A new species of *Desmognathus* (Amphibia: Plethodontidae) from the Southern Appalachian Mountains. *Occasional papers of the Museum of Zoology*. University of Michigan, 651: 1-23.

Tilley, SG and J Bernardo. 1993. Life history evolution in Plethodontid Salamanders. *Herpetologica,* 49(2):154-163

Tilley, SG., and MJ Mahoney. 1996. Patterns of genetic differentiation in salamanders of the *Desmognathus ochrophaeus* complex (Amphibia: Plethodontidae). *Herpetological Monographs,* 10:1-42

Titus, TA, and A. Larson. 1996. Molecular phylogenetics of Desmognathine salamanders (Caudata: *Plethodontidae*): a reevaluation of evolution in ecology, life history, and morphology. *Systematic Biology* 45(4): 451-472

Turner, M. 1997. The ecology, natural history and distribution of *Desmognathus quadramaculatus* in West Virginia. Master's Thesis, Marshall University, Huntington WV

Wake, DB, G Roth and MH Wake. 1983. On the problem of stasis in organismal evolution. *Journal of Theoretical Biology,* 101: 211-224

Walls, SC. 1996. Morphological variation in larval salamanders: Dietary induct of plasticity in head shape. *Oecologica,* 96(2).

Wilder, IW and ER Dunn. 1920. The correlation of lunglessness in salamanders with a mountain brook habitat. *Copeia,* 84:63-68

Whooten, J. 2001. Distribution, morphometrics and tooth morphology of the genus *Desmognathus* in West Virginia. Master's Thesis, Marshall University, Huntington, WV

Appendix 1

Series Collected from Giles County, Virginia

Series collected from Carter County, North Carolina

Series collected from Mercer County, West Virginia

Series collected from Sullivan County, Tennessee

Appendix 2: Gel and Dye Recipes

Zymecicle recipies for *Desmognathus* allozyme electrophoresis

For liquid stains:
Dilute ingrdients to 100 ml with buffer.
2. Divide into 10 ml aliquots and freeze.
3. When ready to use: thaw, dillute to 40 ml with buffer, add PMS and coupling enzymes, and pour over gel.

For agar overlays:
Dilute ingrdients to 150 ml with buffer.
Divide into 15 ml aliquots and freeze.
When ready to use: thaw, add PMS and coupling enzymes, , combine with 15 ml of 1% agar, and pour over gel

System	1 M $MgCl_2$ (ml)	NAD (mg)	NADP (mg)	1% MTT (ml)	Other ingredients		Add at staining	
0.2 M TRIS-HCl, pH 8.0 BUFFER:								
AK	8.0	200.0		5.0	Glucose:	1000 mg	Hexokinase:	80 units
					ADP:	300 mg	G6pdh:	20 units
					Hexokinase:	80 units	1% PMS:	0.5 ml
					G6pdh:	20 units		
					Agar:	0.3 g		
					Buffer:	15 ml		
IDH	2.5		80	10	Isocitric acid:	617 mg	1% PMS:	0.5 ml
LDH		180		10	Substrate solution:	37 ml	1% PMS:	0.5 ml
MDH	1.7	170		10	Substrate solution:	17 ml	1% PMS:	0.5 ml
ME	1.7			10	Substrate solution:	17 ml	NADP:	17 mg
							1% PMS:	0.5 ml
PGDH	10		50	10	6-phosphogluconic acid:	100 mg	1% PMS:	0.5 ml
SOD	3.5			10			1% PMS:	0.5 ml
0.1 M TRIS-HCl, pH 7.0 BUFFER:								
CK (agar)		120		10	Creatine phosphate	3650 mg	Hexokinase:	80 units
					Glucose:	450 mg	G6pdh:	40 units
					ADP:	350 mg		
					Hexokinase:	80 units		
0.5 M PHOSPHATE, pH 7.0 BUFFER:								
GLUD		230		10	Substrate solution:	19 ml	1% PMS:	0.5 ml
					Na pyruvate:	400 mg		

Gel recipies (12.5% hydrolyzed potato starch, 7.5% sucrose):

	250 ml buffer	400 ml buffer
Starch	31.250 g	50.000 g
Sucrose	18.750 g	30.000 g
NAD or NADP	0.625 g	1.000 g

Enzyme and buffer systems used in *Desmognathus* electrophoresis.

Enzyme	No. of loci scored	E.C. number	Buffer system
Aspartate aminotransferase	2	2.6.1.1	2,3
Adenylate kinase	1	2.7.4.3	3
Creatine kinase	1	2.7.3.2	3
Fumarate hydratase	1	4.2.1.2	4
Glucose dehydrogenase	1	1.1.1.47	1
Glutamate dehydrogenase	1	1.4.1.3	2
Glucose-6-phosphate isomerase	1	5.3.1.9	5
Glyceraldehyde-3-phosphate dehydrogenase	1	1.2.1.12	2
Glycerol-3-phosphate dehydrogenase	1	1.1.1.8	2
3-Hydroxybutyrate dehydrogenase	1	1.1.1.30	6
Isocitrate dehydrogenase	2	1.1.1.42	1
L-Lactate dehydrogenase	2	1.1.1.27	4,5
Malate dehydrogenase	2	1.1.1.37	1
Malate dehydrogenase (oxaloacetate-decarboxylating)	1	1.1.1.38	1
Mannose-6-phosphate isomerase	1	5.3.1.8	5
Phosphogluconate dehydrogenase	1	1.1.1.44	2
"Leucyl-glycyl-glycine peptidase"	1	---	4
Superoxide dismutase	1	1.15.1.1	6

Buffer systems:

1. Tris-citrate, pH 8.0 Selander, et al. 1971
2. Tris-EDTA-borate, pH 9.1 Karlin and Guttman, 1986
3. Tris-citrate, pH 6.7 Selander, et al. 1971
4. Poulik Selander, et al. 1971
5. Lithium hydroxide Selander, et al. 1971
6. Tris-versine-borate Selander, et al. 1971
7. Tris-glycine, tris-HCl, E.C. Tech. Bull. No. 134.

Substrate solutions used in *Desmognathus* electrophoresis.

Gel recipies (12.5% hydrolyzed potato starch, 7.5% sucrose):

	1 Liter	500 ml
Alphaketoglutaric acid	0.365 g	0.180 g
Aspartic acid	1.331 g	0.666 g
Polyvinylpolypyrrolidone	2.50 g	1.250 g
Disodium EDTA	0.50 g	0.250 g
Na_2HpO_4	14.20 g	7.1 g

IDH (0.1 M isocitric acid)

DL-isocitric acid	1.47 g
Water to 50 ml	

MDH (2.0 M malic acid, pH 7.0)

DL-malic acid	134.10 g
NaOH	80 g
Water to 500 ml	

Add NaOH ***slowly***
Titrate to pH 7.0 with conc. NaOH

LDH

85% DL-lectic acid	10.60 ml
1 M sodium carbonate	49.00 ml

Addd sodium carbonate ***slowly***
Titrate to pH 7.0 with 0.5 M sodium carbonate

Water to 100 ml

GLUD

Sodium glutamate	4.25 g
0.5 M phosphate pH 7.0 buffer	100 ml

Stain buffers used in *Desmognathus* electrophoresis.

0.2 M tris HCl pH 8.0

Tris	145.24 g
Concentrated HCl	~60 ml
Water to 6 L	

Titrate in the HCl to pH 8.0

0.5 M tris HCl pH 7.1

Tris	60.55 g
Concentrated HCl	~37.5 ml
Water to 1 L	

0.5 M tris HCl pH 7.5

Tris	60.55 g
Concentrated HCl	~30 ml
Water to 1 L	

0.5 M phosphate buffers

Stock solution I:
K_2HPO_4 ($3H_2O$) 57.05 g (43.54 g if anhydrous)
Water to 500 ml

Stock solution II:
KH_2PO_4
Water to 500 ml

Combine and titrate stock solutions to obtain desired pH

Deborah S. Merritt
Curriculum vitae

Current Address: 1578 Washington Boulevard
Huntington, West Virginia 25701

Permanent Address: 4351 Southern Pine Way
Syracuse, New York 13215

Phone: (304)525-5006 (home)
(315)378-9652 (cellular)
Email: merritt31@marshall.edu

Education

2005

MS, Analysis of genotypic and phenotypic differences in *Desmognathus quadramaculatus* across the Southern Appalachians
Thesis Committee: Dr. Thomas Pauley and Dr. Charles Somerville, Dr. Dan Evans

2002

Certificate of Completion, Trinity University, Prague, Czech Republic
Teaching English as a Foreign Language certification

2001

BA, State University of New York at Binghamton, Department of Environmental Studies, Binghamton, New York
Honors thesis: The Effects of Road Runoff on Ecosystem Quality as Determined by the Health of Red-backed Salamanders, *Plethodon cinereus*
Supervising Committee: Dr. Richard Andrus, Dr. Joseph Graney
Senior Thesis: The Transfer of Heavy Metals from Road Drainage areas into the Food Web through Invasive Earthworm Populations
Supervising Committee: Dr. Richard Andrus, Dr. Joseph Graney

2000

Certificate of Completion, School for Field Studies, Center for Marine Fisheries and Coastal Studies, Bamfield, British Columbia
Directed Research: Baseline and Timber Data for the Bamfield Huu-ay-aht Community Forest

Professional Experience

August 2003- May 2005

Principle Investigator, Determination of Species Status of the Black-Bellied Salamander, *Desmognathus quadramaculatus* in West Virginia, Marshall University, Department of Biological Sciences, Huntington ,West Virginia. Studying the speciation of the Black-bellied Salamander in West Virginia comparing (1) genetic differentiation and (2) morphological differences between West Virginia and specimens from other areas of the Appalachians. Conducted

field surveys, genetic analysis using allozymes and compared morphological data statistically using SAS.

August 2003- August 2004

Project Director, Mead Westvaco Landscape Ecology Project, Marshall University, Department of Biological Sciences, Huntington, West Virginia
Responsibilities include, writing protocol to monitor the dispersal rates and distances of juvenile egress in two species of woodland amphibians; participating in mark-recapture study, field surveys, data entry, and data analysis.

January 2002- August 2002

Lab Technician, Resource Ecology and Management Lab, Cornell University, Department of Natural Resources, Ithaca, NY
Research Assistant to Dr. John Maerz, studying the impact of non-indigenous plant invasions to woodland salamander fitness and abundance. Responsibilities include filed survey, participating in mark-recapture salamander study, analyzing gut/ diet samples of woodland salamanders, and data entry

August 2000-December 2001

Honors and theses research. Studied the impact of road runoff on (1) the abundance and water balance of woodland salamanders, and (2) the concentration of heavy metal pollutants in earthworm tissues. Conducted field surveys, mark-recapture studies of salamanders using arrays of artificial cover boards, conducted laboratory studies on salamander dehydration rates, and used an Intercoupled Plasma Mass Spectromoeter (ICMPS) to analyze soil and tissue samples. Wrote two theses and have presented to scientific audiences.

May- August 2001

Research assistant to Mason Ryan, Tropical Forestry Initiative, Tres Piedras, Costa Rica
Studied resource partitioning in two species of Dendrobatid frogs and aided in surveys of Costa Rican herptofauna

Teaching Experience

2005 Graduate Teaching Assistant, *Ecology* and *Ornithology*. Marshall University, Huntington,WV. Lectured, led field trips, and graded assignments in both subjects.

2004 Graduate Teaching Assistant, *Human Physiology* and *Herpetology*. Marshall University, Huntington, WV. Instructed lab portion of course, including grading laboratory reports and class assignment, and lecturing.

2003 Graduate Teaching Assistant, *Introductory Biology for Non-Majors*. Marshall University, Huntington, WV. Instructed lab portion of course

2002 English as a Foreign Language Teacher and Private Tutor, Prague, Czech Republic

2001 Undergraduate Teaching Assistant, *Introduction to the Environment*. State University of New York at Binghamton. Instructed lab portion of course

Undergraduate Teaching Assistant, *Introduction to the Backpacking*. State University of New York at Binghamton. Gave periodic lectures and supervised students on day and weekend trips

Undergraduate Teaching Assistant, *Outdoor Living Skills for Women*. State University of New York at Binghamton. Gave lectures and supervised students on day trip

2000 Assistant Director of Education and Environmental Education Intern, Union of American Hebrew Congregate, Eisner Camp, Great Barrington, Massachusetts. Developed and Implemented curriculum for children ages 7-15.

Awards and Grants

2005 West Virginia Department of Natural Resources Research Grant

Junior Garden Club Botany Scholarship Recipient

2004 Molecular and Cellular Research Travel Award to conduct research at Smith College, Northampton, MA and University of Alabama, Tuscaloosa, AL

Travel grant to attend National Joint Meeting of Ichthyologists and Herpetologists

Summer Award for Graduate Research, Marshall University

2001

Inducted into Golden Key National Honor Society
Inducted into Phi Beta Kappa National Honor Society
Harpur College Undergraduate Independent Research Award (grant), State University of New York at Binghamton to study the affects of road runoff on the accumulation of heavy metal pollution in the tissues of earthworms and its implications for forest foodwebs

2000

Harpur College Undergraduate Independent Research Award (grant), State University of New York at Binghamton to study the affects of road runoff on salamander abundance and water balance.

Presented Papers

"Amphibian use of man-made pools in clear-cut forests". 2005, Association of Southeastern Biologists, Florence, Alabama

“The use of florescence in assessing water quality”. 2004, Graduate Seminar Series, Marshall University, Huntington, West Virginia.