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Distribution and Conservation Genetics of the Cow Knob Salamander, Plethodon punctatus Highton (Caudata: Plethodontidae)

Matthew R. Graham
matthew.graham@unlv.edu

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by

Matthew R. Graham

Thomas K. Pauley, Committee Chairman
Victor Fet, Committee Member
Guo-Zhang Zhu, Committee Member

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MATTHEW R. GRAHAM

Department of Biological Sciences, Marshall University
Huntington, West Virginia 25755-2510, USA
email: mattrgraham@yahoo.com

Summary

Being lungless, plethodontid salamanders respire through their skin and are especially sensitive to environmental disturbances. Habitat fragmentation, low abundance, extreme habitat requirements, and a narrow distribution of less than 70 miles in length, makes one such salamander, *Plethodon punctatus*, a species of concern (S1) in West Virginia. To better understand this sensitive species, day and night survey hikes were conducted through ideal habitat and coordinate data as well as tail tips (10 to 20 mm in length) were collected. DNA was extracted from the tail tips and polymerase chain reaction (PCR) was used to amplify mitochondrial 16S rRNA gene fragments. Maximum parsimony, neighbor-joining, and UPGMA algorithms were used to produce phylogenetic haplotype trees, rooted with *P. wehrlei*. Based on our DNA sequence data, four disparate management units are designated. Surveys revealed new records on Jack Mountain, a disjunct population that expands the known distribution of the species 10 miles west. In addition, surveys by Flint verified a population on Nathaniel Mountain, WV and revealed new records on Elliot Knob, extending the known range several miles south. DNA sequencing of 24 individuals revealed 8 haplotypes. 16 individuals from the main population on Shenandoah Mountain all had the same haplotype, suggesting low genetic variability. Conversely, each individual from all other areas possessed a unique haplotype. Most importantly, a haplotype from Nathaniel Mountain, WV was deeply divergent and has probably been isolated since the early Pleistocene, making the population a conservation priority. It is hoped that this new genetic data will increase the efficacy of Cow Knob salamander conservation efforts by providing the means to implement management plans that conserve intraspecific genetic diversity.

“Nature uses only the longest threads to weave her patterns, so each small piece of her fabric reveals the organization of the entire tapestry.”

Richard Feynman
The Character of Physical Law (1965)

“When we begin to plan how to use a piece of Appalachia, we must set out from the start to save the full complement of native species. Nothing less will do.”

George Constantz
Hollows, Peepers & Highlanders (2004)

DEDICATION

This work is dedicated to my parents, George and Penny Graham, who have remained supportive of my sometimes crazy ventures in academia. Whether it was collecting venomous scorpions from cactus-littered desert floors, cross-country trips in exponentially colder weather than they're used to, or listening to me whine about an organic chemistry exam, they stood by. Mom and Dad, I couldn't have done it without you. This thesis is dedicated, with love, to you.

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I want to thank Dr. Guo-Zhang Zhu for friendly advice regarding molecular techniques and for kindly serving on my committee.

Billy Flint of James Madison University provided helpful Cow Knob salamander expertise and contributed tremendously to this project. I owe Billy much gratitude for his enthusiasm and willingness to collaborate, and for many hours of hard work surveying and collecting tail tips.

While doing field surveys in the remote mountainous corners of West Virginia, I was fortunate enough to enjoy the company of several assistants. Josh Greenwood, one of my greatest friends in WV, spent many hours camping and hiking to isolated salamander locations with me. Ashley Fisher nearly froze to death on an icy cold and rainy day (perfect salamander conditions) while collecting Cow Knob salamanders on Shenandoah Mountain. My girlfriend Jessica Casto, and our trusty sidekick Fajita, braved the West Virginia woods to assist me as well. Jessica and Fajita also kept me sane during the long hours spent writing this thesis and all the associated papers that go with a project like this. I also owe thanks to Jessica's parents, Vernon and Cindy Casto for

graciously letting us use their beautiful cabin in Randolph County, one heck of a field station.

Ally Hogsett, contributed a lot of time meticulously carrying out many of the DNA extractions, amplifications, and purifications.

Mike Brewer and Cassie York kept me up-to-date on the utility of Dr. Fet's DNA lab. Mike, a molecular master, always had valuable advice on molecular techniques and problem solving when things went wrong.

My friend and roommate Conor Keitzer engaged me in interesting conversation and brainstorming that ultimately contributed significantly to this study.

Most of the funding from this project was provided by the West Virginia Division of Natural Resources. Additional support was provided by a gracious donation to the graduate college by Dave Haden. Thank you Dave, your kind contribution made my life a lot easier while working in the field.

Last but not least, many thanks of course go out to the Marshall University Herpetology lab.

Table of Contents

List of Figures

FIGURE

List of Tables

TABLE

Chapter 1

Introduction

Species histories are often greatly influenced by geomorphological changes such as mountain building, fluctuations in sea level, removal of barriers to dispersal, and the coming and going of embayment events. It is occurrences such as these that fabricate the genetic structure of populations across a landscape, and with modern genetic tools it is now possible to infer much of the history of a lineage and then compare it against known geological change (Avise, 2000; Brunsfeld *et al*., 2001; Jockusch & Wake, 2002; Kuchta & Tan, 2006). Along with new genetic techniques, novel or existing statistical tools and more powerful computers have made it even easier to resolve lineage structure across landscapes.

For instance, in the Pacific Northwest the genealogic structure of the Pacific giant salamander, *Dicamptodon tenebrosus* (Baird & Girard, 1852) (family Dicamptodontidae Good & Wake, 1992) based on mitochondrial DNA (mtDNA), revealed distinct north and south clades. These clades were shown to coincide with two large river valleys, which based on molecular clock estimates probably represents mid-Pleistocene refugia (Steele & Storfer, 2006). In another interesting example based on mtDNA sequence analysis, three deep historical subdivisions within a species were revealed by the genetic structure of black ratsnakes (*Pantherophis* sp. – Utiger et al., 2002; family Colubridae Oppel, 1811) (Burbrink et al., 2000; Gibbs et al., 2006), in eastern North America. The three

clades, or ‘phylogroups’, are arranged longitudinally into eastern, central, and western groups. Western and central groups diverged allopatrically due to vicariance caused by the Mississippi River. Eastern and central clades similarly diverged but separation was instead due to a combination of barriers to gene flow. Most of the species’ range in the east was bifurcated by the Appalachian Mountains. However, black ratsnakes also occurred throughout the southeastern coastal plains which lacked the Appalachian Mountain barrier, and vicariance was instead associated with a large embayment event. During the interglacial periods of the Pliocene and Pleistocene, a large bay formed along the Apalachicola River and formed a contiguous barrier with the mountains, thus completely restricting gene flow among eastern and central populations. Studies such as these, in which gene genealogies are interpreted in light of past geographic and paleoclimatic processes are part of a rapidly growing discipline called ‘phylogeography’.

Phylogeography is really just the amalgamation of phylogenetic and biogeographic principles, used together to answer evolutionary questions. The so-called ‘father of phylogeography,’ John C. Avise, defines it as “the study of the processes controlling the geographic distributions of lineages by constructing the genealogies of populations and genes” (Avise, 2000). The primary aim of phylogeography is to use geographic and genetic data to address the relative roles of historical forces in shaping population structure (Cruzan & Templeton, 2000), usually by inferring past events such as range expansion, vicariance, isolation by distance, migration, and bottleneck events.

In addition to answering important evolutionary questions, phylogeography can also be used to aid in conservation efforts. It is well known that amphibian species in particular are experiencing considerable declines across the globe (Lannoo, 2005). Fortunately, many programs (i.e. North American Amphibian Monitoring Program - NAAMP; Terrestrial Salamander Monitoring Program – TSMP) have been initiated in attempts to ameliorate these declines, and more importantly, to prevent extinctions and further loss of biodiversity. As additional aid to these efforts, many phylogeographic studies of amphibian species have recently surfaced (Alexandrino et al., 2002; Eggert et al., 2006; Gabor & Nice, 2004; Kozak et al., 2006; Kuchta & Tan, 2006; Nielson et al., 2006; Sites et al., 2004; Templeton et al., 1995; Weisrock & Larson, 2006;), many of which directly establish conservation units and provide important recommendations regarding the management of species. Populations are often prioritized in phylogeographic studies based on inherent levels of genetic diversity, giving land managers clear goals and direction for conservation efforts. By assessing population structure across a geographical element, phylogeography can identify groups of closely related individuals, quantitate the genetic variability within and between clades, and assign conservation priority to clades based on the prior two analyses, usually in the form of ESUs and MUs (see Conservation Units, section 2.4).

The principal aim of this study was to do just that. I used molecular data in the form of mitochondrial DNA sequences to investigate the genetic structure, at the population level, of the Cow Knob salamander, *Plethodon punctatus* Highton, 1972 (Fig. 1). The Cow Knob salamander is a sensitive species (S1 in WV) with

a small and narrow range and is found only at high elevations in a select few mountains within the Ridge and Valley physiographic province of West Virginia and Virginia (Petranka, 1998). Surveys conducted during this study shed new light on the distribution of *P. punctatus* and genetic analyses suggest how clades within the species should be managed. For those not familiar with the taxa, conventions, or geography examined in this study, I have included a brief overview of such topics (see Background, chapter 2). As a corollary, and based mostly on anecdotal evidence, I suggest my own ideas about possible mechanisms that drove the speciation of this fascinating woodland salamander (Discussion section 5.3).

Figure 1: A Cow Knob salamander, *Plethodon punctatus*, in its natural habitat.

Chapter 2

Background

2.1 *Plethodon* in West Virginia

The genus *Plethodon*, with 55 valid species, comprises a group of North American lungless salamanders of the family Plethodontidae, the largest group of extant salamanders. Members of the genus can be identified by the presence of 4 toes on the front and 5 on the hind feet and a round or oval tail with no constriction at the base. Males usually have a broader head and longer tail than females (Stebbins, 2003) and a more flattened snout (T.K. Pauley, pers. comm).

All *Plethodon* species are nocturnal and adapted to an entirely terrestrial life cycle in woodland habitat; thus they are often called 'woodland salamanders'. During hot, dry weather they either estivate or seek moisture in crevices or underground refugia (Conant & Collins, 1998). Eggs are laid in moist logs or subterranean chambers where they spend their larval stage within the aquatic environment of the egg capsule (Green & Pauley, 1987).

Three major groups of related species exist within the genus *Plethodon*, the western plethodon, the eastern small plethodon, and the eastern large plethodon (Highton, 1962). Members of two of these groups, the eastern small plethodon and the eastern large plethodon, occur in West Virginia. The eastern small plethodon group in West Virginia consists of six species; *Plethodon cinereus*, *P. nettingi*, *P. electromorphus*, *P. richmondi*, *P. hoffmani* and *P. virginia.* The

eastern large plethodon group is made up of five species in the state; *P. glutinosus*, *P. cylindraceus*, *P. kentucki*, *P. wehrlei* and *P. punctatus*. These five species can be identified by various characters provided in Table 1.

This study focused on a member of the eastern large plethodon group in West Virginia. Representatives of this group can be found in every county in the state (Fig. 2), yet certain species have somewhat limited ranges. *Plethodon kentucki*, for example, is restricted to the southwest corner of West Virginia while *P. cylindraceus* and *P. punctatus* are confined to the eastern panhandle. *Plethodon glutinosus* and *P. wehrlei*, on the other hand, have much larger ranges in West Virginia and can be found throughout the large central portion of the state.

Morphologically, these salamanders are somewhat hard to distinguish and it was not until advances in molecular biology techniques that some were even described. Electrophoretic and immunological analyses (Highton, 1989) of genetic variation split one group, the *P. glutinosus* complex, into 16 distinct species. Three of these, *P. kentucki, P. glutinosus* and *P. cylindraceus*, are members of the eastern large plethodon group in West Virginia. It should be noted, however, that many researchers (Frost & Hillis, 1990; Tilley et al, 1990; Petranka, 1998) fail to recognize 13 of the species resulting from the split and consider them all to be *Plethodon glutinosus*. They argue that using genetic distance as the primary criterion for recognizing allopatric or parapatric species assumes that genetic distance tightly correlates with the development of reproductive isolating mechanisms. They suggest that this might not be true in plethodontid salamanders and object to splitting species based solely on

arbitrarily selected genetic distances. Under these criteria, one of the species in West Virginia, *P. cylindraceus*, would not be valid. Conversely, my own morphological investigation (unpublished report) showed *P. cylindraceus* to be morphologically distinct from *P. glutinosus* and *P. kentucki*. I used principal component analysis and canonical discriminate analysis to analyze quantitative morphological characters of the eastern large plethodon of West Virginia, and found all three *glutinosus* complex species to be morphologically, albeit slightly, distinct.

The species investigated in this study is *Plethodon punctatus*, a member of the eastern large plethodon group. This species is the most sensitive of the eastern large plethodon in West Virginia and is listed by the state West Virginia Division of Natural Resources as a species of concern. More specifically, the agency ranks it as an S1 species, meaning that it is considered extremely rare and critically imperiled, or because of some factor(s), it is especially vulnerable to extirpation.

2.2 The Cow Knob Salamander

Two species of eastern large plethodon in West Virginia are not part of the *P. glutinosus* complex. Rather, they group together in their own, the *P. wehrlei* complex. *Plethodon punctatus* and *P. wehrlei* (Fig. 3) are the only two members of this group and can be distinguished from each other by the modal number of trunk vertebrae and color pattern. Most *P. wehrlei* have brassy flecking on the dorsum, have young with orange dorsal spots on the shoulders, and possess 18

modal trunk vertebrae. Neither the orange spots nor the brassy flecking have been observed on the dorsum of *P. punctatus*, which has 19 modal trunk vertebrae (Highton, 1972). Superficially these species can sometimes be identified by their slight difference in dorsal base color as well; *P. punctatus* is usually dark and almost black where *P. wehrlei* has a lighter base color and appears more grayish. None of these characters are completely diagnostic, however, as variation in each species causes overlap. For instance, the number of costal grooves ranges from 17-19 in *P. wehrlei* and 18-19 in *P. punctatus*. Overlapping characters like this make it markedly difficult to identify these two species based on morphology and color patterns alone. Therefore the treatment of this group as two distinct species has been debated.

On the contrary, biogeographic and genetic evidence suggests that *P. punctatus* were derived from ancestral *P. wehrlei* stock that became geographically isolated from the main population (Highton, 1972). Highton (1995) hypothesized that the dry periods of the Pliocene probably isolated many populations due to reduction of forests at lower elevations, which led to allopatric speciation. Then, once the wetter climates returned, *P. punctatus* only survived in their restricted mountain habitat, and now have a very limited range.

Unfortunately, Highton (1995) didn't mention a possible mechanism for his allopatric speciation theory or why Cow Knob salamanders remained in their high-elevation mountain refugia. His estimate of time since divergence of *P. punctatus* and *P. wehrlei* is that it occurred in the Pliocene epoch, which on a geologic timescale extends from 5.332 million to 1.806 million years before

present. One goal of this study is to provide a more accurate molecular clock estimate of the species split, thus testing Highton's hypothesis.

Cow Knob salamanders have been documented to be restricted to a strict set of habitat parameters (Tucker, 1998). They are all generally found above 2,500 feet with most being found above 3,000 feet, and prefer the cool and moist habitats of north-facing aspects, where they emerge and forage when humidity reaches 100% (Fig. 4). Cover objects, especially rocks (Fig. 5), are important for identifying habitat, and they exist in 10 different forest types. They occur with greatest densities in Oak-Birch-Hemlock forests, much of which is fragmented by forest service roads (Fig. 6).

2.3 Distribution of *P. punctatus*

In his original description of *P. punctatus*, Highton (1972) had to rely solely on his own records to estimate the distribution of the species. He collected 11 individuals which he designated as types (USNM 190224-190234), and examined specimens from 8 sites; 7 of which were from Shenandoah Mountain, the other from Great North Mountain (Fig. 7). Since the ridges of these mountain ranges form the border between West Virginia and Virginia, Highton reported the species as occurring in 5 counties despite its small range: Pendleton and Hardy counties in WV, and Augusta, Rockingham [*sic* - MRG], and Shenandoah counties in Virginia. He admits, however, that field surveys at the time were not sufficient and that populations on Shenandoah Mountain are possibly isolated from those on Great North Mountain (Highton, 1972).

Over 20 years passed before the first intensive surveys were finally conducted for *P. punctatus*. In 1995, Thomas Pauley (Marshall University) and his graduate students surveyed the West Virginia portion of the George Washington National Forest for the species. In all they discovered 42 *P. punctatus* at 16 different sites. All 42 specimens, however, were observed on Shenandoah Mountain, and none were discovered on Great North Mountain, despite their efforts. All were found at elevations above 3,100 feet, mostly from north-facing aspects (Pauley, 1995).

The following year, one of Pauley's graduate students, Robert Tucker, conducted surveys for *P. punctatus* under Hemlock stands within the George Washington National Forest (Tucker, 1996). He observed 22 *P. punctatus* at 9 sites. Tucker's results didn't expand the known range of *P. punctatus* but they did increase the known elevational range since individuals were observed down to 2,950 feet in elevation; 150 feet lower than previously encountered.

Tucker went on to complete a master's thesis on the Cow Knob salamander at Marshall University, during which he studied the natural history and ecology of the species (Tucker, 1998). He found 122 individuals and expanded the elevational minima to 2,540 feet. Tucker also discovered that *P. punctatus* are considerably more abundant at higher elevations and found a strong correlation between north-facing aspects and density, supporting the results of Pauley (1995).

Concurrently, Pauley conducted more surveys for *P. punctatus* in West Virginia, this time outside of the George Washington National Forest (Pauley,

1998). Much of the land he tried to survey turned out to be private, so he and his graduate students were mostly limited to sites open to the public. He discovered and searched 17 sites from 1997 to 1998 and found *P. punctatus* at only two of them. Both new sites were in Hardy County (one on Shenandoah Mountain near the Pendleton county border and one on Helmick Rock near Lost River State Park) and expanded the known range of the species to the northeast.

In the most recent work with Cow Knob salamanders, William Flint from James Madison University, completed another thesis on this species and added even more to our knowledge of its distribution (Flint, 2004). Flint and his assistants added an astonishing 215 presence/absence location records. He focused on the southern and eastern regions of the range (the Virginia side) and found several new localities. More importantly, Flint extended the range 6.5 km south along the Shenandoah Mountain ridgeline and provided the most accurate distribution maps to date (Fig. 8).

Overall, what started out as a distribution roughed out by 8 documented sites, has turned into a much clearer, albeit not perfect, picture of the Cow Knob salamander's true geographic range. Several individuals, mostly from unaffiliated institutions, sometimes collaborating and sometimes not, built a nice foundation for the potential distribution. It stood out to me, however, that much was still left uncertain. For instance, nobody had since verified the Great North Mountain site published by Highton in his original description of the species. Furthermore, problems with access to private land in the west, and still

insufficient surveys in areas not accessible by roads leave a lot of this species' true range unknown.

Part of the purpose of this study was to address the abovementioned issues and to provide additional insight to the actual distribution of this unique and sensitive species. Understanding where a rare species like *P. punctatus* resides across a landscape is crucial, and probably the most important step towards implementing plans for its conservation.

2.4 Conservation Units

With the intention of discussing species' survival programs, a group of conservation biologists met at the Zoological Society of Philadelphia in 1985, and tried to construe new ways to identify populations that possess genetic attributes important to the preservation of present and future generations of species. The emphasis of the meeting was on subspecies, and the problems with taxonomy of the time in determining which subspecies actually represented legitimate examples of adaptive variation. It was decided, however, that subspecies should not be the issue and that conservationists ought to instead address *evolutionarily significant units* (ESUs) within species (Ryder, 1986). Essentially, as outlined by Ryder (1986), the idea was that ESUs should be established when differing techniques produce concordance among data sets. Geographic distribution data, such as that which indicates discrete geographical populations, could therefore be used in conjunction with genetic techniques to assess which groups or gene pools are traveling though evolutionary time independently.

The ESU acronym quickly caught on and in 1994 was given attention by the now esteemed University of California, Berkeley professor, Craig Mortiz. In a 1994 paper, he points out some potential shortcomings of the ESU idea; like the important fact that preserving variants adapted to previous conditions may counteract current natural selective pressures, thus negating the evolutionary process. Moritz (1994) also strengthened the utility of ESUs by defining new criteria that all factions defined as so must meet. Chiefly, ESUs "should be reciprocally monophyletic for mtDNA alleles and show significant divergence of allele frequencies at nuclear loci." Reciprocally monophyletic groups are clades of related individuals or haplotypes that do not contain any portion of the other (see Fig. 9); a product of long term restriction of gene flow. Moritz (1994) admits that his criteria seem overly restrictive and dealt with this by contrasting ESUs to '*management units*.' These management units, or MUs, were noted as fundamental for the management of the more inclusive ESUs. Moritz defined MUs with less constraint, as populations with significantly divergent alleles at either nuclear or mitochondrial loci. Therefore, according to Moritz (1994), ESUs deal with the history of population structure and MUs address current structure, making them more useful in addressing short-term management issues.

In the most recent and in-depth discussion about ESUs and MUs, Crandall et al. (2000) critically reanalyzed the Moritz definitions and pointed out a few flaws. The main problem with Moritz's rationale is that functional divergence does not always necessitate a long history of isolation. In fact in many instances speciation can occur even among sympatric species (see Dieckmann & Doebeli,

1999; Doebeli, 1996; Hedin, 1997; Turner & Burrows, 1995). Crandell et al. (2000) propose a different approach, this time taking into account both “genetic exchangeability” and “ecological exchangeability,” or the idea that individuals can occupy the same ecological niche. The shortcoming of this method is that the null hypothesis of ecological exchangeability should be tested using the statistical procedures of Templeton & Sing (1993), which requires broad knowledge of ecological life history in the form of quantitative data. Such data are usually not readily available or realistically obtainable for many species.

There is debate over which concept to use, but due to the lack of ecological genetic data for the Cow Knob salamander, the criteria of Crandell et al. (2000) could not be used in this study. Instead, like other authors of similar salamander studies (Miller et al., 2005; Pabijan et al., 2005), I use the Moritz criteria of reciprocal monophyly for ESUs and divergence of mtDNA loci for MU designation, with the overall ambition of defining units helpful to the maintenance of genetic diversity. Conservation units, in the form of MUs, are defined but also prioritized based on the amount of genetic differentiation, under the assumption that the loss of a more diverse population would be more detrimental to the conservation of the species as a whole. This is a crucial step in management, since the loss of diversity at the genetic level, often coupled with increased genetic load, diminishes the long-term prospects of populations (Frankham, 2003; Row & Beebee, 2003) and may lead to extinction (Saccheri et al. 1998).

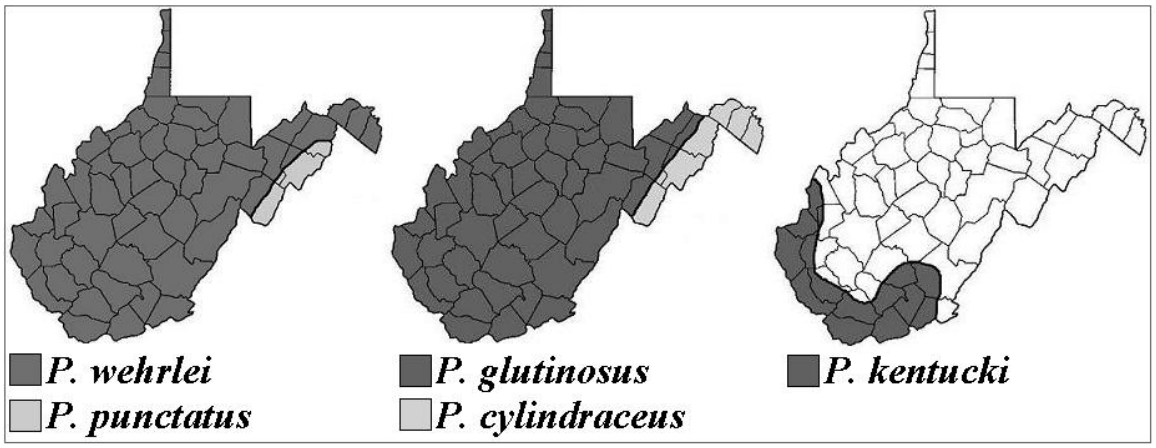

Figure 2: Rough estimates of the distribution of the eastern large plethodon in West Virginia (based on Petranka, 1998; Pauley, pers. comm.).

Figure 3: *Plethodon wehrlei*. Note the grayish dorsal color, as opposed to the dark black back of *P. punctatus*.

Figure 4: A Cow Knob salamander foraging during a rain. (photo courtesy of William D. Flint)

Figure 5: Typical cover object refuge of a Cow Knob salamander. Caliper placement to show scale.

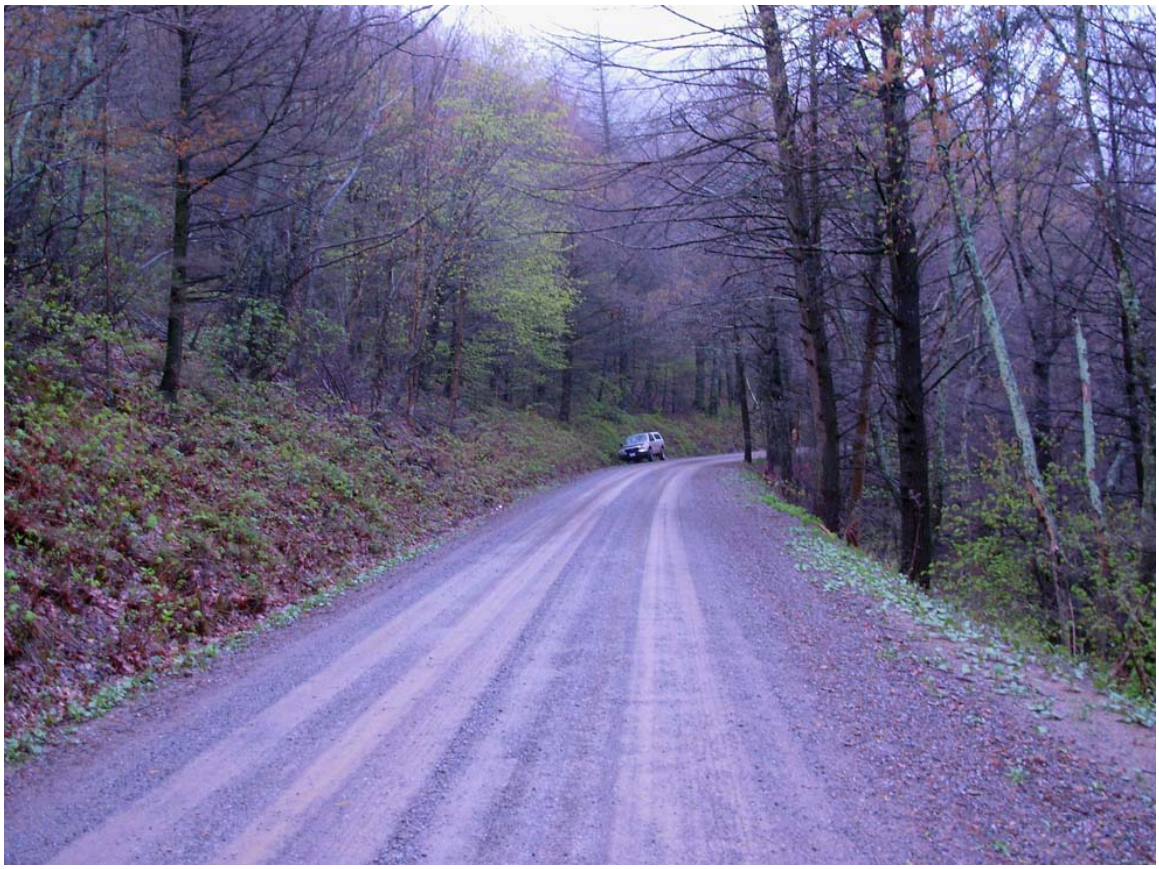

Figure 6: Example of a forest service road fragmenting ideal Cow Knob salamander habitat on the west side of Shenandoah Mountain, WV.

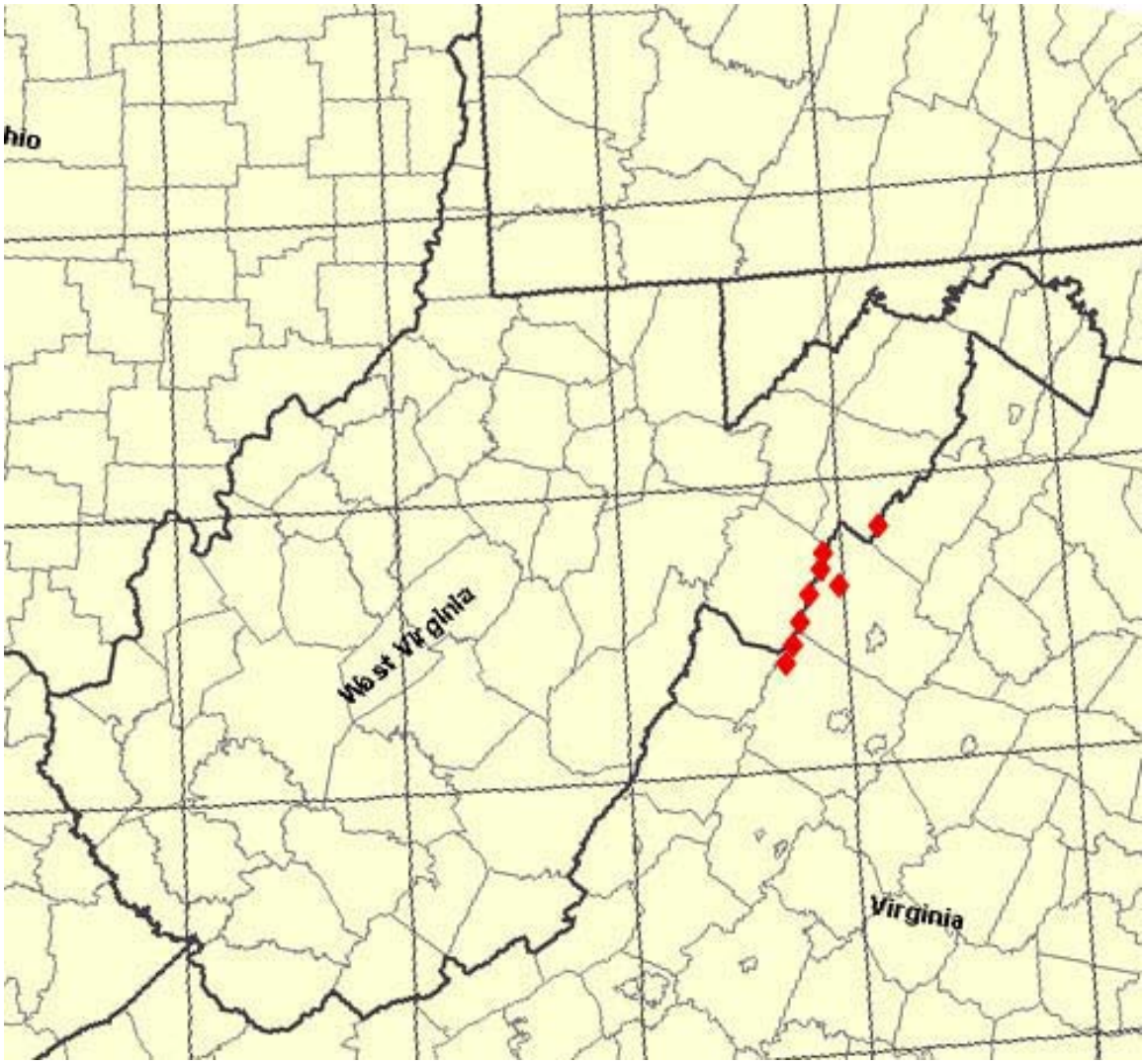

Figure 7: Originally reported distribution of *P. punctatus* as estimated by 8 sites (red diamonds) examined in the type description. (adapted from Highton, 1972)

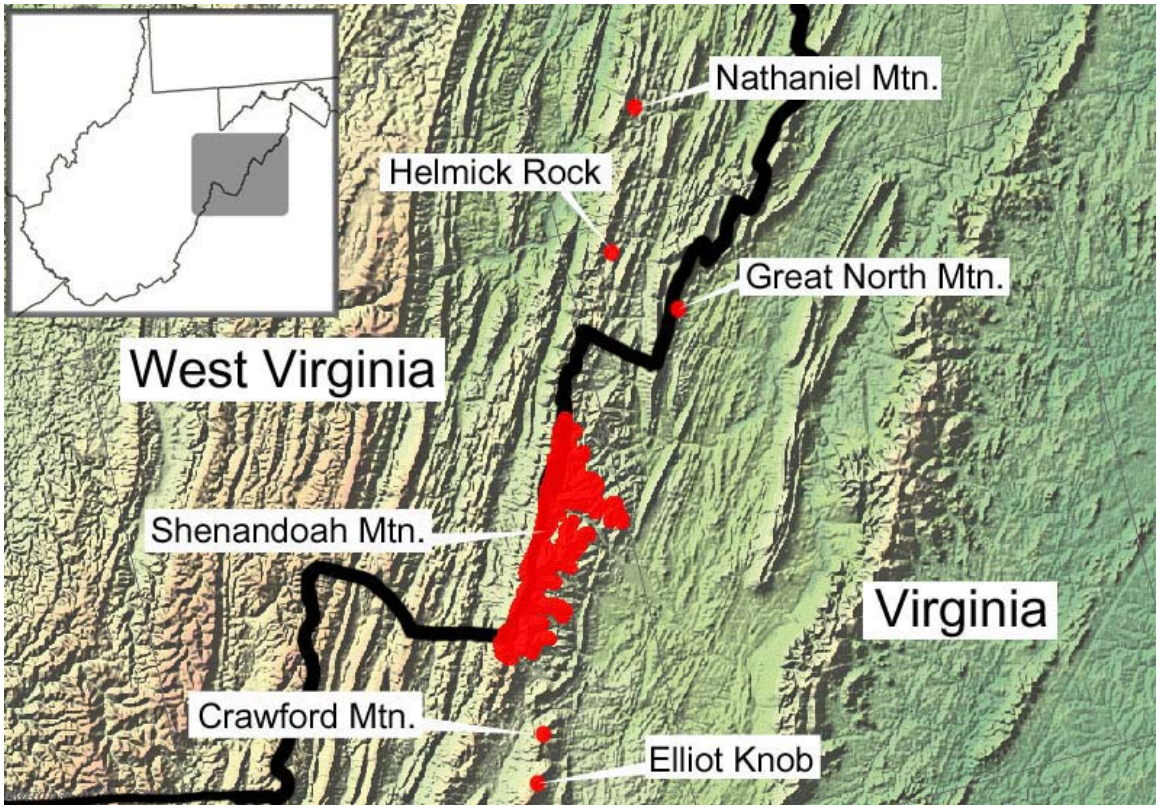

Figure 8: Known distribution of *P. punctatus* after Flint (2004). At the time, most records besides that of the main population had not been verified.

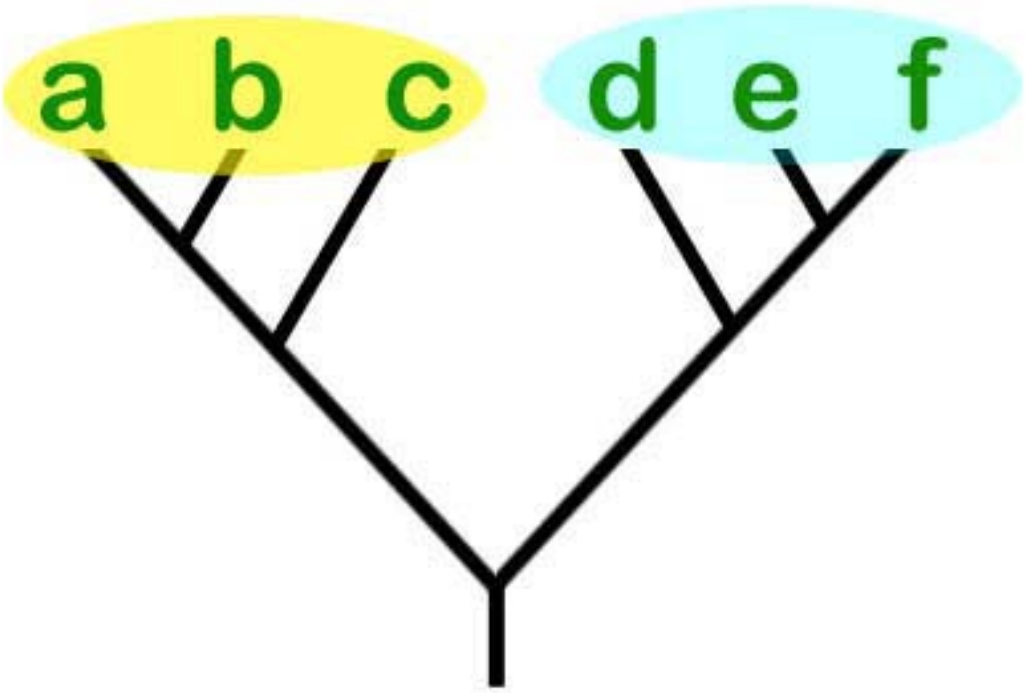

Figure 9: An example of reciprocal monophyly. According the criteria of Moritz (1994), clades *abc* and *def* could represent evolutionarily significant units (ESUs).

Table 1. Key to the eastern large *Plethodon* of West Virginia (from an unpublished report by the author)

1. Strong webbing on feet…….*wehrlei* complex (2)
   Feet with no webbing to moderate webbing……(3)
2. Brassy flecking dorsally, red spots dorsal to shoulders or medium to small white spots concentrated laterally…….……***wehrlei***
   Lack of brassy dorsal flecking, no red spots and medium to large white spots, with most concentrated laterally with a few large spots on back ……....***punctatus***
3. Throat light……..……(4)
   Throat dark…….……***glutinosus***
4. Small white spots concentrated mostly on sides…….***kentucki***
   Large white dorsal and lateral spots ……..***cylindraceus***

Chapter 3

Methods

3.1 Geographic and Taxonomic Sampling

All sampling was conducted at sites within the Ridge and Valley physiographic province of the middle Appalachians in Virginia and West Virginia. The ridge and valley region consists of extensively folded and thrust-faulted Paleozoic strata that form level-crested ridges that run in a southwest-northeast direction (Stephenson & Saxena, 1994).

Survey techniques included visual encounter surveys, or VES (Flint & Harris, 2005; Crump & Scott, 1994), and daytime cover object surveys through ideal habitat. VES were conducted at night during or just after rain events by walking through habitat with flashlights. Daytime cover object searches involved turning over objects such as flat rocks and logs. Habitat searched was chosen by the following parameters specified by Tucker (1998); elevation (>2,500'), aspect (preferably north-facing), cover objects present, and major vegetation type (Oak-Birch-Hemlock overstory preferred).

When a specimen was found, latitude and longitude were recorded with a Garmin Etrex Legend™ GPS unit and the animal was placed in a Ziploc™ bag and carried back to the survey vehicle for data collection. Individuals were identified as either male, female, or juvenile based on size and presence/absence of a small circular gland at the distal tip end of the chin called

the mental gland. Size was recorded by using calipers to measure snout to vent length (SVL), measured from the tip of the snout to the posterior margin of the vent (Petranka, 1998). Tips of the tails, 10 to 20 millimeters in length, were removed with sterile razorblades and immediately placed in a 1.5 mL Eppendorf™ containing 95% ethanol. Tail tips were kept on ice until they were returned to the lab at Marshall University where they were stored in a freezer at minus 20 C.

To maximize efficiency, I collaborated with another researcher, William Flint, who was also conducting surveys for Cow Knob salamanders. Flint focused his searches, using primarily VES, on putative disjunct and unverified populations and conducted extensive surveys in Virginia and the extreme southern portion of the range. My time, as well as that of my various assistants, was spent mostly doing daytime cover object searches in potential habitat west of the known range.

Surveys conducted by my party are cataloged into three separate units; the main population on the Shenandoah Mountain ridgeline, Great North Mountain ridgeline, and western ridges and knobs. Our Shenandoah Mountain ridgeline surveys included sites along the ridge of Shenandoah Mountain that forms the border between West Virginia and Virginia. These were conducted on the western side of the ridge (the WV side) in Pendleton and Hardy counties in areas where *P. punctatus* were already known to occur, running from U. S. Forest Road 85 near Ugly Mountain northeast to Helmick Rock.

The Great North Mountain ridgeline portion of our surveys included areas west of the ridgeline that comprises the WV/VA border in Hardy County. We searched several sites as far south as County Road 20 northeast to Long Mountain and Big Schloss.

The western ridges and knobs region posed the greatest challenge for my survey team. This region consists of areas of high-elevation west of the known populations on Shenandoah Mountain. Most of this area is private land and an unusual amount of landowners posted no trespassing signs around their property, but we searched as many accessible areas as possible. This included sites as far west as North Fork Mountain, as far north as Spring Mountain near Grant County, and south to the borders of Highland and Augusta Counties, VA.

Working together with Flint, our efforts made for an intensive survey of the entire known range of this species. Since *P. punctatus* is such a sensitive species, the exact locations of survey sites will not be revealed. West Virginia specimens were collected under scientific collecting permit number 2006.063 issued by the West Virginia Division of Natural Resources.

3.2 DNA Extraction, Amplification, and Purification

In the lab, tissue samples (10-20 millimeters tail tips) were sliced into small fragments by hand with sterilized razor blades. Genomic DNA was then isolated from the tissue samples using a Qiagen DNeasy™ extraction kit and protocol. A fragment of the mitochondrial gene encoding 430–450 base pairs of LSU (large ribosomal subunit) 16S rRNA, was amplified by polymerase chain reaction

(PCR). The gene fragment was targeted using the universal primer 16Sbr, or LR-J-12887, with a reverse primer designed by Victor Fet, known in the lab as '40'; both were used in a number of phylogeographic and phylogenetic studies (see Fet et al., 2002; Teruel et al., 2006).

I attempted to amplify another gene as well, cytochrome *b* (a subunit of coenzyme Q – cytochrome c reductase, a transmembrane lipoprotein involved in cell respiration), but ran into problems with amplification. Essentially the gene appeared to amplify, but non-specific priming of the cytochrome *b* primers, as evident by the appearance of numerous bands on agarose gels, made most of these results insufficient to sequence. Several techniques could have been employed to deal with the non-specific priming problem (such as nested PCR or gel extractions), but due to time and financial limitations I focused my work on the 16S gene which yielded excellent results nearly every time. Primers used to attempt amplification of cytochrome *b* are L14841 (Table 2) and H15149 after Kocher et al. (1989) and several salamander studies (Caccone et al., 1997 ; Murphy et al., 2000; Shunqing et al., 2004; Riberon et al., 2001; Riberon et al. 2002).

A Perkin Elmer Genamp ® PCR 2400, version 2.10 thermocycler was used to amplify the 16S rRNA gene fragments using a standard protocol as outlined in Table 3. Positive PCR products were verified using a 1% agarose electrophoretic gel (Fig. 10) and then purified with a Quiagen™ purification kit. Automated Sanger dideoxy sequencing of the double-stranded PCR product was

conducted at the Sequencing and Services Facility, University of Georgia (Athens, GA), on the ABI 9600 Sequencer.

3.3 Phylogenetic analysis

DNA sequences were initially aligned using ClustalX 1.81 (Thompson et al., 1997) and then further aligned by eye (Fig. 11). The alignment consisted of 383 aligned positions (bases) which was saved as a Nexus (*.nex) output file compatible with PAUP* 4.0.

Maximum parsimony (MP), neighbor-joining (NJ), and unweighted pair group method with arithmetic means (UPGMA) analyses were implemented in PAUP* 4.0b10 (Swofford, 1998). Maximum parsimony searches were conducted with all characters equally weighted and employed the heuristic search option. Gaps were treated as “missing” for all analyses. Non-parametric bootstrapping was used to assess nodal support (Felsenstein, 1985) with 1,000 replicates. Transition to transversion ratio (TR:TV) was set to 3:1 and DNA sequence data from *P. wehrlei* was used to root the trees.

Since traditional phylogeny reconstruction, such as parsimony, neighbor-joining, and maximum-likelihood make assumptions that are sometimes invalid at the population level (Clement et al., 2000), I have also used a haplotype network approach called TCS, or statistical parsimony. This method calculates the probability of parsimony and is often used to infer population level genealogies. TCS algorithms, as outlined in Templeton et al. (1992), were implemented using the TCS version 1.21, a free program available online

(http://darwin.uvigo.es/software/tcs.html). The maximum number of steps that haplotypes can differ from each other was estimated using a 95% confidence limit. Gaps were treated as a fifth base pair.

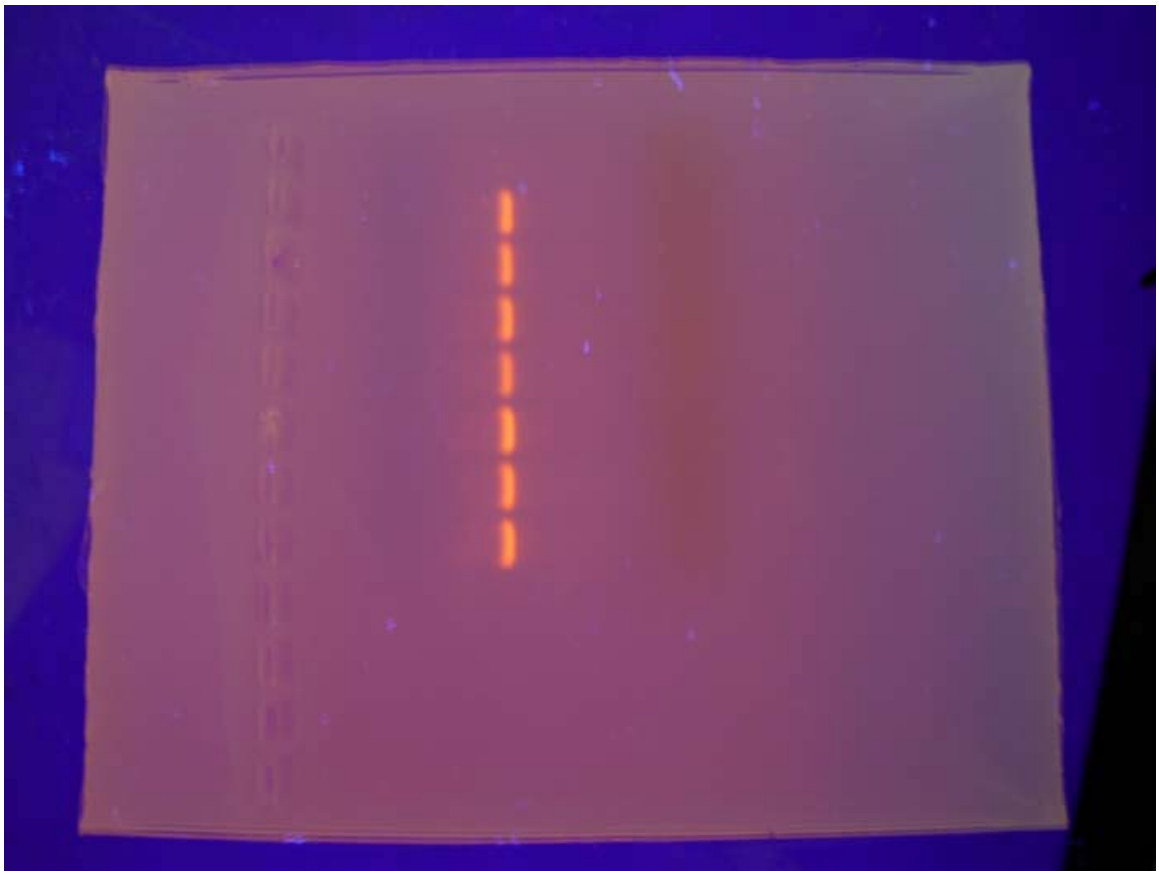

Figure 10: Agarose gel showing 16S rRNA PCR products.

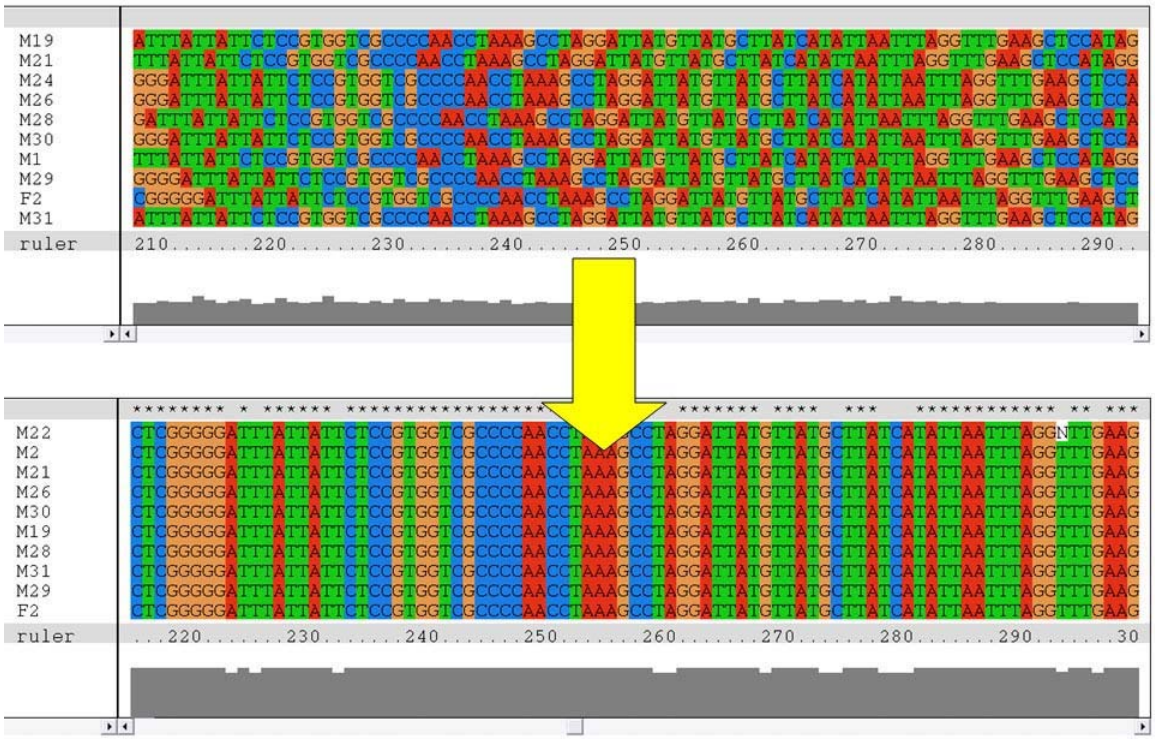

Figure 11. Clustal X interface demonstrating a multiple DNA sequence alignment.

Table 2. Primers use to amplify mitochondrial cytochrome *b* and 16S rRNA genes.

Cytochrome *b*
Forward (L14841): 5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3'
Reverse (H15149): 5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3'
16S rRNA
Forward (16Sbr): 5’-CGATTTGAACTCAGATCA-3’
Reverse (40): 5’-GTGCAAAGGTAGCATAATCA-3’

Table 3: Protocol used to amplify 16S rRNA gene fragments.

	Time		Temperature
Denaturing	0:45		94
Annealing	0:45		50
Extension	0:45		72
Pre-cycling	5 :00		94
Cycles		32	
Post-cycling	~		72

Chapter 4

Results

4.1 Distribution and Survey Results

William Flint, I, and our assistants conducted surveys spanning the entire known range and most of the potential range of the Cow Knob salamander from early April to September of 2006. Our searches were most productive during the rainy month of May, but many specimens were found throughout the other summer months as well. To minimize impact on *P. punctatus*, the two survey parties only collected 20 tail tips each, even though many more individuals were captured.

Graham surveys

Several assistants and I surveyed intensively for *P. punctatus* during May and June, 2006. We searched 33 sites in 3 counties in West Virginia, Pendleton, Hardy, and Hampshire. Of these, *P. punctatus* were observed at 9 sites. Much time was spent attempting to access remote sites or sites on private land and we were never able to obtain access to an additional 17 sites.

We observed 33 *P. punctatus* from the 9 sites, of which we measured 31 and collected tail tips from 20. At least 1 but no more than 3 tail tips were collected from each site. Twenty-six measured individuals were mature adults, comprising

11 males and 15 females. Males tended to be longer with a mean SVL of 66.5 mm, while females averaged 61.2 mm SVL (Fig. 12).

All but one site where we discovered *P. punctatus* were along the Shenandoah Mountain ridgeline. This included 3 sites along the southern end of the ridgeline, 5 sites located centrally around High Knob and Cow Knob, and no sites along the northern extent of the ridge. Several attempts were made to obtain specimens from the northern locations, especially Helmick Rock, but conditions were usually rather dry. The sites in this region where *P. punctatus* were observed were all at or near sites where they have historically been found, so our records there did not expand the species' range.

Several trips were made to the Great North Mountain in attempt to verify Highton's record of individuals from the area in his 1972 type description of *P. punctatus*. We searched three sites east of Basore, and several areas of ideal habitat near Long Mountain and Big Schloss. Despite our extensive surveys there, which even included long hikes into remote expanses, no individuals were observed.

In the western ridges and valleys region we had the opportunity to be the first ever to search many areas for Cow Knob salamanders. Most of the region consisted of private property, but among the sites searched we only verified the presence of *P. punctatus* at one. This was surprising because at many sites that seemed like they should have Cow Knob salamanders, we found none and often could not find many salamanders at all. Examples of this are Ant Knob near the border of Hardy County, and Heavener Mountain just north of Route 33. Both of

these are high-elevation peaks (>2,800 feet), or knobs, located directly adjacent to Shenandoah Mountain where *P. punctatus* populations reside. Both locations are remote and require steep uphill hikes to reach, and are thus relatively untouched by human activity, yet no Cow Knob salamanders were found there.

In stark contrast to these knobs is a site where *P. punctatus* were found, Jack Mountain. Jack Mountain is a ridgeline running parallel to Shenandoah Mountain but set 12.5 miles to the west and bifurcated by several low-laying valleys where Cow Knob salamanders can not survive. The area includes high peaks, such as Pine Tree Knob at 3,196 feet, but these peaks were inaccessible. Instead, I was forced to survey a low section of Jack Mountain on its eastern side, the slope of Botkin Ridge at only 2,850 feet in elevation. The first attempt at this site proved futile. During a second attempt right after a hard rain, however, three salamanders were observed, two of which were captured. Upon inspection, they initially looked more characteristic of *P. wehrlei* (Fig. 13) but did possess some of the distinguishing features of *P. punctatus*. Tail tips were removed and after DNA analysis they turned out to in fact be *P. punctatus*, a new record that expands the range of the species 10 miles to the west.

Another exciting incident happened during our searches in a region of hills and ridges that run directly inline with Jack Mountain to the northeast. It was at Brushy Mountain, a peak 2,980 feet in elevation that requires a short hike, that we might have discovered another western population of *P. punctatus*. Two individuals were observed here directly after a strong rain under large flat rocks set into the soil. Unfortunately, before we could capture these animals, both

salamanders escaped down holes deep into the ground and could not be dug out. I observed these salamanders only briefly yet I feel that they could represent another population of isolated Cow Knob salamanders. Both individuals were large and looked just like the specimens found a few miles to the south on Jack Mountain.

Flint surveys

Efforts to find Cow Knob salamanders in Virginia were left completely up to my colleague William Flint. There, he and his assistants were able to expand the known distribution of *P. punctatus* 11 miles to the southwest and southeast with two new sites. In the southwest, Flint found populations on the west northwest face of Northeast Peak, and in the southeast he found populations at Elliot Knob.

Flint surveyed other areas as well, and like I, could not find any individuals on Great North Mountain. Flint did have more luck than I at Helmick Rock where he used VES to observe several *P. punctatus,* from which he removed two tail tips. Even further north northeast he verified unconfirmed reports of a population on Nathaniel Mountain. There he managed to find a single individual which appeared to him as "*wherlei*-like" in appearance (pers. comm.).

In all, Flint collected 20 tail tips from 13 different sites, many of which were used in the molecular genetics portion of this study.

4.2 Phylogenetics

Mitochondrial DNA sequencing of 23 individuals resulted in 7 different haplotypes that mostly corresponded to different geographic populations. Sixteen individuals from throughout the range of Shenandoah Mountain all shared the same haplotype, suggesting low genetic variability within the population. In contrast, only two individuals were sequenced from Helmick Rock and each possessed a distinct haplotype.

Phylogenetic reconstruction using an exhaustive search yielded a single MP tree and nearly identical NJ trees. The MP tree (Fig. 14) displayed clear geographic structure with the three northern haplotypes (Nathaniel Mountain and two Helmick Rock haplotypes) grouping together in a polytomic clade, and southern haplotypes (Shenandoah Mountain and Elliot Knob) also grouping together. Most interesting, however, is the Jack Mountain haplotype. The branching of this haplotype, from specimens originally thought to be *P. wehrlei*, was right between the northern and southern haplotypes, suggesting that the specimens actually represent another population of *P. punctatus*.

Six different neighbor-joining cladograms were examined based on the following distance measures: absolute, Kimura 2-parameter, Jukes-Cantor, F81, HKY85, and Tamura-Nei. All methods produced nearly identical trees so the Jukes-Cantor tree in Figure 15 is representative of them all. Most strikingly, in this phylogeny the Nathaniel Mountain haplotype is strongly separated from the other *P. punctatus* haplotypes, depicting a deep divergence between this population and all other Cow Knob salamander populations.

UPGMA of the 7 haplotypes produced a cladogram with similar results (Fig. 16). Two separate clades are clearly shown, one with northern haplotypes (Nathaniel Mountain and Helmick Rock) and one with Jack Mountain and southern haplotypes (Shenandoah Mountain and Elliot Knob).

Results of TCS analysis (Fig. 17) also show structure that corresponds to geography. The outgroup haplotype is 17 mutational steps from the Shenandoah Mountain haplotype. Northern haplotypes are several steps more divergent from the outgroup, with the Nathaniel Mountain haplotype the most divergent of all.

A genetic distance matrix of the 24 specimens used in this study is presented in Figure 18.

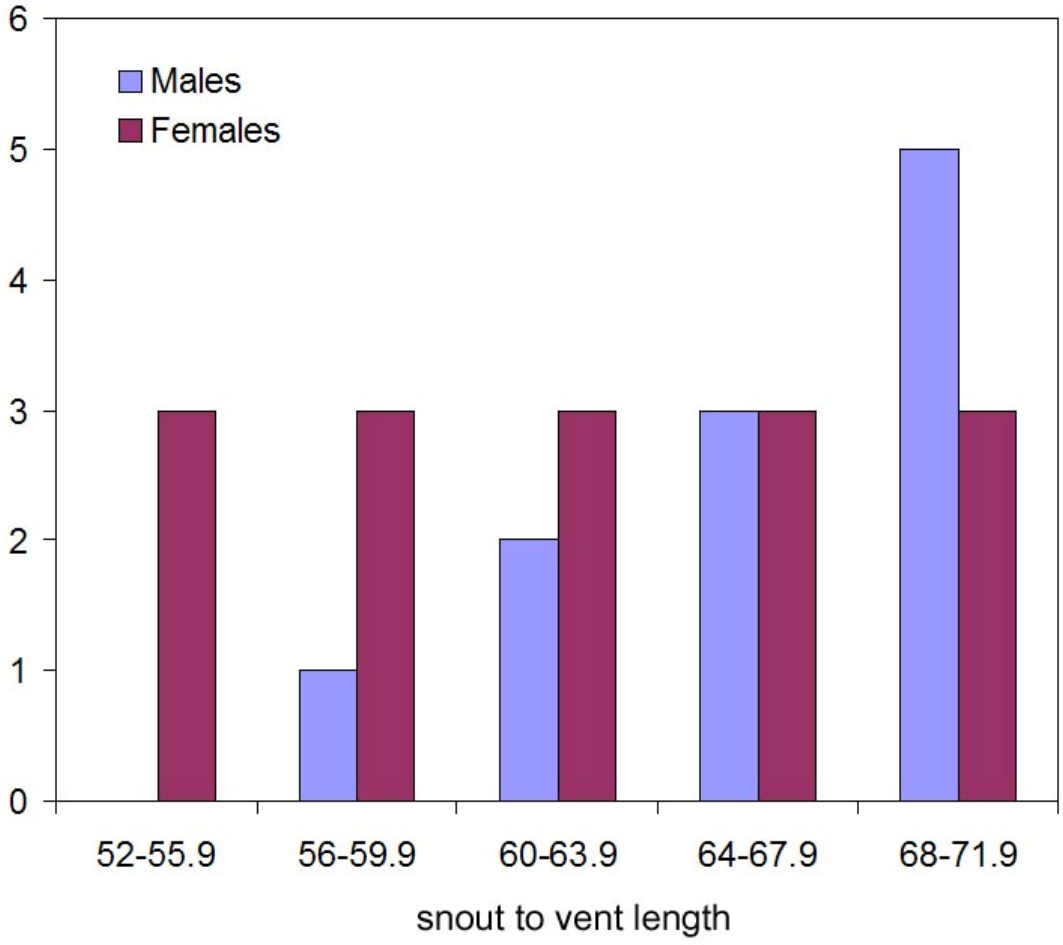

Figure 12. Snout to vent length (in millimeters) of *Plethodon punctatus* observed during 'Graham' surveys.

Figure 13: *Plethodon punctatus* from Jack Mountain, WV. Specimens from this locality looked very similar to Werhle's salamander, *P. wehrlei*, which is abundant in the nearby Allegheny Mountain physiographic province.

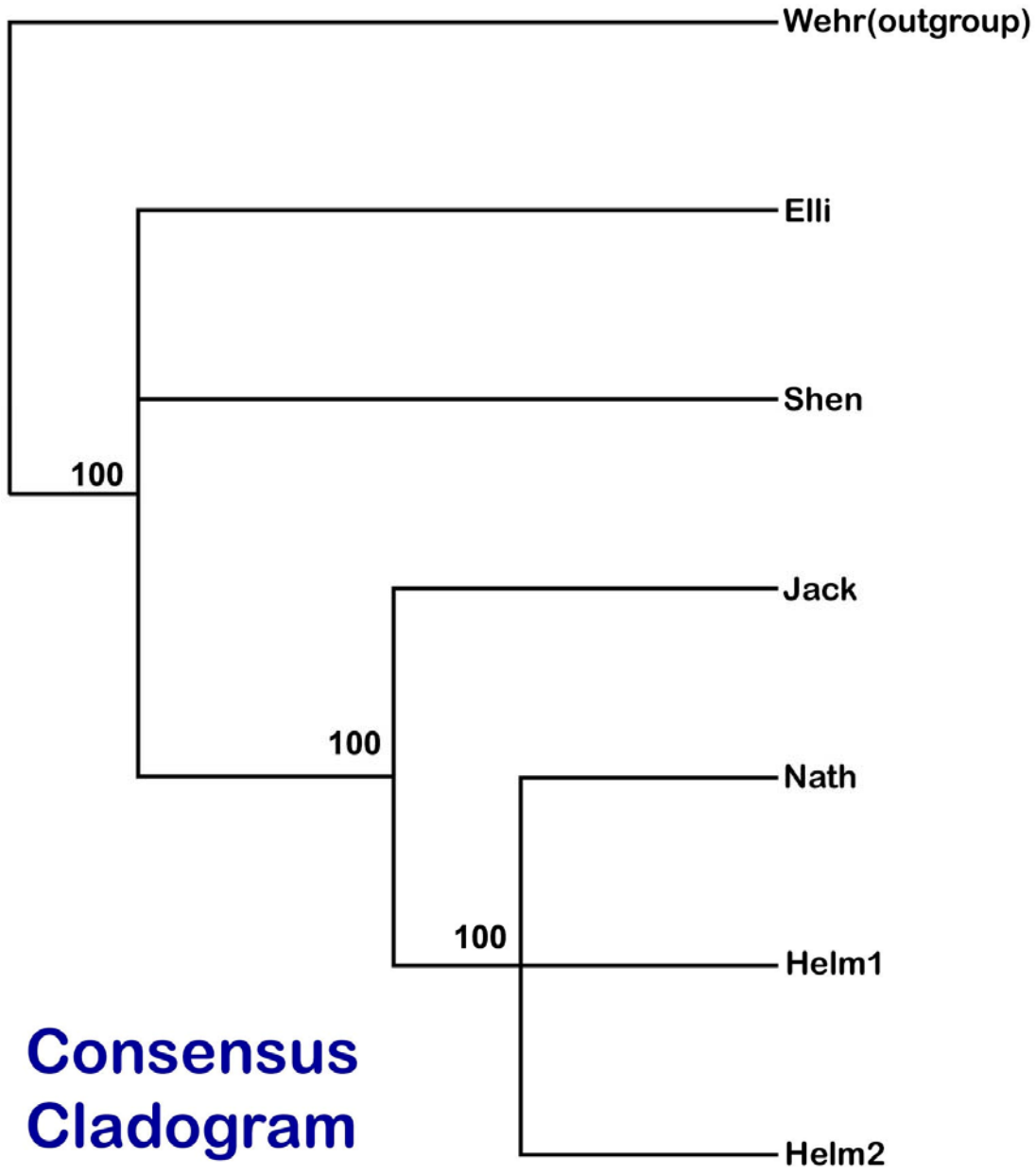

Figure 14: Maximum parsimony consensus cladogram of *P. punctatus* populations. Nodes are supported by bootstrap values of 100%.

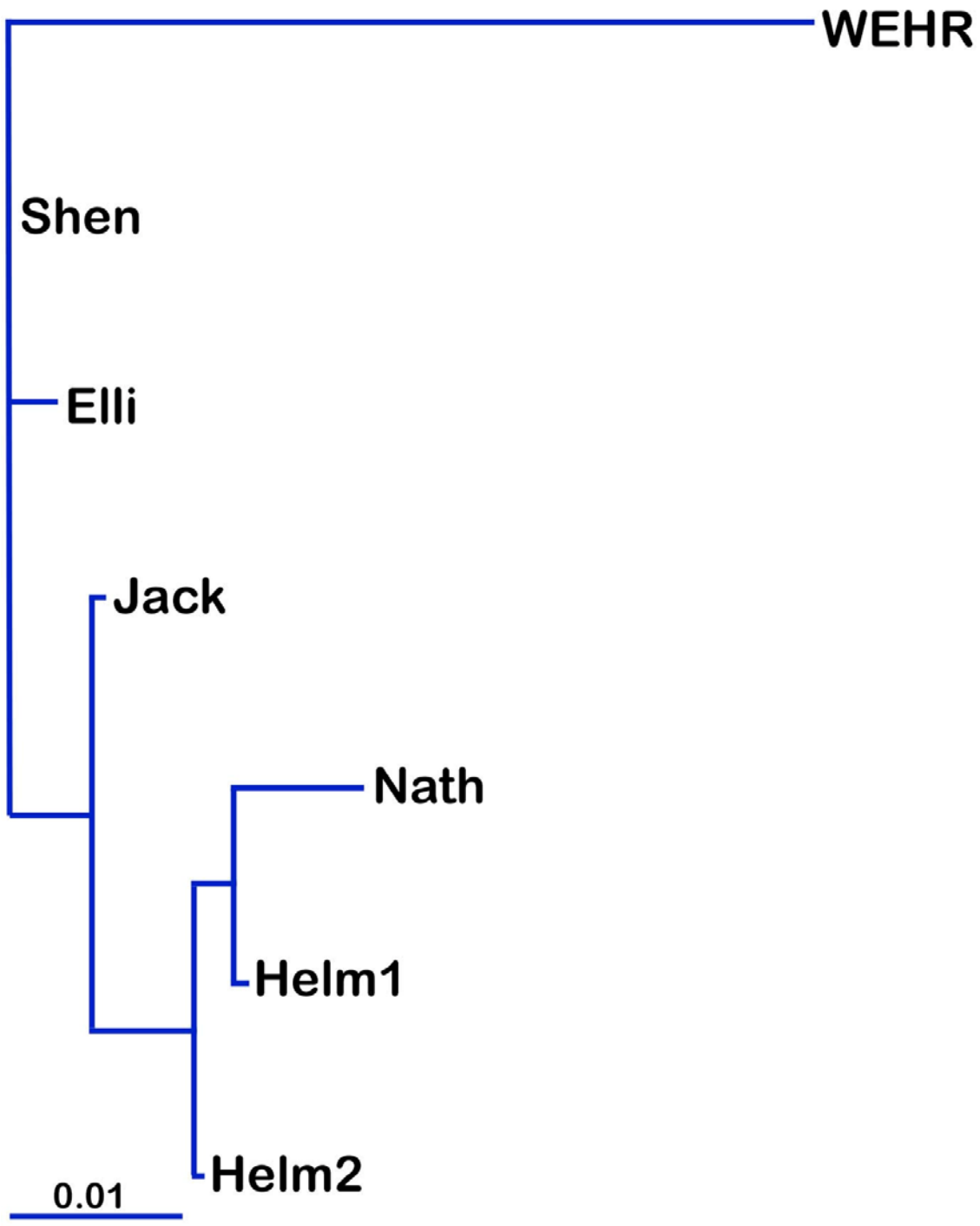

Figure 15: Jukes-Cantor neighbor-joining cladogram of *P. punctatus* populations. Abbreviations represent haplotypes from the following locations: Shenandoah Mountain (Shen), Elliot Knob (Elli), Jack Mountain (Jack), Helmick Rock (Helm1 and Helm2), and Nathaniel Mountain (Nath). Tree is rooted with *P. wehrlei* (WEHR).

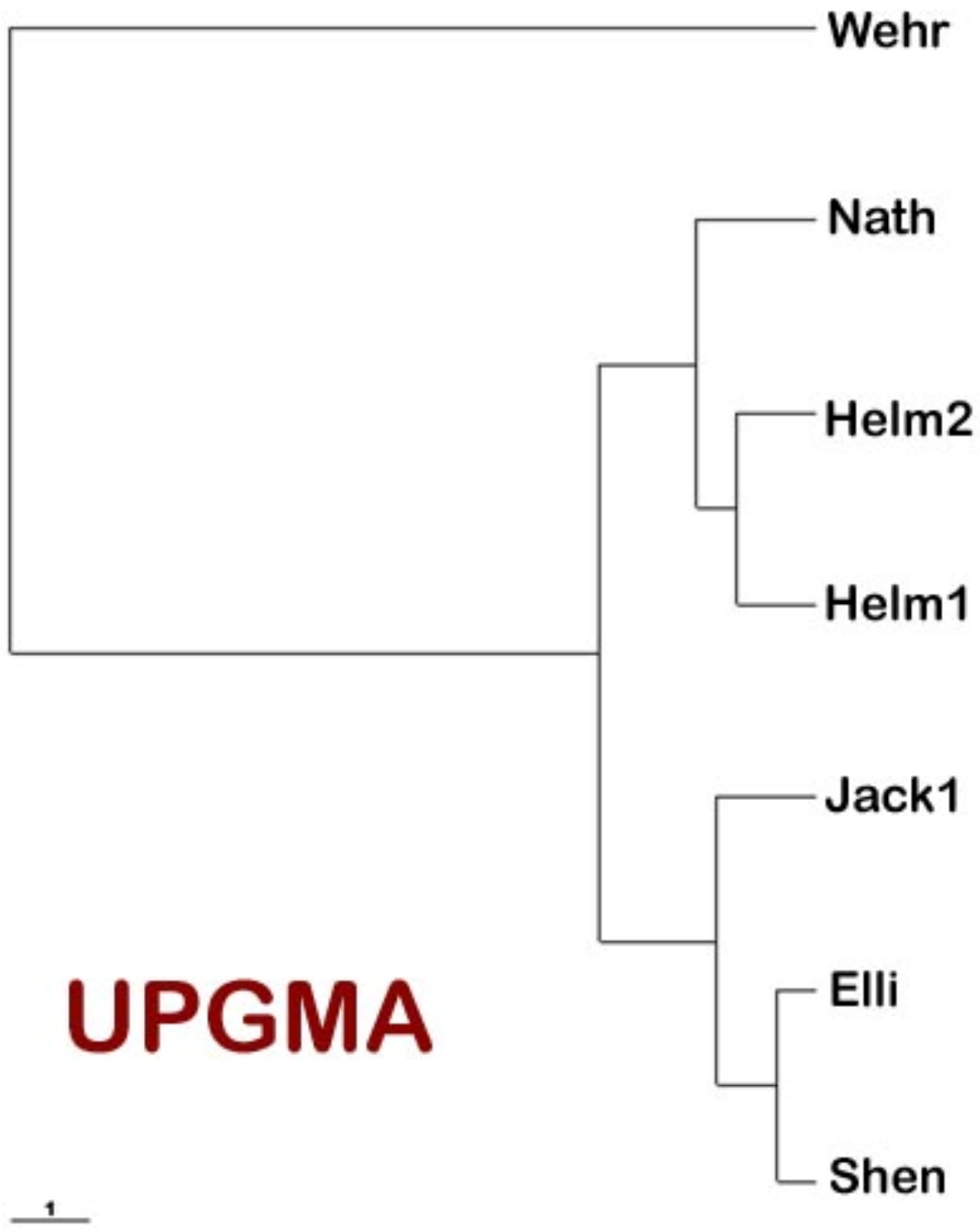

Figure 16: UPGMA phylogram of *P. punctatus* populations.

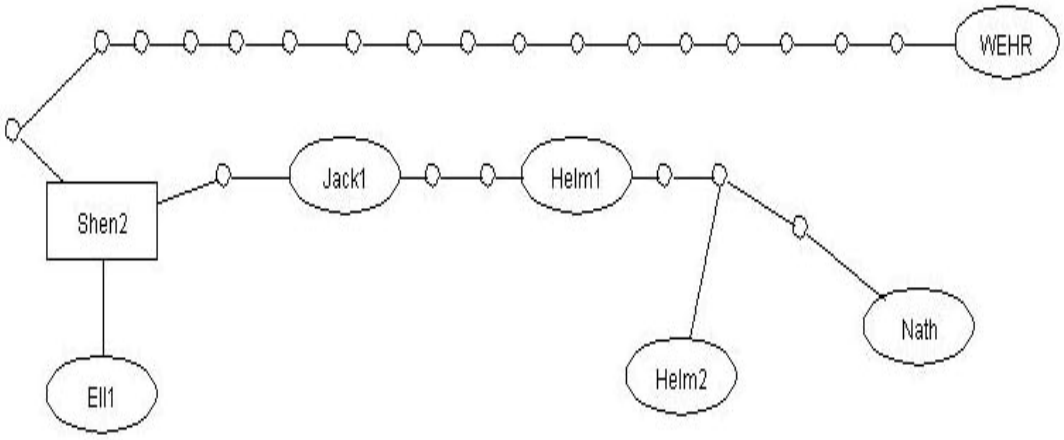

Figure 17: Haplotype parsimony network produced by TCS version 1.21. The ovals and square, except WEHR, represent *Plethodon punctatus* haplotypes from the following locations: Shenandoah Mountain (Shen2), Elliot Knob (Ell1), Jack Mountain (Jack1), Helmick Rock (Helm1 and Helm2), and Nathaniel Mountain (Nath). Small circles represent hypothetical intermediate haplotypes.

	1	2	3	4	5	6	7
1 Helm2	-						
2 Helm1	0.00529	-					
3 Nath	0.00799	0.00808	-				
4 Jack1	0.00271	0.00264	0.01609	-			
5 Jack2	0.00271	0.00268	0.01602	0.00000	-		
6 Nor1	0.00271	0.00532	0.01866	0.00264	0.00265	-	
7 Ell1	0.00805	0.01055	0.02131	0.00792	0.00528	0.00264	-
8 Shen2	0.00538	0.00792	0.01865	0.00528	0.00264	0.00000	0.00264
9 Tom1	0.00538	0.00792	0.01865	0.00528	0.00264	0.00000	0.00264
10 CR25	0.00538	0.00792	0.01865	0.00528	0.00264	0.00000	0.00264
11 Redd1	0.00537	0.00793	0.01862	0.00527	0.00262	0.00000	0.00264
12 PR87b	0.00538	0.00792	0.01865	0.00528	0.00264	0.00000	0.00264
13 PR87	0.00538	0.00792	0.01865	0.00528	0.00264	0.00000	0.00264
14 WildO2	0.00538	0.00792	0.01865	0.00528	0.00264	0.00000	0.00264
15 Shen3	0.00538	0.00792	0.01865	0.00528	0.00264	0.00000	0.00264
16 CowK1	0.00538	0.00792	0.01865	0.00528	0.00264	0.00000	0.00264
17 Shen4	0.00538	0.00792	0.01865	0.00528	0.00264	0.00000	0.00264
18 Tom2	0.00538	0.00792	0.01865	0.00528	0.00264	0.00000	0.00264
19 Shen1	0.00538	0.00792	0.01865	0.00528	0.00264	0.00000	0.00264
20 WildO1	0.00538	0.00792	0.01865	0.00528	0.00264	0.00000	0.00264
21 Waln1	0.00538	0.00792	0.01865	0.00528	0.00264	0.00000	0.00264
22 High1	0.00538	0.00792	0.01865	0.00528	0.00264	0.00000	0.00264
23 Ell2	0.00271	0.00268	0.01602	0.00000	0.00000	0.00265	0.00528
24 WEHR	0.05109	0.05328	0.05901	0.05031	0.04771	0.04508	0.04767

Uncorrected ("p") distance matrix (continued)

	8	9	10	11	12	13	14
8 Shen2	-						
9 Tom1	0.00000	-					
10 CR25	0.00000	0.00000	-				
11 Redd1	0.00000	0.00000	0.00000	-			
12 PR87b	0.00000	0.00000	0.00000	0.00000	-		
13 PR87	0.00000	0.00000	0.00000	0.00000	0.00000	-	
14 WildO2	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	-
15 Shen3	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
16 CowK1	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
17 Shen4	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
18 Tom2	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
19 Shen1	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
20 WildO1	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
21 Waln1	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
22 High1	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
23 Ell2	0.00264	0.00264	0.00264	0.00262	0.00264	0.00264	0.00264
24 WEHR	0.04504	0.04504	0.04504	0.04509	0.04504	0.04504	0.04504

Uncorrected ("p") distance matrix (continued)

	15	16	17	18	19	20	21
15 Shen3	-						
16 CowK1	0.00000	-					
17 Shen4	0.00000	0.00000	-				
18 Tom2	0.00000	0.00000	0.00000	-			
19 Shen1	0.00000	0.00000	0.00000	0.00000	-		
20 WildO1	0.00000	0.00000	0.00000	0.00000	0.00000	-	
21 Waln1	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	-
22 High1	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000	0.00000
23 Ell2	0.00264	0.00264	0.00264	0.00264	0.00264	0.00264	0.00264
24 WEHR	0.04504	0.04504	0.04504	0.04504	0.04504	0.04504	0.04504

Uncorrected ("p") distance matrix (continued)

	22	23	24
22 High1	-		
23 Ell2	0.00264	-	
24 WEHR	0.04504	0.04771	-

Figure 18: Distance matrix (uncorrected “p”)

Chapter 5

Discussion

5.1 Phylogeography

Based on the new data from our surveys for *P. punctatus* and the genetic work conducted in this study, we now have a better idea of the actual distribution of the species. Instead of being thinly distributed along the ridges of Shenandoah Mountain and Great Mountain, it is now evident that the range of this species is much more complex. It seems that all current populations are derived from a single ancestral population, evident by the relatedness of populations in relation to geography (Fig. 19). Dispersal and/or vicariance do to climatic oscillations are likely to have since sundered this ancestral population into the isolated populations that exist today.

The most significant portion of this animal's occurrence definitely occurs on Shenandoah Mountain. All historical surveys and the 2006 surveys conducted by Flint and myself revealed healthy populations along the ridgeline. Cow Knob salamander numbers appear dense here, from the southern peaks in Highland, Bath, and Augusta counties, VA, all the way north to the type locality, Cow Knob, WV. I consider this stretch the main population which is 45 miles long running southwest to northeast. Although relatively long, this is still a geographically tiny size for the significant portion of a species' range. Most of the range is extremely

narrow, under 8 miles at its widest point and usually spanning less than 5 miles in width.

Our new record on Jack Mountain, which represents the western most location for this species, is a curious one. Contrary to our expectations, the genetic data proved these individuals to be *P. punctatus* and not the more common and nearby *P. wehrlei*. As shown in Figures 14-16, these individuals group right in between northern and southern clades. In my opinion, the Jack Mountain population should be further investigated. Most of the land on Jack Mountain is private property and my surveys were thus restricted to a site at only 2,800 feet in elevation, so it would be interesting to conduct surveys along the much higher ridgelines and peaks. According to US Forest Service maps, there is a road that runs south along the ridgeline of Jack Mountain from CR 25 near Moyer Gap. A gate blocks the road in the north, but the land is owned by the paper company MeadWestvaco, who might give access to researchers (Pauley, pers. comm.). I recommend that those who survey for *P. punctatus* on Jack Mountain do so after a good rain event. I spent considerable time looking for salamanders at the Jack Mountain site and had just about given up on it, but happened to be in the area during a very hard rain and salamanders actually turned out to be plentiful there.

Probably the most perplexing result of this study is the genetic distances of two specimens from Elliot Knob, Virginia. One of these specimens, Ell2, shares the exact same haplotype as the Jack Mountain specimens, while the other is a haplotype that is actually the most genetically dissimilar to those. This brings into question the validity of the sequence data of the Elliot Knob specimens, which

clearly should be much more genetically similar than they are. I cannot say for sure whether this difference represents natural genetic variation within the population, but I doubt it, and believe that the sequence data from this population should be resampled.

When I had first heard that Nathaniel Mountain represented a potential population of Cow Knob salamanders, I was skeptical. Sure enough, however, Flint managed to find an individual during his surveys and sent me a tail tip. The resulting haplotype grouped in with all the other haplotypes yet was still somewhat distinct. All of the pairwise genetic distances with this haplotype were greater than 0.016 substitutions per site. According to the molecular clock estimates (1.3%/Myr) employed by Weisrock et al. (2001), gene flow among the Nathaniel Mountain population has been restricted from the main population for 1.32–1.62 million years. This puts the split sometime in the early to middle Pleistocene, a time of extreme climatic oscillations (Barnosky, 2005). It could be that *P. punctatus* inhabited a much larger range that went north all the way to Nathaniel Mountain, but was sundered by fluctuations in climate. I believe that the Nathaniel Mountain population represents a relict population that has been isolated on its northern habitat for about 1.5 million years, left on its own evolutionary trajectory.

The most closely related Individuals to Nathaniel Mountain, were from Helmick Rock. Tail tips from two specimens at this location were collected during William Flint's VES surveys. Genetic data from these specimens shows them as distinct,

but still groups them together with the Nathaniel Mountain specimen, forming a northern clade.

5.2 Conservation Implications

Cow Knob salamanders face various threats across their range. Flint (2004) found that roads in particular have a major influence on the abundance of *P. punctatus*. Roads and their peripheries, especially areas downhill of roads (up to 74 meters), experience greatly reduced numbers of salamanders, likely caused by eroded loose soil filling in interstitial spaces.

Despite the impact of roads, Cow Knob salamanders and their habitat are still in pretty good shape. Most of the range of this species lies within USDA Forest Service land, so it has received considerable attention. In fact, in 1993, a 43,000 acre area in the George Washington National Forest was created in part to preserve the forest habitat required by *P. punctatus*. The designated area, the Shenandoah Mountain Crest – Special Interest Area (SMC-SIA), includes all land on Shenandoah Mountain above 914 meters within the Dry River Ranger District (Flint, 2004). This reserve preserves an area with the greatest abundance of Cow Knob salamanders and is a great step toward the conservation of the species.

Conversely, mere numbers of individuals being preserved is not always the most important consideration with developing a conservation plan. A more effective strategy, especially for a species with a small range like this one, is to protect as much of the underlying genetic diversity within the species as possible.

Environmental change, both anthropogenic and natural, is an ongoing process, and genetic diversity provides populations with the means to adapt and evolve to such change. Furthermore, loss of genetic diversity usually results in increased inbreeding and overall reduction of reproduction and survival (Frankham et al., 2004). Therefore, genetic results provided by this study are especially important to the future management of *P. punctatus*.

Based on my genetic data, I suggest that populations on Helmick Rock, Jack Mountain, Shenandoah Mountain, and Nathaniel Mountain all be treated as separate management units (MUs). All four populations meet the Moritz (1994) criteria for management units and are all likely on separate evolutionary trajectories. In addition, the Nathaniel Mountain population represents a special concern and may even deserve an ESU designation. Conclusions and recommendations for each population in this study are outlined below.

Shenandoah Mountain **(Fig. 20)**

The majority of Cow Knob salamanders almost certainly occur in the Shenandoah Mountain region. Therefore, this population is inherently important simply due to the sheer number of individuals it represents. On the other hand, specimens throughout this region, represented by 1 haplotype from 16 individuals in this study, possess especially low genetic variability (see Figs. 13–16). I therefore recommend that management of the Shenandoah Mountain region remain as is. The current population appears healthy as surveys year after year have produced consistent records of many individuals. Furthermore,

Cow Knob salamander habitat in much of this region is already being protected, and in some cases local populations are even being monitored. In summary, this region represents a large proportion of global *P. punctatus* abundance, is a monophyletic group, but contains low genetic diversity. It should be managed as a single large MU of medium importance.

Helmick Rock (Fig. 21)

Based on haplotype diversity from Helmick Rock, Cow Knob salamanders there vary genetically from all other populations by 0.26–1.10 substitutions per base. Based on molecular clock estimates (1.3%/mya) they have not been isolated longer than about 0.85 mya, and although they are an insular species now, they might have even experienced very recent gene flow. This taken in regard to the population's geographic location is not very surprising. The main ridgeline of Shenandoah Mountain, where many individuals occur, extends north to only a few miles southwest of Helmick Rock. Recent climatic fluctuations during the Pleistocene could have easily allowed individuals to disperse at lower elevations when the climate was cool. It was only within the last couple of thousand of years when, as temperatures rose, a population could have become isolated, and would not have had time to undergo significant divergence at the genetic level.

Although recent gene flow is likely, the Helmick Rock population still represents the second most divergent population and should be valued from a conservation perspective for the genetic variation that it is able to contribute. Diversity is still

much higher than that found within the large Shenandoah Mountain population, so this population should be managed as an additional MU also of medium importance to conservation.

Jack Mountain (Fig. 22)

Sequence data from two individuals collected on Jack Mountain produced identical haplotypes, and thus low genetic diversity within the population can be expected. These haplotype differ from all other populations by 0.26–0.79%, meaning that the Jack Mountain population has undergone even more recent gene flow with the Shenandoah Mountain population than the Helmick Rock population has. This is astonishing since dispersal of specimens on Jack Mountain is blocked from Shenandoah Mountain individuals to the east by a series of low-laying valley barriers. The valleys experience high temperatures and the rain shadow effect produces areas so dry that a native cactus species even occurs there. It is therefore hard to imagine a moisture dependent species like *P. punctatus* traversing such extreme valleys.

It must have been that times of cooler climates in the Pleistocene allowed corridors between the ranges. For the meantime, and as long as the earth's temperature continues to increase, Jack Mountain individuals will remain isolated from other individuals and maintain their separate conduits through evolutionary time. I suggest that this population be considered a MU of low to medium importance to the overall conservation of the species.

Nathaniel Mountain (Fig. 23)

Pabijan et al. (2005) states that "rationale behind preserving conservation units stems from a compromise between limited resources and a need to preserve as much genetic diversity as possible in a given species." One way to preserve a hugely significant portion of genetic diversity within Cow Knob salamanders, and without putting limits on many resources, is to immediately and efficiently protect the habitat of individuals from Nathaniel Mountain, WV. This population, unfortunately represented in this study by only one individual, contains a haplotype deeply divergent from all other Cow Knob salamander populations. Based on genetic distance measures, the Nathaniel Mountain haplotype is 0.79–2.13 substitutions per site different from the other populations. Using molecular clock estimates (1.3%/mya) the population diverged no sooner than 0.6 mya and maybe as late as 1.64 mya, or the late Pliocene to mid Pleistocene.

The loss of any population as differentiated as the Nathaniel Mountain population would result in the loss of an appreciable amount of genetic diversity in the species and should be avoided at all costs. Unfortunately, this population is probably restricted to a very small range on Nathaniel Mountain in West Virginia, and rough estimates of abundance seem very low. Several survey attempts in the area have proved futile (Pauley & Fisher, pers. comm.), and even Flint's surveys only resulted in one tail tip for the study.

I believe that the population of *P. punctatus* inhabiting Nathaniel Mountain is in dire need of additional study. Individuals there represent by far the most divergent population of Cow Knob salamanders, making them of extreme

importance to the preservation of the species. Furthermore, additional genetic data should be gathered to determine if the population is reciprocally monophyletic and life history data should be quantitated for analysis of ecological exchangeability. Such analyses might reveal that Nathaniel Mountain individuals actually represent 'evolutionary significant units' (ESUs), or even more importantly could even be a new species. For now, the Nathaniel Mountain population of Cow Knob salamanders should be considered a MU of high importance. This group, more than all of the others, warrants additional study, and could prove to be an important resource in the struggle to secure the preservation of the species as a whole.

5.3 *Plethodon punctatus/wehrlei* Divergence

Over the duration of this study, I spent a lot time in the field critically analyzing Cow Knob salamander habitat, ecology, and behavior and took extensive notes on syntopic, sympatric, and parapatrically occurring salamander species. This experience, along with the newly derived genetic data (mentioned above), spurred my own ideas regarding the reasons and mechanism that drove the divergence of *Plethodon punctatus* and *P. wehrlei*.

Essentially, I agree in part with Highton 1995 who hypothesized that the split resulted from climatic oscillations. Highton suggested that the split took place during the Pliocene, which agrees with the molecular clock estimates of 3.5-4.5 mya since divergence (based on 1.3%/mya) from this study, and the results of Weisrock et al. (2001). On the contrary, Highton puts an emphasis on the "dry

periods of the Pliocene," but I believe that the cause was more a result of overall temperature fluctuations that forced mesic habitat up and down mountainsides. Highton is probably right that populations were isolated from each other on different mountaintops, but why would they speciate allopatrically? And what is the mechanism?

It seems more feasible to me that the ancestral *P. wehrlei/punctatus* ancestor was at one time abundant with a large range but was isolated on mountaintops during a "dry spell". The speciation event must have occurred when things became much cooler again. As cooler temperatures returned, ideal habitat was then forced back down the mountainsides. At one time, when temperatures were decreasing, the mesic forests which comprise such ideal habitat for these salamanders were centralized neither at the top nor at the bottom of the mountains, but somewhere in between. Therefore, the maximum resources were at mid elevations and lots of species were competing for the same resources. This idea of course relies on the assumption that these salamanders are constrained by their habitat niches; an idea termed "niche conservatism" (Wiens, 2004). The general idea of niche conservatism is that dominant vegetation types, which comprise the overall countenance of a habitat, are adapted to certain climate regimes and adapt to changing climate by moving to different latitudes and elevations. Smaller species found within these habitats, such as salamanders, are thus more likely to follow the moving habitat than to remain where they are and adapt to entirely different habitat and climate. It is niche

conservatism then, which defines the geography and much of the genetic distribution of populations as landscapes change.

One species specifically adapted to the mesic mountain habitats must have been an especially formidable competitor, the white-spotted slimy salamander, or *P. cylindraceus*. This salamander is much larger than both *P. punctatus* and *P. wehrlei* and must have used a significant amount of their resources. Interspecific competition is known to influence niche differentiation as inferior competitors are driven from contested resources through exploitation or interference (Gall et al., 2002; Hairston, 1987). Influences of competition have been documented in *P. punctatus*, which as juveniles are the same size as adult sympatric *P. hoffmanni* (now *P. virginia*) and utilize the same food and surface habitat. Interspecific competition drove these two species to partition their food niches temporally, a phenomenon called noncoincident feeding (Fraser, 1976). It is reasonable then, that even stronger competition with large and aggressive *P. cylindraceus* would have operated as a negative selective pressure on the *P. werhlei/punctatus* ancestor and forced them into less optimal conditions. With this in mind, I propose the following scenario.

When optimum resources were located at central elevations, selection must have been for individuals tolerant of the colder climates at the elevationally higher edges of resource availability, and for those tolerant of hot and dry conditions at the other end of the spectrum at low elevations. One of the ways higher populations could have adapted to a much colder climate was by following cracks and crevices (interstitial spaces) of talus slopes deep underground into

the mountains. Changes in phenology would then follow as these populations were forced into shorter active periods but benefited greatly by little competition for resources. As the climate cooled even further, competitors might have followed resources down the mountains and became isolated long enough to speciate allopatrically. Then, upon returning warmer temperatures, the high-elevation individuals (*P. punctatus*) remained better adapted to the high elevation habitats and competitively excluded any returning individuals whose ancestors had followed the low elevation resources (*P. wehrlei*), thus shaping today's distribution of the *wehrlei* complex.

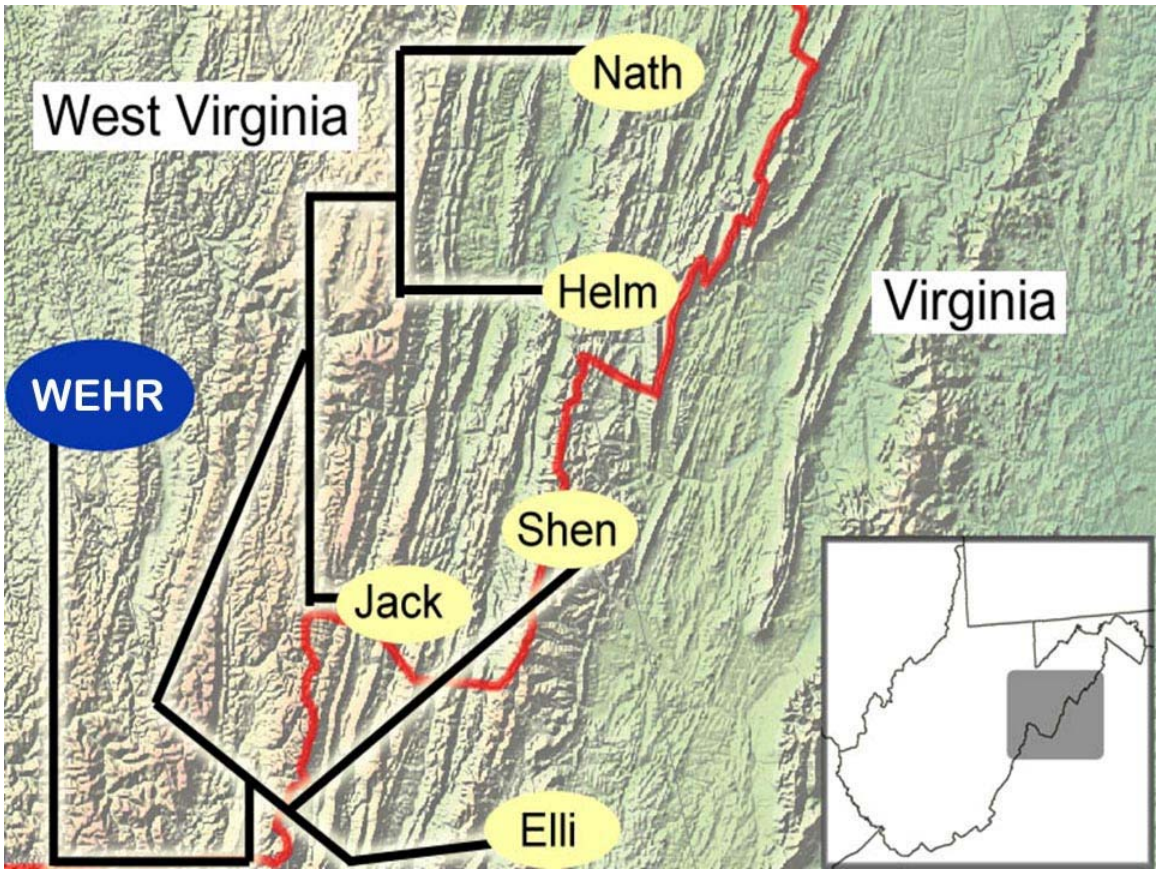

Figure 19. Geographical overlay of maximum parsimony consensus tree. Each haplotype is representative of a distinct *Plethodon punctatus* population; Nath = Nathaniel Mountain, Helm = Helmick Rock, Jack = Jack Mountain, Shen = Shenandoah Mountain Range, Elli = Elliot Knob. Tree is rooted with *P. wehrlei* (WERH).

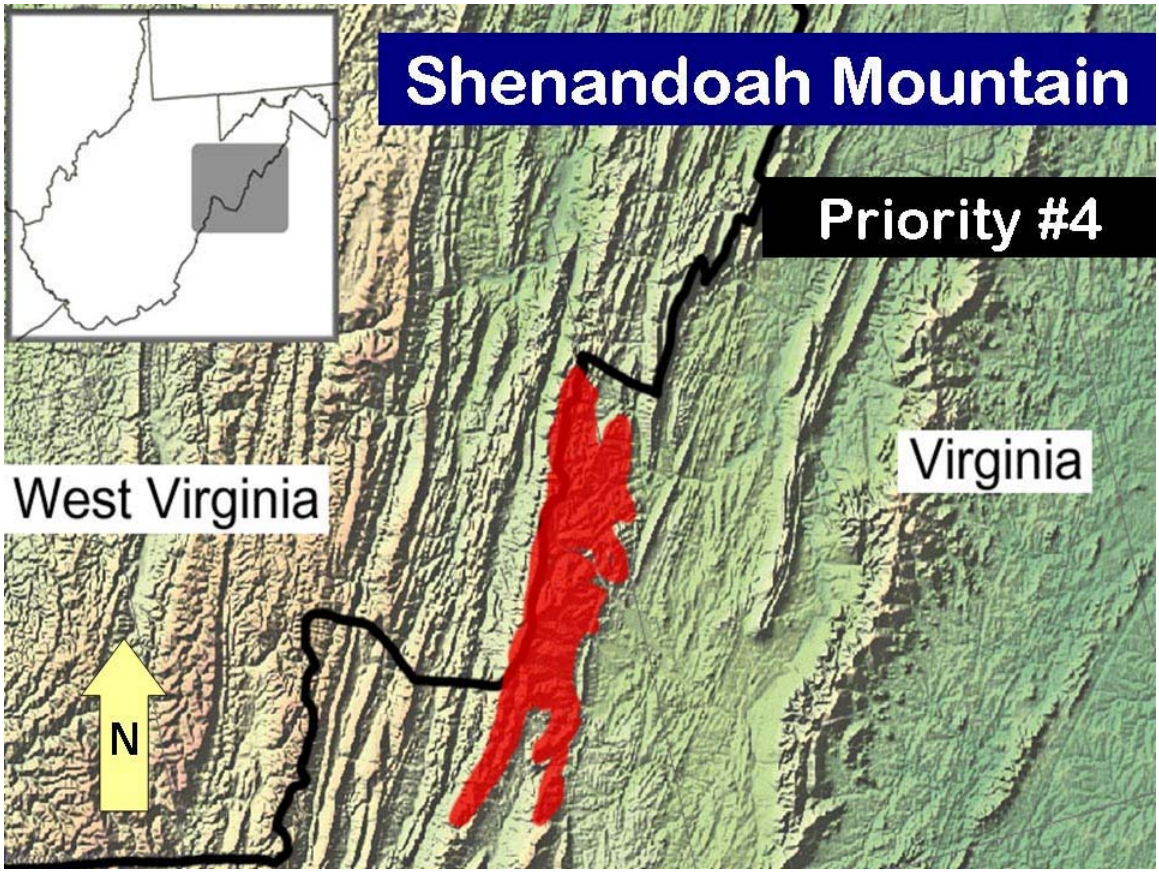

Figure 20. Distribution of *P. punctatus* on the Shenandoah Mountain range.

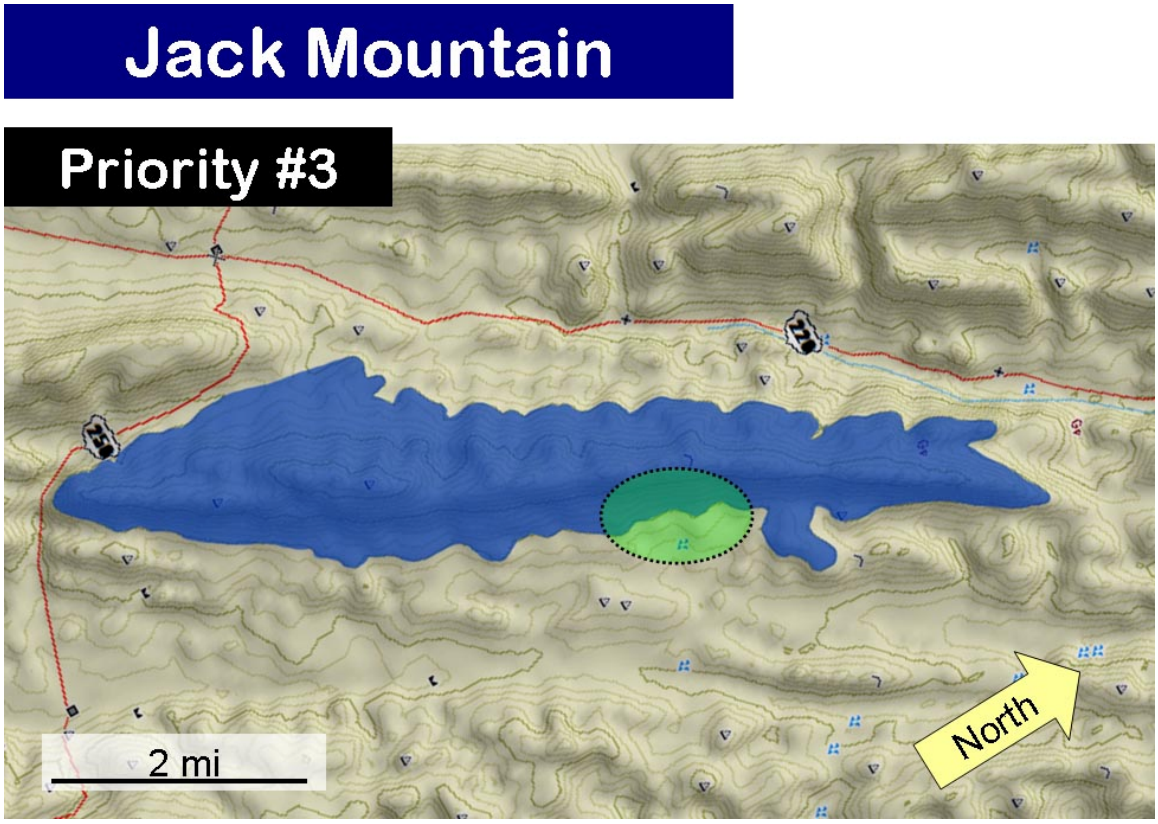

Figure 21. Relief map of Jack Mountain. Two *P. punctatus* individuals were discovered here at 2’800 feet in elevation in the area highlighted by the green oval. Area in blue represents elevations over 3’000 feet, ideal habitat for the species.

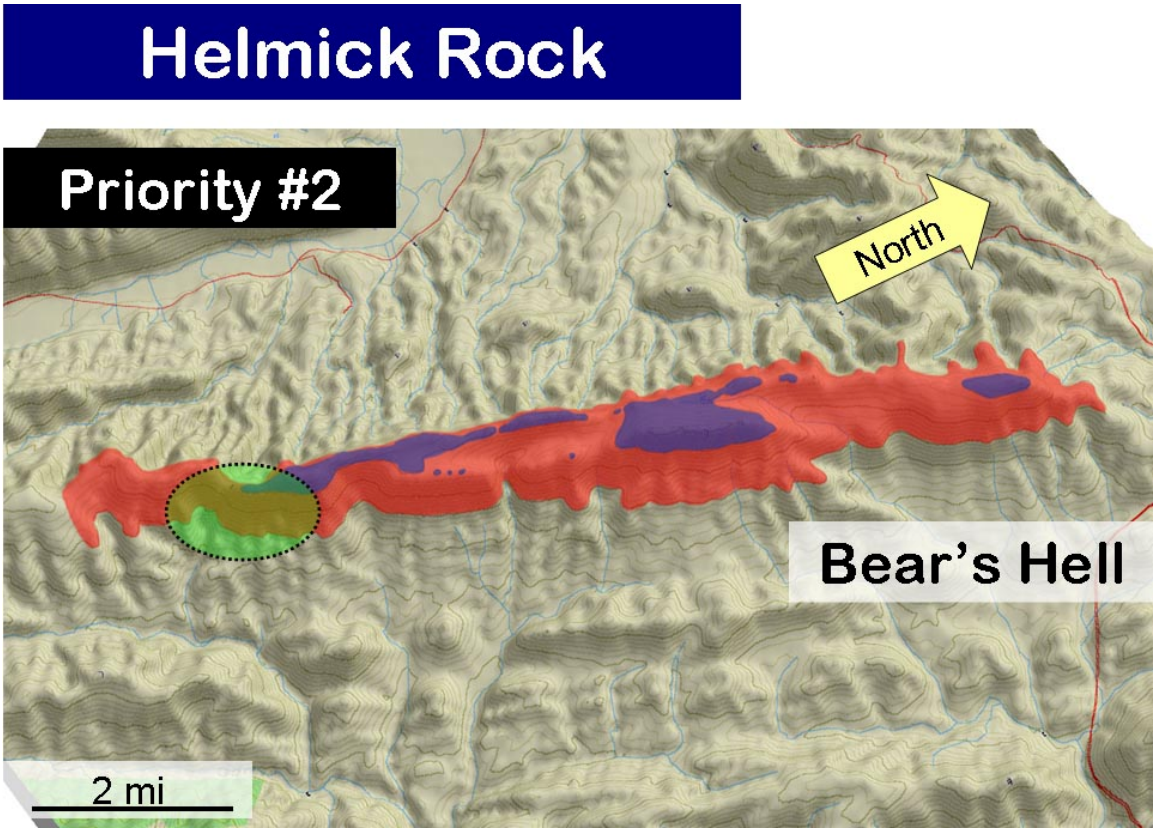

Figure 22. Potential distribution of *P. punctatus* at Helmick Rock (south side of ridgeline) and Bear's Hell (north side). Areas in red are elevations above 2,500 feet (potential habitat) and blue areas are above 3,000 feet (ideal habitat). Specimens from this study were collected from the area highlighted by the green oval.

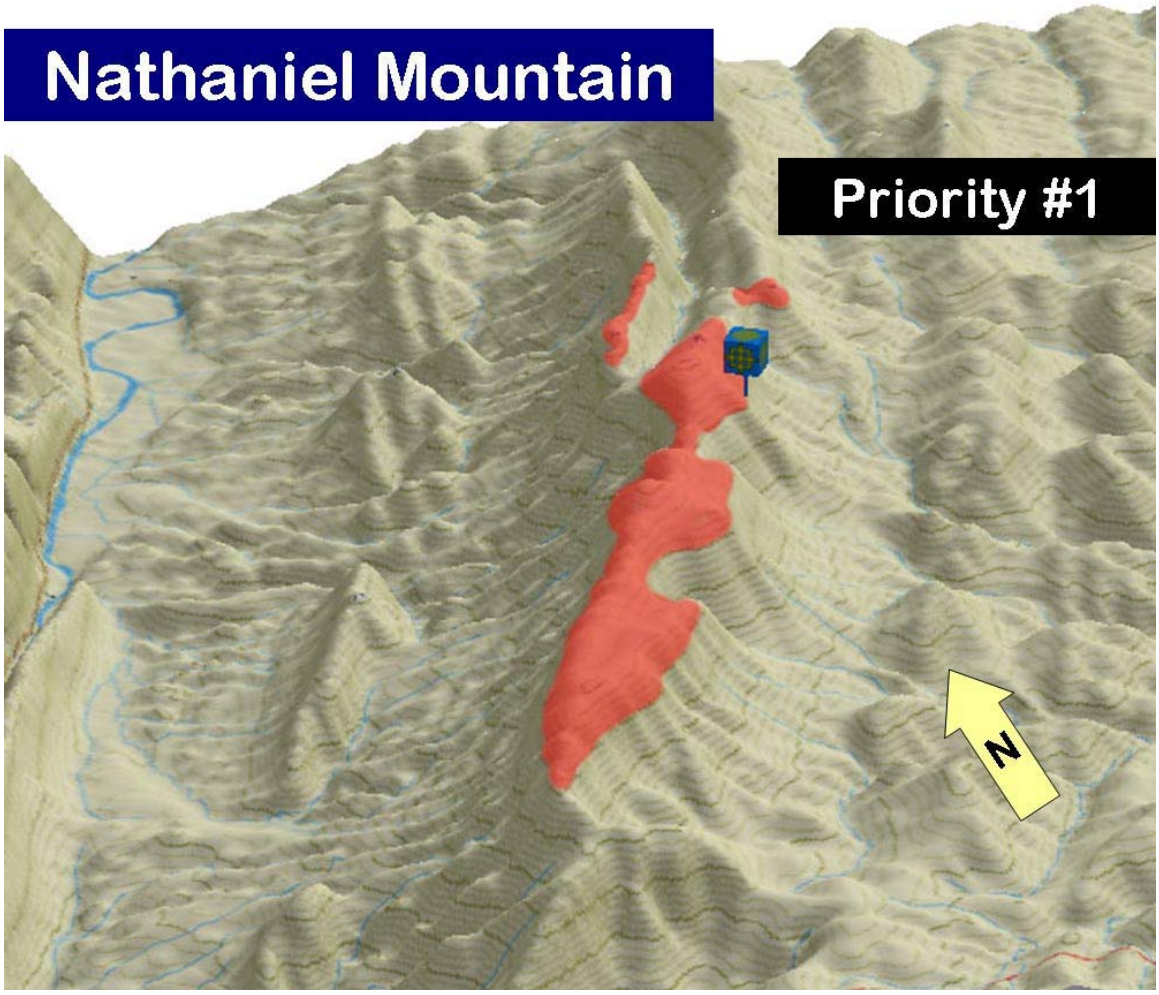

Figure 23. Potential distribution of *P. punctatus* on Nathaniel Mountain, WV. Red areas are elevations above 2,500 feet and indicate potential habitat for the species. The specimen used in this study was collected at the point indicated by the blue cube.

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Appendix Biographical Sketch & CV

My interest in science dates back to when I was a child. Growing up around chaparral, montane, and desert ecosystems, I became enthralled by the native fauna that surrounded me. I was especially curious about reptiles and amphibians, so my room was often cluttered with terrariums, heat lamps, and animals that clambered and slithered about. Then in 1999, I came across a strange arachnid hiding under a boulder in Mission Gorge, San Diego. It was a tiny and strikingly colored scorpion that turned out to be an exceptionally rare find and spurred an interest in scorpion biology. Before I knew it I was at Marshall University studying scorpions with Dr. Victor Fet.

During my undergraduate years, Dr. Fet and I collaborated on many projects, and I became involved in the projects of other professors like Dr. James Joy and Dr. Guo-Zhang Zhu as well. It was during my final semester that I met Dr. Pauley while enrolled in his ornithology class.

Post graduation, I worked with birds as an intern for Point Reyes Bird Observatory Conservation Science (PRBO) at the Palomarin Field Station in northern California, but soon ended up back at Marshall for graduate school. This time, however, I wanted to work with reptiles and amphibians and Dr. Pauley graciously accepted me into his lab.

Presently, I have accepted a position in the doctoral program at the School of Life Sciences of the University of Nevada, Las Vegas, There I will be pursing both my passion for herpetology and scorpiology simultaneously. Two professors, Drs. Jef Jaeger and Brett Riddle, will co-advise me on dissertation work concerning scorpion biogeography. Financial support is to be supported by a research assistantship working toward the conservation of the endangered relict leopard frog, *Rana onca*.

My long term goals include uncovering historical biogeographic patterns in North American scorpions, assisting in the revision of the North American scorpion family Vaejovidae, and completing a field guide on western United States scorpion fauna.

MATTHEW R. GRAHAM

CURRICULUM VITAE

DEPARTMENT OF BIOLOGICAL SCIENCES
MARSHALL UNIVERSITY
HUNTINGTON, WV 25755-2510, USA

(415) 272-2110
mattrgraham@yahoo.com

EDUCATION AND TRAINING

M. S. in Biology	Marshall University, May 2007
B. S. in Biology (minor in chemistry)	Marshall University, December 2004

PROFESSIONAL EXPERIENCE

Teaching

Teaching Assistantship	BSC 121 – Principles of Biology Lab (for majors) BSC 120 – Principles of Biology Lab (for majors) BSC 104 – Introduction to Biology Lab (for non-majors)
Instruction	2003-2006 – Taught DNA extraction, amplification, and purification techniques to five students; four undergraduates and one graduate student
Graduate Assistantship	Fall 2005 – Autism Services - Academic tutor and assistant for students with Asperger's syndrome

Experience

Thesis Research	Distribution and conservation genetics of the Cow Knob Salamander, *Plethodon punctatus* Highton (Caudata: Plethodontidae)
Principle Investigator	Spring 2006 – Morphological investigation of the West Virginia large woodland salamanders (Plethodontidae)
Webmaster	2005-2006 – Marshall University Herpetology Lab webpage. (www.marshall.edu/herp)
Research Assistant	2005 –Stream salamander species and their potential as indicators of stream health.
Research Assistant	2006 – Turtle assemblages of the eastern West Virginia panhandle – identified turtles and used telemetry to track red-bellied turtles.
Principle Investigator	2000-2006 – The Scorpions of California – conducted

	scorpion surveys of the southwest using daytime cover object surveys and night ultraviolet light detection surveys.
Principle Investigator	2006 – Comprehensive examination of the scorpion collection of the San Diego Museum of Natural History: new records, identifications, and a list.
Research Assistant	2001-2006 – Mitochondrial DNA extraction, amplification, and purification of scorpions and salamanders.
Intern (Nest Searcher)	Spring & Summer 2005 – Point Reyes Bird Observatory Conservation Science (PRBO) – nest searched and banded songbirds in the Point Reyes National Seashore, CA.
Research Assistant	2005-2006 – Snake assemblages of West Virginia – assisted in snake surveys in the Monongahela National Forest, WV.
Research Assistant	Spring 2006 – Surveying for eastern hellbenders (*Cryptobranchus a. alleganiensis*) in West Virginian streams to determine current distribution and assessing habitat parameters that maybe affecting this distribution.
Co-founder	2006 – Marshall Herpetology Journal Club: Weekly deliberations on various aspects of reptile and amphibian study.
Research Assistant	2003 – Mosquito surveys of West Virginia: tracked the spread of *Anopheles* species.
Research Assistant	2004 – Identification of a novel male germ cell-specific protein: assisted in primer design; DNA extraction, amplification, and purification; northern blot; cloning via plasmid vectors; germ cell isolation; and DAPI staining.
Research Assistant	2007 – *Ambystoma* monitoring project: constructed drift fences and monitored movement of salamanders to and from breeding ponds.

Service

Referee	*Euscorpius – Occasional Publications in Scorpiology*
Volunteer Work	Farallon Island Patrol – April 2005 Habitat for Humanity - 2004

	Science Fair Judge - Fairland High School, Fairland, OH – Spring 2003 & 2004 Santa Barbara Museum of Natural History – Identification of scorpion specimens - 2001

Scholarships/Fellowships

Summer Research Grant ($500)	Marshall University Graduate School – Summer 2006
West Virginia DNR Grant ($2,200)	Spring 2006
Summer Undergraduate Research Fellowship (S.U.R.F.) ($3,600)	Marshall University – Summer 2004
Robert J. Profant Memorial Scholarship ($500)	Santa Barbara City College – Spring 2001

Publications

GRAHAM, M. R. 2007. Sky Island *Vaejovis*: two new species and a redescription of *V. vorhiesi* Stahnke (Scorpiones: Vaejovidae). *Euscorpius*, 51: 1–14.

GRAHAM, M. R. & V. FET. 2006. Serrula in retrospect: a historical look at scorpion literature (Scorpiones: Orthosterni). *Euscorpius*, 48: 1–19.

TERUEL, R., V. FET & **M. R. GRAHAM**. 2006. The first mitochondrial DNA phylogeny of Cuban Buthidae (Scorpiones: Buthoidea). *Boletín de la S.E.A.*, 39: 219-226

GRAHAM, M. R. 2006. Redescription and lectotype designation of *Vaejovis lapidicola* Stahnke, 1940 (Scorpiones: Vaejovidae). *Euscorpius*. 46: 1-6.

GRAHAM, M. R. 2006. Malformed pedipalp finger dentition of the scorpion *Superstitionia donensis* (Scorpiones: Superstitioniidae). *Euscorpius*, 42: 1-4.

FAN, J., **M. R. GRAHAM**, H. AKABANE, L. L. RICHARDSON & G. ZHU. 2005. Identification of a novel male germ cell-specific gene TESF-1 in mice. *Biochemical and Biophysical Research Communications*, 340(1): 8-12.

FET, E. V., D. NEFF, **M. R. GRAHAM** & V. FET. 2003. Metasoma of *Orthochirus* (Scorpiones: Buthidae): are scorpions evolving a new sensory organ? *Revista Ibérica de Aracnologia*, 8: 69-72.

Presentations

GRAHAM, M. R., W. D. FLINT, A. A. HOGSETT & T. K. PAULEY. 2007. Distribution and conservation genetics of the Cow Knob salamander, *Plethodon punctatus*. *Association of Southeastern Biologists 68th Annual Meeting*. April 21. (Presentation)

GRAHAM, M. R., W. D. FLINT, A. A. HOGSETT & T. K. PAULEY. 2007. Distribution and conservation genetics of the Cow Knob salamander, *Plethodon punctatus*. *82nd meeting of the West Virginia Academy of Sciences*. March 31. (Presentation)

BREWER, M., **M. R. GRAHAM**, V. FET & M. E. SOLEGLAD. 2007. Scorpion serrula: an

enigmatic structure under SEM. *82nd meeting of the West Virginia Academy of Sciences*. March 31. (Poster)

(1st Place Poster) **GRAHAM, M. R.**, T. E. BALDWIN, M. B. WATSON & T. K. PAULEY. 2006. Herpetofaunal species richness of four West Virginia national parks. *West Virginia Academy of Science 81st Annual Meeting*. Shepherd University, Shepherdstown, WV.

GRAHAM, M.R. 2006. Distribution and conservation units of two rare salamanders in West Virginia: a thesis progress seminar. *Marshall University Seminar Series*, October 24th (presentation)

GRAHAM, M.R. 2006. Distribution and gene flow of the Cow Knob Salamander (*Plethodon punctatus*) in West Virginia. *Marshall University Seminar Series*, Spring Semester (presentation)

GRAHAM, M. R., N. GRAHAM, J. FAN & G. ZHU. 2004. Construction of a GFP-tagged TESF-1 Expression Vector. *S.U.R.F.* Marshall University, Huntington, West Virginia. (poster & presentation)

FET, E. V., D. NEFF, **M. R. GRAHAM** & V. FET. 2003. Metasoma of *Orthochirus* (Scorpiones: Buthidae): are scorpions evolving a new sensory organ? *American Arachnological Society 27th Annual Meeting*, Denver, CO, 24-28 July 2003. (poster)

FET, E. V., **M. R. GRAHAM**, V. FET & S. G. STRAIT. 2003. Quantitative analysis of sexual dimorphism in *Euscorpius* (Scorpiones: Euscorpiidae). *Sigma Xi Research Day*, Marshall University, Huntington, West Virginia. (poster)

R. TERUEL, V. FET, J. L. GREENWOOD, **M. R. GRAHAM**, E. V. FET & D. HUBER. 2003. First data on the DNA phylogeny of some Cuban Buthidae (Scorpiones). *American Arachnological Society 27th Annual Meeting*, Denver, CO, 24-28 July 2003. (poster)

E. V. FET, W. I. TOWLER, S. THOMPSON, A. COEHRAN, J. GREENWOOD, E. PRICE, A. SINK & **M. R. GRAHAM**. 2002. Mitochondrial DNA markers and scorpion evolution (Arachnida: Scorpiones). *Sigma Xi Research Day*, Marshall University, Huntington, West Virginia (poster)

Published Abstracts

BALDWIN, T. E., **M. R. GRAHAM**, M. B. WATSON & T. K. PAULEY. 2006. Herpetofuanal species richness of four West Virginia national parks. *Association of Southeastern Biologists 67th Annual Meeting*. University of Tennessee, Gatlinburgh, TN. Abstract published in Southeastern Biology, 53(2):225:271.

GRAHAM, M. R., R. TERUEL & V. FET. 2004. Mitochondrial DNA data on phylogeny of *Centruroides* (Scorpiones: Buthidae) from the Caribbean and North America. *Association of Southeastern Biologists 65th Annual Meeting*. University of Memphis, Memphis, TN, 14-17 April 2004. Abstract published in Southeastern Biology, 51(2): 211.

FET, V., M. E. SOLEGLAD, M. C. ESTEP, R. N. HENSON, M. U. CONNELL, K. BOST, M. D. BARKER & **M. R. GRAHAM**. 2002. Preliminary analysis of 16S rRNA gene in the North American Vaejovidae (Arachnida: Scorpiones). *Association of Southeastern Biologists 63rd Annual Meeting*. Appalachian State University, Boone, NC, 10-13 April 2002. Abstract published in Southeastern Biology, 2002, 49(2): 200.