MORPHOLOGICAL AND GENETIC VARIATION BETWEEN *PLETHODON LONGICRUS* AND *PLETHODON YONAHLOSSEE*

A Thesis

by

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ABSTRACT

MORPHOLOGICAL AND GENETIC VARIATION BETWEEN *PLETHODON LONGICRUS* AND *PLETHODON YONAHLOSSEE*

(August 2005)

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In recent years, the systematics of plethodontid salamanders has been revised through the use of molecular markers and multivariate statistics to reveal a more complex array of evolutionary relationships and a greater degree of biodiversity, especially in the Southern Appalachian Mountains. One question that remains is the taxonomic status of *Plethodon longicrus*, the Crevice Salamander. Although being described as a distinct species by Adler and Dennis in 1962, it is now considered a geographical variant of *P. yonahlossee*. The present study included both a phylogenetic analysis and a morphological analysis to help resolve this issue. For the morphological analysis, twenty-one measurements were collected from 160 specimens. Each character was regressed on snout-vent length, and the residuals from these regressions were analyzed. The principal component analysis and discriminant function analyses revealed significant species differences between *P. longicrus* and *P. yonahlossee*. These

analyses also found significant differences between populations within species, with the Bat Cave population being distinct from the other southern *P. longicrus* populations and the Powderhorn Mountain and Howard's Knob populations of *P. yonahlossee* displaying morphological variation. In addition, significant morphological differences were detected among populations between species. Bat Cave specimens were distinct from both *P. yonahlossee* populations. The other *P. longicrus* populations, Shumont, Grant and Bearwallow Mountain, were also morphologically different than Powderhorn Mountain and Howard's Knob. For the phylogenetic analysis, a 832 base pair portion of the mitochondrial gene, *cytochrome* b, was sequenced and analyzed in PAUP* 4.0. Genetic variation was observed within *P. longicrus* and *P. yonahlossee*. The Bat Cave population was separated from all other populations and showed distinct genetic divergence in the phylogram. The Shumont Mountain and Rock Creek Road populations formed a clade separate from a larger clade, which included a subclade of the remaining southern populations. These results, along with the morphological evidence, suggest that, under the evolutionary species concept, the Bat Cave population is distinct from *P. yonahlossee* should be recongized as *P. longicrus*. Other southern populations are also morphologically distinct from both *P. yonahlossee* and *P. longicrus*. Genetic analysis indicated two morphologically similar new species within the southern populations. One species occurs at Shumont Mountain and Rock Creek Road, while the second includes the remaining southern populations. State and federal endangered status is needed to ensure the protection of these three species because of their restricted ranges.

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DEDICATION

For my grandfather, Thomas Sanderson Coleman, and grandmother, Rebecca Fondren Coleman, who was not able to be here to see the completion of this degree, but whose love is still felt.

For my parents, Mark and Connie Coleman, who despite my best efforts, never allowed me to become an island unto myself. I thank God for the opportunity to call these two people, whom I greatly love and admire, Dad and Mom.

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INTRODUCTION

The Crevice Salamander, *Plethodon longicrus* Adler and Dennis, is currently recognized by the state of North Carolina as a species of special concern due to its limited range in and around the Hickory Nut Gorge of western North Carolina. However, this organism is classified by the scientific community as a geographical variant of *Plethodon yonahlossee* Dunn and is listed by the Peterson Field Guide to Reptiles and Amphibians of Eastern North America as the Bat Cave variation, referring to its type locality (Conant and Collins, 1998). Past studies have examined the taxonomic status of this salamander (Guttman *et al.*, 1978; Blaesing and Hagen, 1983; Justice, 1983) but the question remains as to its evolutionary relationship with *P. yonahlossee.* To understand this problem and its implications, additional historical and technical background is needed.

The twentieth century witnessed a boom in salamander systematics, with morphology providing most of the features for discriminating new species (Arnold, 2000). In the early 1960's, only 16 of the 43 currently named species of the Genus *Plethodon* were recognized in the eastern U.S. (Highton, 1995). It was only during the last 25 years that molecular markers provided powerful new tools for analyzing relationships phylogenetically (Arnold, 2000). Recently, allozyme data for proteins (Highton, 1983; Highton and MacGregor, 1983; Highton, 1999; Highton and Peabody, 2000; Tilley, 2000; Camp *et al.*, 2002) or

DNA sequence data (Moritz *et al.*, 1992; Hillis *et al.*, 2001; Bonett and Chippindale, 2004) have been utilized in these analyses. Molecular markers revealed cryptic species, parapatric distributions, and ancient lineages that were not apparent when morphology alone was used for analyzing variation (Arnold, 2000).

Numerous studies have used molecular markers to examine speciation and evolutionary relationships of salamanders. Richard Highton has championed the use of protein electrophoresis data and genetic distances as the best tools for recognizing species (e.g. Highton, 1989; Highton and Peabody, 2000). Highton (1989) used allozyme data to study 135 populations of the widespread species *Plethodon glutinosus*, and found it to be a complex of 16 distinct species instead of one (Highton, 1989). He argued that these groups have been independent for at least two million years and should be considered sixteen separate species (Highton, 1989). Only two of these species, *P. kentucki* (Mittleman, 1951; resurrected by Highton and MacGregor, 1983) and *P. aureolus* (Highton, 1983), had been described earlier or retrieved from synonymy based on allozyme variation and morphology. Genetic data allowed Highton to determine that no hybridization was taking place when *P. kentucki* was in sympatry with a *P. glutinosus* species. He also concluded that populations of *P. kentucki* had undergone long periods of divergence based on the high amount of genetic distances between them (Highton, 1983). Highton and his colleagues used similar techniques to recognize three species in what was once *P. dorsalis*

(Highton, 1997) and at least six species in what was once *P. jordani* (Highton and Peabody, 2000).

Highton (1999) has also used allozymes to examine variation and speciation in two salamanders of the *Plethodon cinereus* group. He found two distinct groups within both *P. hoffmani* and *P. richmondi*, with the groups in each species showing Nei distances above 0.20, well above his cut off of 0.15 for species level distinction. In *P. hoffmani*, he named the new species *P. virginia*; and in *P. richmondi*, the new species name was *P. electromorphus* (Highton, 1999). Highton (1999) hypothesized that *P. virginia* diverged in isolation from *P. hoffmani* on Shenandoah Mountain since the Pliocene. The New, Kanawha and Teays rivers might have been barriers to diverging populations of *P. richmondi* and *P. electromorphus* promoting speciation in these salamanders during the Pliocene (Highton, 1999). Highton (1999) argued that each species in the *P. cinereus* group was isolated in one or a few of the mountain ranges where they probably arose.

Despite the ability of enzyme electrophoresis to differentiate between closely related species, the phylogenetic analysis of a mitochondrial gene whose product is involved in respiration can provide a finer degree of discrimination due to its conserved nature. Sequence variation has been utilized in numerous systematic studies. Hillis *et al.* (2001) reported a new species of blind subterranean salamander, *Eurycea waterlooensis,* in Barton Spring in Austin, Texas. This morphologically distinct species occurred in the same spring inhabited by *E. sosorum*, another salamander with larval form as an adult

(paedomorphic) described by Chippindale *et al.* in 1993. The analysis of an eleven hundred base pair fragment of the *cytochrome* b gene allowed better reconstruction of the position of *E. waterlooensis* within the group of paedomorphic and metamorphosing species in the Central Texas *Eurycea* (Hillis *et al.*, 2001)

Bonett and Chippindale (2004) clarified the systematic relationships of the salamanders of the *Eurycea multiplicata* complex using variation in two mitochondrial genes, *cytochrome* b and ND4. Previously, the complex was composed of two species, *E. tynerensis* and *E. multiplicata*, which contained two subspecies, *E. m. multiplicata* and *E. m. griseogaster*. The two subspecies were grouped together due to morphology but the two subspecies had different ranges. *E. m. multiplicata* was from the Ouachita Mountains and *E. m. griseogaster* lived in the Ozark Mountains (Bonett and Chippindale, 2004). A cave species, *Typhlotriton spelaeus,* occurred with both *Eurycea* species in the Ozarks (Bonett and Chippindale, 2004). However, the phylogenetic analysis indicated that the evolutionary relationships of the group followed geography instead of morphology. Populations of *E. m. griseogaster* were closer to *E. tynerensis* than they were to *E. m. multiplicata*, so these populations were included under the older name, *E. tynerensis* (Bonett and Chippindale, 2004). Since *T. spelaeus* clustered within *Eurycea*, Bonett and Chippindale (2004) argued for the synonymy of *Typhlotriton* under *Eurycea* and its inclusion in the *E. multiplicata* species group.

Parra-Olea *et al.* (2002) described two new species of salamanders in the *Bolitoglossa macrinii* group based on the phylogenetic analysis of two mitochondrial genes, *cytochrome* b and 16S rDNA. Genetic divergence had been previously observed in unique populations, using allozymes, but the possibility that intermediate populations between these and *B. macrinii* might display genetic continuity with *B. macrinii* prevented its description. The discovery of a population located in the hypothesized intermediate zone enabled the authors to examine the evolutionary relationship (Parra-Olea *et al.*, 2002). The intermediate population clustered with *B. macrinii*, and this clade was highly divergent from the unique populations, described as *B. oaxacensis* (Parra-Olea *et al.*, 2002).

Although the use of molecular markers has gained prominence over morphological analyses in systematic studies, morphometrics has proven successful in discriminating between closely related species. Wilson and Larsen (1999) utilized multivariate statistics to find morphological differences between closely related salamanders of the *P. vandykei* species group. *P. idahoensis* was described as a separate species (Slater and Shipp, 1940) from the older *P. vandykei* (Van Denburgh, 1906) and this distinction was supported by molecular studies despite past contradictory morphological studies (Wilson and Larsen, 1999). Wilson and Larsen (1999) used discriminant function analysis and canonical discriminant analysis and found greater morphological differences between populations of the two species than within either of them. They

recognized both *P. vandykei* and *P. idahoensis* as species (Wilson and Larsen, 1999).

Carr (1996) performed a morphological analysis, incorporating both principal component analysis and discriminant function analysis, of the 16 distinct groups of *Plethodon glutinosus* species complex described by Highton (1989). The results of the principal component analysis suggested that the variation present in the complex is due to overall size, and the author observed the existence of two morphological groups distinguished mostly by those size differences (Carr, 1996). The small-bodied group included all species from the Coastal Plains and *P. aureolus* from the mountains. The large-bodied group included all species from the mountains and the interior areas. *P. kentucki* was morphologically distinct from all others in the *P. glutinosus* species group (Carr, 1996). Even though the discriminant function correctly identified only 61.5% of the specimens, it was able to detect the morphological groups and thus had a higher degree of success in assigning the specimens into the correct morphological group. Of the misclassified Coastal Plains specimens, 77.1% of individuals were classified to another Coastal Plain species. The same was true for 86.3% of the misclassified non-Coastal Plain specimens (Carr, 1996).

Camp *et al.* (2002) discovered a new species similar to and occurring with (sympatric) *Desmognathus quadradmaculatus* using morphometrics and genetics. Their principal component analyses indicated slight but consistent differences in body proportions between the two forms. Their discriminant function analyses were able to identified correctly 91% of the specimens tested.

Covariance analyses found significant difference between the two forms for more than half of the characters measured (Camp *et al.,* 2002). The authors also examined allozyme variation and found fixed differences at four loci between the large and small forms with no genetic exchange. They argued that based on both the molecular and morphological differences the smaller of the two salamanders should be recognized as *D. folkertsi*, a separate species from the larger *D. quadramaculatus* (Camp *et al.,* 2002).

One systematic question that has remained unresolved despite several attempts is the taxonomic status of *Plethodon longicrus*. In 1962, Adler and Dennis described this large plethodontid salamander found in a small area near the Bat Caves at Bat Cave in Rutherford County, N.C. The authors cited two morphological characters, long hind limbs and the number of costal grooves between adpressed limbs, which easily distinguished *P. longicrus* from other large *Plethodon* like *P. glutinosus, P. jordani,* and *P. yonahlossee* (Adler and Dennis, 1962). *P. longicrus* seemed to be the largest species of *Plethodon* with a snout to vent length of up to 101 mm and total length up to 221 mm in the type series. It had large eyes and a flattened head and body that corresponded to its use of crevices at that locality as retreats (Adler and Dennis, 1962). It was a black salamander with scattered white speckling and a variable number of small, dark brown patches on its back. The authors described it as having proportionately the longest legs and the highest number of vomerine teeth of any member in the genus. Adler and Dennis (1962) argued that *P. longicrus* was

most similar to *P. yonahlossee,* another species of *Plethodon* with long legs and a chestnut colored stripe that runs the entire length of the dorsum. The proximity of the salamanders' ranges also indicated their close evolutionary relationship to Adler and Dennis (1962) (Figure 1).

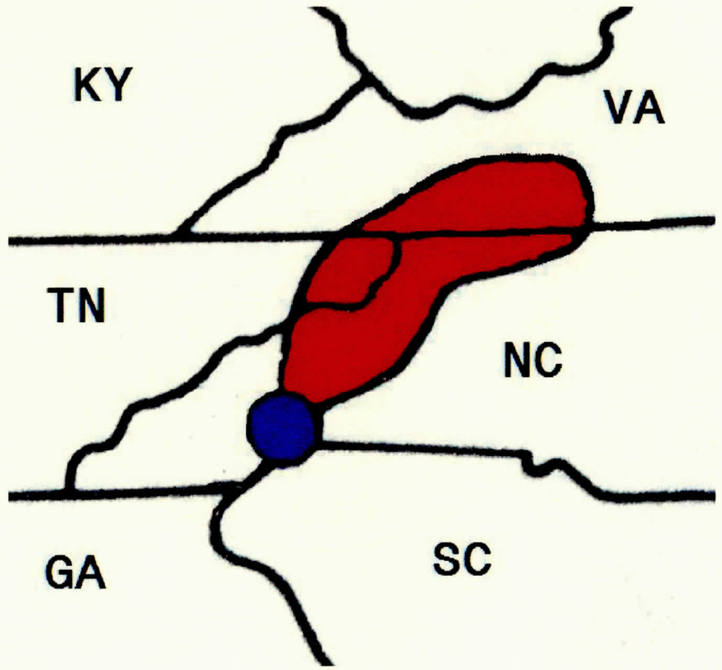

Figure 1. Ranges of *Plethodon longicrus* (blue) and *P. yonahlossee* (red). The map is modified from Conant (1975).

Pleththodon yonahlossee was described by E.R. Dunn (1917) based on animals from Old Yonahlossee Road in Linville, Avery County, North Carolina. The species was a large species of *Plethodon* with long legs, light throat, and a wide chestnut stripe down the back. Dunn (1917) thought *P. yonahlossee* was one of the most primitive species of *Plethodon* because of its low number of costal grooves, striped dorsal pattern, large number of vomerine teeth, and large size. By 1975, it was well known that *P. yonahlossee* occupied moist forests at moderate elevations from southern Virginia to just north of Asheville, Buncombe County, North Carolina, where its range approached that of *P. longicrus* (Conant, 1975).

Highton (1972) argued that *Plethodon longicrus* should be included in synonymy with *P. yonahlossee* (Highton, 1972). He found intermediate animals between Bat Cave and the southernmost *P. yonahlossee* locality in the Swannanoa Mountains. Highton (1972) argued that significant gene flow still occurred between the two salamanders. Highton (1972) stated that, although *P. longicrus* differed in morphology and pigmentation from *P. yonahlossee*, the two salamanders are conspecific.

Several studies in the late 1970's and early 1980's attempted to clarify the systematic relationship between *Plethodon longicrus* and *P. yonahlossee* with limited success (Guttman *et al.*, 1978; Blaesing and Hagen, 1983; and Justice, 1983). Guttman *et al.* (1978) and Blaesing and Hagen (1983) used protein electrophoresis to characterize the genetic structures of populations attributed to the two species but found contradictory results. Guttman *et al.* (1978) compared

three *P. longicrus* populations (Bat Cave in Rutherford County and Grant Mountain and Bearwallow Mountain in Henderson County) and three southern *P. yonahlossee* populations (Lakey Gap in Buncombe County, Grandfather Mountain in Avery County and Unaka Mountains in Unicoi County, Tennessee). Eighteen of their 21 loci were fixed in all populations and similar alleles of the three polymorphic loci were seen in most of the populations. One allele had frequencies that varied with altitude (Guttman *et al.*, 1978). In a critical comparison between sympatric populations of the two species at Bearwallow Mountain, no differences were evident in the proteins examined. Guttman *et al.* (1978) concluded that all populations should be called *P. yonahlossee*. In his The Salamanders of United States and Canada, Petranka (1998) followed Guttman *et al.* (1978) and placed *P. longicrus* in synonymy with *P. yonahlossee*.

Blaesing and Hagen (1983) compared protein variation of animals from the type locality of *Plethodon longicrus* (Bluerock Mountain which included the Bat Caves in Rutherford County) with that found in three northern populations of *P. yonahlossee* (Iron Mountain, Tennessee, Mount Jefferson, North Carolina and Comers Rock, Virginia). They found nine of the thirteen loci to be polymorphic (Blaesing and Hagen, 1983), including three loci reported by Guttman *et al.* (1978) to be monomorphic. The authors concluded that the two groups were undergoing speciation and they should be classified as either subspecies or two distinct species (Blaesing and Hagen, 1983). Blaesing and Hagen (1983) argued that the polyacrylamide gel electrophoresis employed in their study had greater

resolving power than the starch gel electrophoresis utilized by Guttman *et al.* (1978).

Although Guttman *et al.* (1978) also performed a morphological analysis, they included only two characters (snout-vent length and the number of costal folds between adpressed limbs) and found no differences between the two named species. Justice (1983) performed a more comprehensive morphological analysis including 16 characters. He separated the data based on sex and site and then performed an analysis of covariance (ANCOVA) on each character with snout-vent length as the covariate. Characters that differed significantly between the species were then analyzed using discriminant function analysis (DFA). When the data were partitioned into North (*Plethodon yonahlossee*) and South (*P. longicrus*), over eighty percent of all specimens were correctly identified by the discriminant function (Justice, 1983). Based on the morphological variation observed, Justice (1983) argued that *P. longicrus* should be considered a distinct species.

Although these studies have endeavored to elucidate the relationship between *Plethodon longicrus* and *P. yonahlossee*, each of these studies had its own shortcomings and none have resolved the relationship of these two salamanders. Guttman *et al.* (1978) suggested in their study that their use of starch gel electrophoresis might not have had the discriminating power that polyacrylamide gel electrophoresis does. In addition, their morphometric analysis only examined two morphological characters (Guttman *et al.*, 1978). Justice's (1983) morphological analysis was much more extensive; however, he

included only one *P. yonahlossee* population. Although Blaesing and Hagen (1983) performed their electrophoretic study with starch gel electrophoresis, they included *P. yonahlossee* populations only from the northern part of their range. A study that does not contain considerable methodological problems should provide a more reliable answer to the taxonomic status of *P. longicrus*.

The present study attempts to clarify the evolutionary relationship between *Plethodon longicrus* and *P. yonahlossee*. The combination of both a molecular analysis of a conserved gene segment and an extensive morphological analysis of several populations should provide further insight into the taxonomic status of *P. longicrus*.

Materials and Methods

Morphological analysis

Preserved specimens from four *Plethodon longicrus* (the Bat Caves in Rutherford County, Bearwallow Mountain near the county line between Buncombe and Henderson counties, Grant Mountain in Henderson County, and Shumont Mountain at the county line between Buncombe and Henderson counties) and two *P. yonahlossee* (Howard's Knob and Powderhorn Mountain in Watauga County) populations were analyzed (Figure 2). *Plethodon longicrus* and *Plethodon yonahlossee* specimens were borrowed from the Appalachian State University Vertebrate Collection (APPSU) for the morphological analysis. Additional *P. longicrus* specimens from the Bat Cave population were also borrowed from the North Carolina Museum of Natural History Herpetology Collection (NCMNH).

Each specimen was transferred individually to a tray of alcohol for measurement of the characteristics shown in Figure 3 and listed in Table 1. These include measures of head shape (head length, head depth, head width, interocular distance, internarial distance, eye-snout length and eye length) body shape (snout-vent length, interlimb distance, body length and pelvic width), tail shape (tail width and tail height), and parts of limbs (right hind limb length, femur length, fibula length, fourth toe length, tarsal to 4^{th} toe length, 4^{th} toe length,

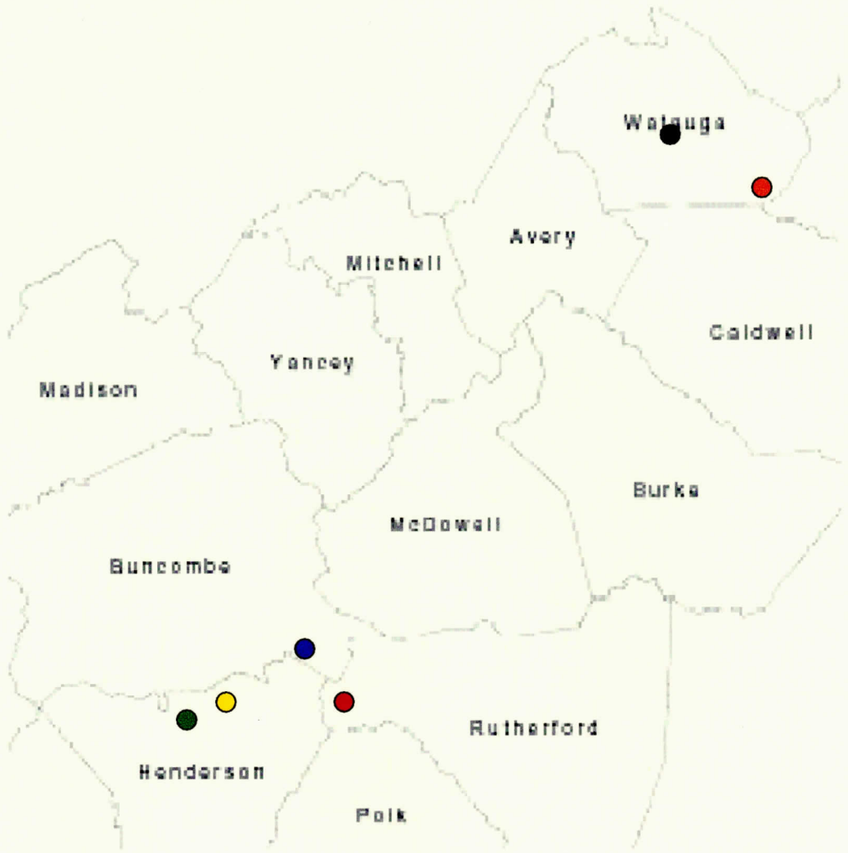

Figure 2. Map showing approximate locations of populations of *Plethodon longicrus* and *P. yonahlossee* sampled for morphological analysis. Populations and colors are: Bat Caves (red), Shumont Mountain (blue), Grant Mountain (yellow), Bearwallow Mountain (green), Howard's Knob (black) and Powderhorn Mountain (orange). Map is modified from http://wind.appstate.edu/maps/wnc.gif.

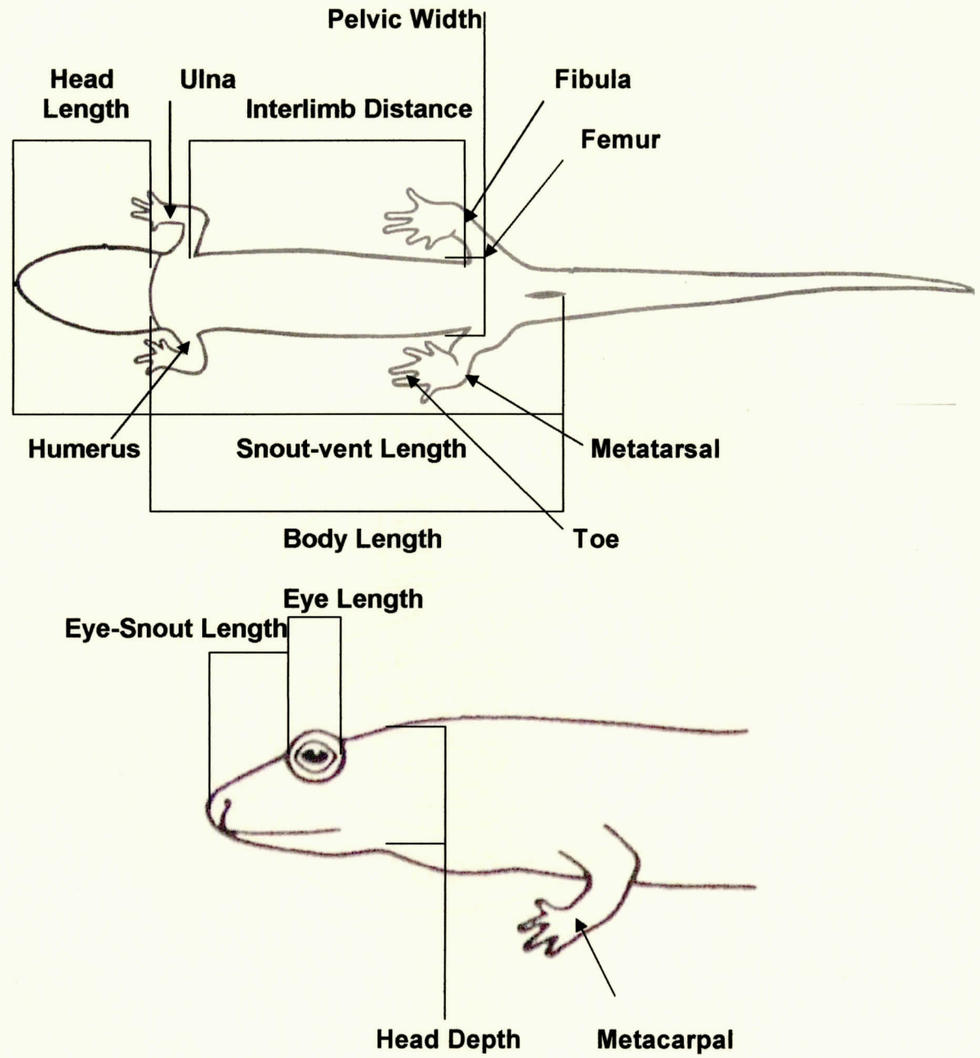

Figure 3. Diagram displaying reference points for the measurements used in the morphological analysis. Characters not shown are head width, interocular distance and internarial distance, tail width at base and tail depth at base. Figures modified from Conant and Collins (1998).

Table 1. Morphological characters and abbreviations collected from each specimen.

Body length (BL)	Interlimb distance (ILD)
Carpal to 3rd finger length (CTFL)	Internarial distance (IN)
Eye length (EL)	Interocular distance (IO)
Eye-snout length (ES)	Pelvic width (PW)
Femur length (FEL)	Right hind limb length (RHL)
Fibula length (FIL)	Snout-vent length (SVL)
4th toe length (FTL)	Tail depth at base (TDB)
Head depth (HD)	Tail width at base (TWB)
Head length (HL)	Tarsal to 4th toe length (TFTL)
Head width (HW)	Ulna length (UL)
Humerus length (HUL)	

humerus length, ulna length and carpal to 3rd finger length). Measurements were made with both digital and manual calipers with an accuracy of .01 mm.

Tail width and height were measured at the first costal fold behind the posterior edge of the vent. The right hind limb length was calculated by adding the femur length, fibula length, tarsal to 4th toe length, and half of the pelvic width. The pelvic width was measured as the distance between the hind limbs' attachment to the body. Head width was measured at the posterior angle of the jaw. Snout-vent length (SVL) was distance from anterior edge of snout to posterior edge of the vent. Body length was measured from gular fold to posterior edge of the vent, and the head length was measured from gular fold to anterior edge of snout. Distance between the inner parts of the swelling of the eyelids constituted interocular distance, while distance between nostrils was internarial distance. The eye-snout length was measured from the anterior edge of the snout to the anterior edge of the eyes. The 4th toe length was distance

from the distal end of the toe to the beginning to the tarsal. Some specimens were measured more than once to ensure accuracy and confidence in data collection.

Each morphological character was regressed against snout-vent length to obtain the residuals of these regressions. Separate regressions were calculated for data sets including: 1) all specimens to test for species level differences, 2) all specimens of a particular sex to test for species level differences for each sex and 3) all specimens of *Plethodon longicrus* to test for differences among populations of this species. Residuals about each regression were separated according to species, sex and population and compared statistically for differences. The residuals of the regressions of all characters were analyzed in the following statistical analyses instead of the original measurements to control for differences in sizes (Camp *et al.*, 2002). Comparisons, excluding within *P. longicrus*, used residuals from all specimens regressions.

Principal component analysis (PCA) was used because it is a multivariate statistical analysis that allowed comparison of correlated data, such as morphological data. PCA utilized the correlated data set in the production of a data set, called principal components, that was uncorrelated (Wiley, 1981). Because the principal components were uncorrelated, they were analyzed independently of each other. Percentage of variance of the original data set related to each component was additive (Wiley, 1981). The effect of each character's contribution to the principal component value was termed its character loading or eigenvector. Varying levels of contribution existed, with

some characters loading more strongly than others on each principal component (Wiley, 1981). A correlation matrix was used in the principal component analyses since the residuals of the characters' regressions were analyzed (Wiley, 1981).

A one-way analysis of variance (ANOVA) was run on the least squared means of the groups' principal component values of first three principal components to determine the presence of significant differences. Plots of the first three principal components were produced to observe how the members of the species or populations clustered on each axis. Degree of separation between species or populations was correlated to morphological differences. Ten PCA's were performed to examine morphological variation between: 1) all specimens, all males and all females separated into *P. longicrus* and *P. yonahlossee*, 2) all specimens of individual populations within species, and 3) all specimens of individual populations between species.

Discriminant function analysis (DFA) was used because it is a statistical analysis that classifies organisms (Wiley, 1981). Since this study attempted to determine if *P. longicrus* is distinct from *P. yonahlossee*, a linear DFA was utilized because this analysis had the ability to detect the presence of two distinct groups within a set of individuals and also to discern which individuals belonged to each group (Wiley, 1981). Initially, each specimen was assigned a priori to either *P. longicrus* or *P. yonahlossee* based on collection site. A discriminant axis that best differentiated between the two groups was produced based on the groups' bivariate means (Wiley, 1981). A discriminant score was then calculated

based on the location on the axis for each group and each specimen. The specimens were assigned to the group whose mean discriminant score was closest to the specimens' discriminant score (Wiley, 1981). Both residuals and original measurements were analyzed with DFA's to determine which data set had a greater ability in differentiating between species or populations. Ten DFA's were run that corresponded to the PCA's. A stepwise DFA was run on the data to find which of the characters or their residuals possessed significant discriminating abilities. All statistical analyses were conducted in SAS (1999) with significance evaluated at $P < 0.05$.

Genetic Analysis

Tail tips were collected from *Plethodon longicrus* individuals from six sites, Bat Cave, Rutherford County, N.C., Shumont Mountain, Buncombe County/Henderson County, N.C., Grant Mountain, Henderson County, N.C., Bearwallow Mountain, Buncombe/Henderson County, N.C. and a rock outcrop on N.C. State Road 74-A, Henderson County, N.C (Figure 4). *P. yonahlossee* tail tips were collected from specimens from four populations: Deck Hill Road, Watauga County, N.C., Old Turnpike Rd., Watauga County, N.C., Howard's Creek Road, Watauga County, N.C., Old Yonahlossee Road, Avery County, N.C. and Beech Mountain, Watauga County, N.C. Tissue was taken from individuals representing two populations that occur within the geographical gap between the known ranges of *P. longicrus* and *P. yonahlossee*: Hebron Road, McDowell County, N.C. and Rock Creek Road, Buncombe County, N.C. Tissue was also collected from two *P. cylindraceus* specimens from Bearwallow Mountain,

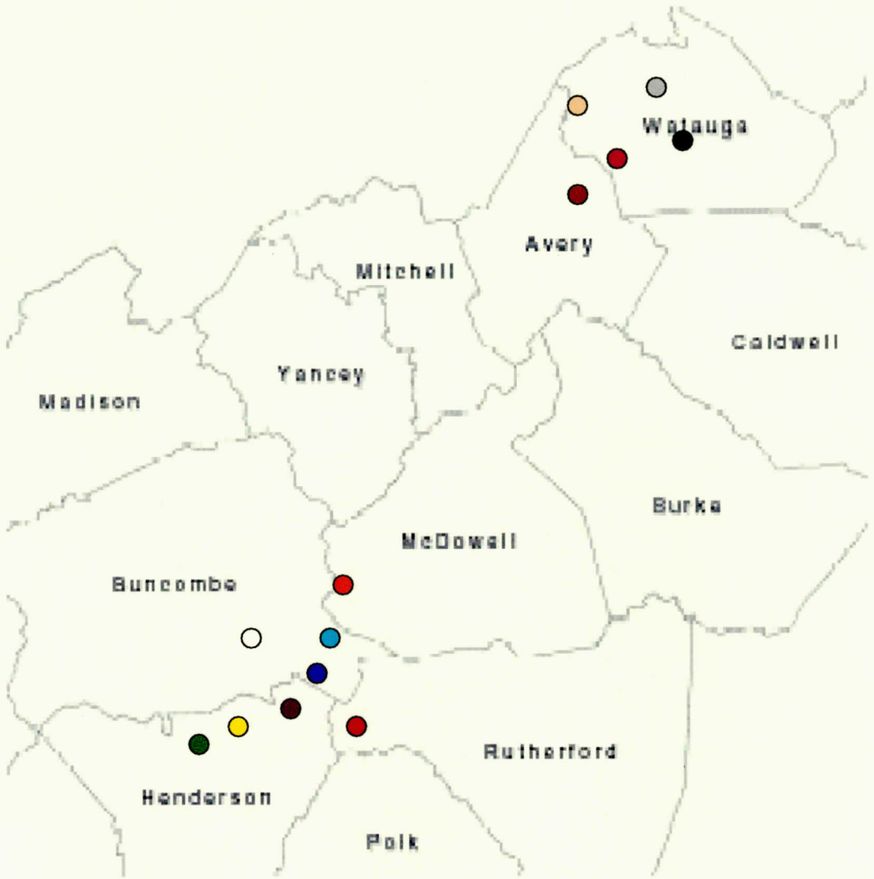

Figure 4. Map showing approximate locations of populations of *Plethodon longicrus*, *P. yonahlossee*, and *P. cylindraceus* used for the genetic analysis. Populations and colors are: Bat Caves (red), Shumont Mountain (blue), Grant Mountain (yellow), Bearwallow Mountain (green), rock outcrop on U.S. 74-A (purple circle), Rock Creek Road (teal), Sand Branch Road (white), Hebron Road (orange), Old Yonahlossee Road (brown), Old Turnpike Road (pink), Deck Hill Road (black), Howard's Creek Road (gray) and Beech Mountain (peach). Map is modified from http://wind.appstate.edu/maps/wnc.gif.

Buncombe/Henderson County, N.C. and Sand Branch Road, Buncombe County, N.C. Tissue were transferred immediately to 95% un-denatured ethanol and stored at room temperature and at -20^0 C.

DNA was extracted using Qiagen DNEasy kits (Qiagen, Inc., Valencia, CA). A small amount of tissue was placed in distilled water for ten minutes. After being soaked, the tissue samples were transferred to a 1.5 ml Eppendorf tube, and 180 µl of ATL buffer and 20 µl of Proteinase K were added to each tube. The tubes were vortexed and incubated overnight in a Precision Scientific water bath (Precision Scientific, Inc., Chicago, Ill.) set at 55°C. Following incubation, 200µl of AL buffer was added, gently mixed, and placed in a 70°C water bath for ten minutes. Next, 200 µl of 100% ethanol was added and mixed. Each sample was then transferred to a DNEasy column and centrifuged at 14,000xg for one minute in an Eppendorf Microcentrifuge 5417C (Brinkmann Instruments, Inc., Westbury, NY). Five hundred microliters of AW1 buffer was added and recentrifuged as above. Then, 500 µl of AW2 buffer was added, and the tubes were centrifuged at 14,000xg for three minutes. The spin columns were then transferred to de-capped 1.5 ml Eppendorf tubes, 200 µl of AE buffer was added, and the tubes were allowed to stand for one minute prior to centrifuging at 14,000xg for one minute. Extracted DNA was transferred to a 1.5 ml Eppendorf tube, and a small amount of each sample was checked on a 1% agarose gel stained with Ethidium Bromide (2ug/ml) and visualized using the Alpha Innotech Digital Imaging and Analysis System (Alpha Innotech Corp., San Leandro, CA.) to check extraction success.

A PCR reaction was performed on the extracted DNA using MVZ 15 (GAACTAATGGCCCACACAorTAorTTACGNAA) and MVZ 16 (AAATAGGAARTATCAYTCTGGTTTRAT) primers for the amplification of an 832 base pair fragment of the *cytochrome* b gene using a TAKARA© PCR kit (TAKARA BioInc., Madison, WI). A master mix was produced with 10.7 µl of sterile distilled water, 2 µl of PCR buffer, 1.6 µl of deoxyribonucleotide triphosphates, 2 µl of MVZ 15, 2 µl of MVZ 16, and .1 µl of *Taq* polymerase for each sample. Each PCR tube received 18 µl of master mix and 2 µl of the appropriate DNA sample. A negative control containing only 20 µl of master mix was also run to check for contamination of the reactions. Reactions were carried out in a Perkin Elmer© GeneAmp PCR 9600 System (Norwalk, CT). Parameters for all PCR reactions were 1 cycle of 95°C for 3 minutes, 45°C for 1 minute, and 72°C for 1 minute, 44 cycles of 95°C for 30 seconds, 45°C for 45 seconds, and 72°C for 1 minute, and 1 cycle of 72°C for 20 minutes. Reaction tubes were then held at 4^0C until removed.

The PCR products were assessed on a 1% agarose gel with *PhiX*174/*Hae*III size markers (Promega Corp., Madison, WI) stained and visualized as above to check for successful reactions. If the negative control had no PCR products, other lanes were checked for presence of a band in the 800 base pair range. Samples with these bands were assumed to have the desired *cytochrome* b DNA fragment. The PCR products were gel extracted using Montage Millipore© (Millipore Corp., Bedford, MA) gel extraction kits following manufacturer's instructions and suspended in 20 µl of TE buffer (Tris, EDTA).

The PCR products were cloned using Invitrogen© pCR 2.1 or pCR 4.0 cloning vector and chemically competent One Shot TOPO10 *Escherichia coli* (*E. coli*) cells (Invitrogen Life Technologies, Carlsbad, CA). For each cloning reaction, a solution of 1 ul of vector, 1 µl of salt solution, and 4 µl of the DNA sample was added to a sterile PCR tube. The PCR tubes were incubated at room temperature for twenty minutes, then 2 µl of each solution was added to 25 µl of TOPO 10 *E. coli* cells in 1.5 ml Eppendorf tubes and placed on ice for twenty minutes. Cells were then heat shocked at 42°C for 30 seconds and returned to the ice. Two hundred and fifty µl of S.O.C. medium was added to the cells before incubation for 90 minutes at 37° C and 200 rpm in an INNOVA 4000 incubator shaker (New Brunswick Scientific, Edison, NJ). Transformed cells were plated on Luria Broth Agar plates containing ampicillin (LBAmp) (0.05 mg Amp/ml of LBagar) previously spread with 40 µl of 5-bromo-3-indoyl-beta-D-galactoside (X-gal) (Acros Organics, Geel, Belgium). Plates were incubated overnight at 37°C in an air Isotemp Incubator (Fisher Scientific, Atlanta, GA) and screened for white colonies.

Four white colonies from each plate were streaked onto new LBAmp agar plates and cultured as above. To check these colonies for the insert containing the *cytochrome* b fragment, a group of cells was picked and placed in an Eppendorf tube containing 100 µl of PCR quality water. Plasmid DNA was extracted using the boiling miniprep method (Ausubel *et al.*, 1989). Tubes were placed in a boiling water bath for five minutes and spun in the Eppendorf Microcentrifuge 5417C at 13,000xg for 2 minutes. Ninety µl of the supernatant

was transferred to another 1.5 ml Eppendorf tube and the DNA precipitated using 237 µl of 100% ethanol and 9 µl of 3M NaOAc. Tubes were placed in the -20°C freezer overnight or in the -80°C freezer for thirty minutes. Plasmid DNA was pelleted by centrifugation at 17,000xg in the Eppendorf Microcentrifuge 5417C for 15 minutes, washed in 237 µl of 70% ethanol, and centrifuged at 17,000xg in the Eppendorf Microcentrifuge 5417C for five minutes. DNA was dried in the DNA speed vacuum (Savant Instruments Inc., Farmingdale NY) for five minutes and suspended in 20 µl of TE buffer.

A restriction digest was run on the isolated DNA to confirm the presence of the cloned insert. Plasmid DNA was digested for three hours at 37°C with 2 µl of *Eco*RI in 2 µl of restriction buffer and 16 µl of suspended DNA. The presence of an 832 bp band was confirmed on a 1% agarose gel using *Phi*X174/*Hae*III size markers.

A sizable proportion of colonies of cells confirmed to have cloned inserts were cultured overnight in LBAmp broth at 37°C with shaking in an INNOVA 4000 incubator shaker (New Brunswick Scientific, Edison, NJ). Plasmid DNA was isolated using the Alkaline Lysis method (Ausubel *et al.*, 1989) and 2 ml of cell suspension. Cells were pelleted by centrifugation at 13000xg in an Eppendorf Microcentrifuge 5417C, resuspended in GTE (50mMGlucose; 25mM Tris, pH 8.0; 10mM EDTA), lysed with SDS/NaOH(1% Sodium dodecyl sulfate; 0.2M Sodium hydroxide). Potassium acetate (3M KOAc) was added, and the tubes incubated on ice for five minutes. The plasmid DNA was precipitated with Isopropanol and pelleted by centrifugation at 13,000xg in an Eppendorf

Microcentrifuge 5417C. The DNA was washed two times using 100% followed by 70% ethanol and dried in a DNA speed vacuum. Isolated plasmid DNA was suspended in 30 µl of TE (Tris /EDTA) buffer and stored in a -20°C freezer until it could be sequenced.

Cloned DNA was sequenced using the Sanger Method (Ausubel *et al.*, 1989), an Epicentre Thermosequenase kit (Epicentre Biotechnologies, Madison, WI), and IR-labeled primers (LI-COR Biosciences, Lincoln, NB). Each sequencing reaction consisted of 6.1 µl sequencing master mix and 2.5 µl of sample DNA. Sequencing master mix contained 3.6 µl reaction buffer, 2 µl of IR_{700} and IR_{800} forward and reverse sequencing primers (2.5mM), and 0.5 µl of thermosequenase for each sample to be sequenced. Two µl aliquots of sequencing reaction mix were added to each of four tubes containing 1 µl of the appropriate chain terminator mix. The sequencing reaction was carried out in the Perkin Elmer GenAmpPCR 9600 System with the following parameters: an initial melt cycle at 92°C for three minutes followed by thirty-three cycles of 92°C for thirty seconds, 54°C for forty-five seconds, and 72°C for two minutes. Upon completion, 3 µl of LI-COR stop solution was added to each tube and the tubes were stored at -20°C until sequencing.

Sequencing was performed using the Global Edition IR DNA Analyzer Gene ReadIR 4200 (LI-COR Biosciences). Sample sequences were analyzed using e-Seq DNA Sequencing and Analysis Software (LI-COR Biosciences) and edited using Align IR v2.0 (LI-COR Biosciences). Multiple copies of each sequence were compared for accuracy and sequences edited where appropriate.

Sequence identity was confirmed using a BLAST search at the National Center for Biotechnology Information Website (http://www.ncbi.nlm.nih.gov). Sequences from all sampled specimens were aligned using Align X (Vector NTI Suite, Informax, Bethesda, MD) and sequences trimmed to the same length. Aligned sequences were loaded into McClade 4(Sinauer Associates, Inc., Sunderland, MA) and analyzed using PAUP v4.06b (Phylogenetic Analysis Using Parsimony, Swofford, 2000). A maximum parsimony heuristic search was performed and produced a 50% majority-rule consensus trees from a group of equally parsimonious trees. A bootstrap analysis was also performed with 100 replicates, and the maxtrees were set at 50,000.

RESULTS

Morphological Analysis of *Plethodon longicrus* and *P. yonahlossee* Populations

A total of 160 specimens from six populations were measured (Table 2, Appendix A). These included 108 animals from four populations called *Plethodon longicrus* because of their southern distribution, dorsal white spotting and interrupted, brownish dorsal stripes. With the exception of the Bat Cave site, 13 males and 13 females were measured for each population.

Table 2. Samples sizes for each population measured for morphological analysis.

Population	Total	Female	Male
Plethodon longicrus	108	58	50
Bat Cave	30	19	11
Bearwallow Mountain	26	13	13
Grant Mountain	26	13	13
Shumont Mountain	26	13	13
Plethodon yonahlossee	52	26	26
Powderhorn Mountain	26	13	13
Howard's Knob	26	13	13
Total	**160**	**84**	**76**

In the PCA examining morphological variation between all specimens of *Plethodon longicrus* and *P. yonahlossee*, there was a significant difference between species only with respect to principal component (PC) 1 (F = 8.00, P = 0.047), but this difference accounted for 24.11% of the total variation. Least squared means for PC 1 were 0.470 for *P. longicrus* and -1.040 for *P. yonahlossee*. Most of the variation was associated with those characters that contributed most heavily to the eigenvalue describing PC 1 (Table 3). *P. longicrus* populations had longer legs and heads and wider snouts than did the *P. yonahlossee* populations, but there was considerable overlap between populations in the plot of PC 1 and PC 2 (Figure 5).

A significant species difference was observed in the PCA for females in relation to PC 1 (F = 8.01, P = 0.047). Least squared means for PC 1 were 0.494 for *P. longicrus* and -1.270 for *Plethodon yonahlossee*. Based on the characters with the highest eigenvectors for PC 1 (Table 3), *P. longicrus* females had longer hind and fore limbs and longer toes. No significant species difference was detected in the PCA for males with respect to any of the principal components.

The PCA's examining morphological variation between populations within species found two significant site differences. Significant variation was detected in the PCA that compared Bat Cave population to the other *Plethodon longicrus* populations (Bearwallow Mountain, Grant Mountain and Shumont Mountain) with respect to PC 1 (F = 10.21, P = 0.002) and PC 3 (F = 5.57, P = 0.020). The two principal components accounted for 33% of the

Table 3. Highest three eigenvectors that contributed to significant principal components in analyses of *Plethodon longicrus* and *P. yonahlossee* populations.

Analysis	n	Significant PC	Character	Eigenvectors
All	160	1	RHL	0.429
			IND	0.318
			HL	0.307
Female	84	1	RHL	0.367
			UL	0.331
			FTL	0.322

total variance within the data set. Population means for PC 1 were 1.014 for Bat Cave and -0.390 for other *P. longicrus* populations, and population means for PC 3 were 0.515 for Bat Cave and -0.198 for other *P. longicrus* populations. Based on these values and highest three eigenvectors for PC 1 and PC 3 (Table 4), the Bat Cave specimens displayed wider and longer heads as well as longer bodies and limbs than the other *P. longicrus* specimens. No significant site differences were observed in the PCA that examined variation between the Shumont Mountain population and Bearwallow Mountain plus Grant Mountain populations.

Significant morphological variation was detected in the PCA between the *Plethodon yonahlossee* populations in relation to PC 2 (F = 9.81, P = 0.003) and PC 3 (F = 10.06, P = 0.003). Over 28% of the total variance in the data set was explained by these principal components. Population means for PC 2 was 0.695 for Powderhorn Mountain and -0.695 for Howard's Knob. The characters that

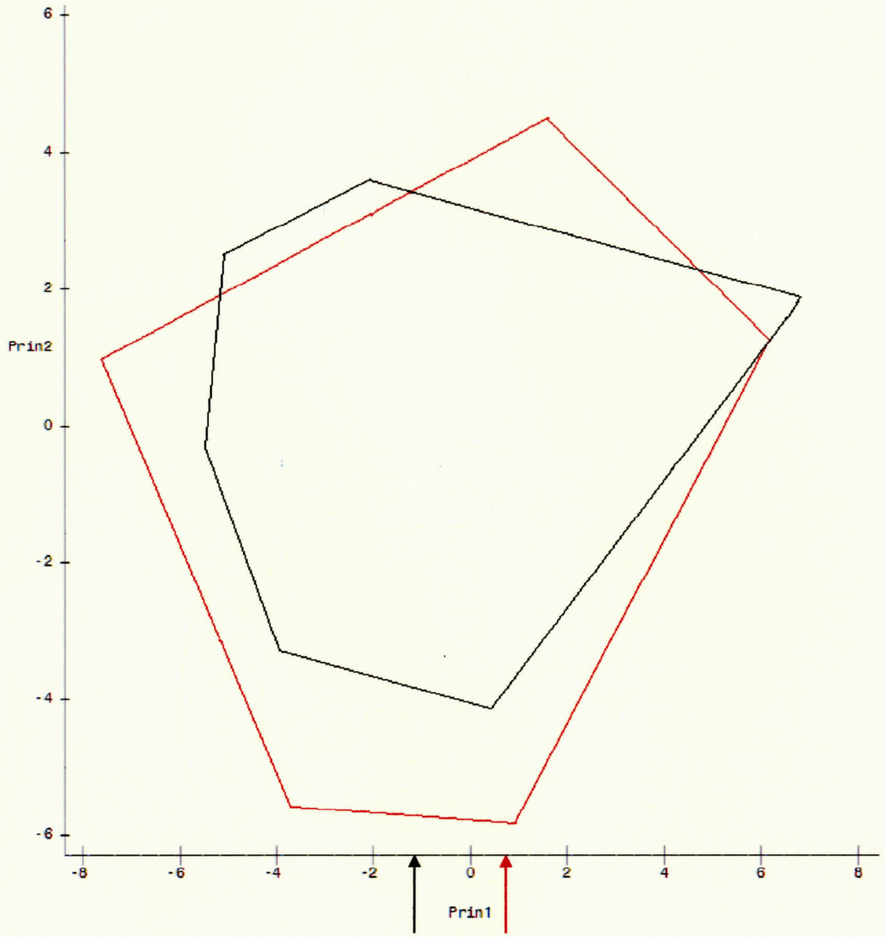

Figure 5. Plot of first and second principal components of PCA of 160 specimens of *Plethodon longicrus* (Red) and *P. yonahlossee* (Black). A significant species difference was found with respect to PC 1 (P = 0.047). Arrows indicate population least squared means. No significant difference was found in relation to PC 2.

contributed most strongly to the eigenvalue of PC 2 were HD, TDB and TWB (Table 4). Powderhorn Mountain specimens displayed thicker heads and tails and wider tails. Population means for PC 3 were 0.670 for Powderhorn Mountain and -0.670 for Howard's Knob. Based on the eigenvectors of PC 3 (Table 4), the Howard's Knob specimens had shorter hind limbs and bodies compared to the Powderhorn specimens. Separation of the two populations was evident on both significant principal components (Figure 6).

Table 4. Highest three eigenvectors of significant principal components examining variation among populations within species.

Analysis	n	Significant PC	Character	Eigenvectors
Bat Cave vs. other *Plethodon longicrus*	108	1	HW	0.339
			HW	0.316
			IN	0.309
		3	BL	0.461
			ILD	0.398
			RHL	0.309
Howard's Knob vs. Powderhorn Mountain	52	2	THB	0.489
			TDB	0.457
			HD	0.384
		3	RHL	0.373
			ILD	0.373
			FIL	0.357

The PCA's also detected significant site differences among populations between species. Significant morphological variation was observed in the comparisons of Bat Cave separately to each *Plethodon yonahlossee* populations.

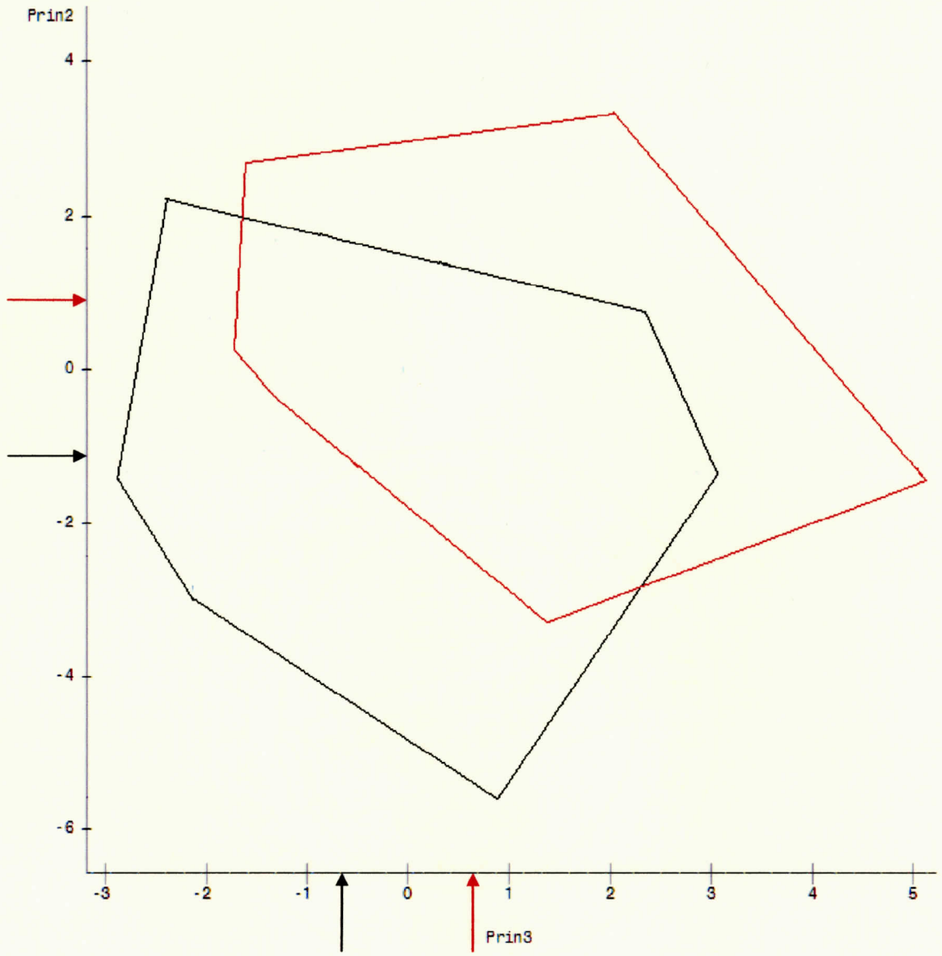

Figure 6. Plot of second and third principal components of PCA of 52 specimens of Powderhorn Mountain (Red) and Howard's Knob (Black). A significant site difference was found with respect to PC 2 (F = 9.81, P = 0.003) and PC 3 (F = 10.06, P = 0.003). Arrows indicate population means.

In the first comparison, the PCA found a significant species difference between Bat Cave and Powderhorn Mountain with respect to PC 1 (F = 13.83, P = 0.0005), which accounted for 25% of the original variance. Population means for PC 1 were 0.938 for Bat Cave and -1.082 for Powderhorn. The characters that had the highest eigenvectors for PC 1 were IN, TFTL and RHL (Table 5).

A significant species difference was also observed with respect to PC 1 (F = 30.20, P <0.0001) when specimens of Bat Cave and Howard's Knob were compared in PCA. The amount of variance explained by this principal component was 25.84%. Population means for PC 1 were 1.256 for Bat Cave and -1.450 for Howard Knob specimens. The characters IN, RHL and HW displayed the highest eigenvectors for PC 1 (Table 5). Pronounced separation of the populations on the first principal component axis in the plot of PC 1 and PC 2 demonstrated the significant morphological differences (Figure 7). In both comparisons, the Bat Cave specimens had longer legs and wider snouts than the specimens of the two *P. yonahlossee* populations.

The PCA comparing the southern "*Plethodon longicrus*" populations of Shumont, Grant and Bearwallow Mountain, with Powderhorn Mountain population found significant morphological differences in relation to PC 2 (F = 7.27, P = 0.042). This principal component accounted for over 15% of the total variance in the original data. Population means for PC 2 were -0.407 for *P. longicrus* and 1.222 for Powderhorn Mountain. The characters that had the highest eigenvectors were TWB, THB and HD (Table 5), so Powderhorn

Mountain specimens had thicker heads and tails in addition to wider tails than Shumont, Grant and Bearwallow specimens. No significant species difference was detected by the PCA when Shumont, Grant and Bearwallow Mountain populations were compared to Howard's Knob.

Table 5. Highest three eigenvectors of significant principal components in analyses examining variation among populations between species.

Analysis	n	Significant PC	Character	Eigenvectors
Bat Cave vs. Powderhorn Mountain	52	1	IN	0.336
			TFTL	0.304
			RHL	0.300
Bat Cave vs. Howard's Knob	52	1	IN	0.345
			RHL	0.325
			HW	0.305
Shumont, Grant and Bearwallow Mountain vs. Powderhorn Mountain	52	2	THB	0.466
			TWB	0.443
			HD	0.397

Four to seven characters contributed significantly to the DFA's that examined morphological variation between species (Table 6). When the residuals of all specimens were analyzed, the discriminant function correctly classified over 77% of the specimens as opposed to over 81% when the original measurements were analyzed (Table 7). In the male DFA, the use of the original measurements again resulted in a higher percentage of specimens correctly classified, but over 80% of the male specimens were correctly classified no

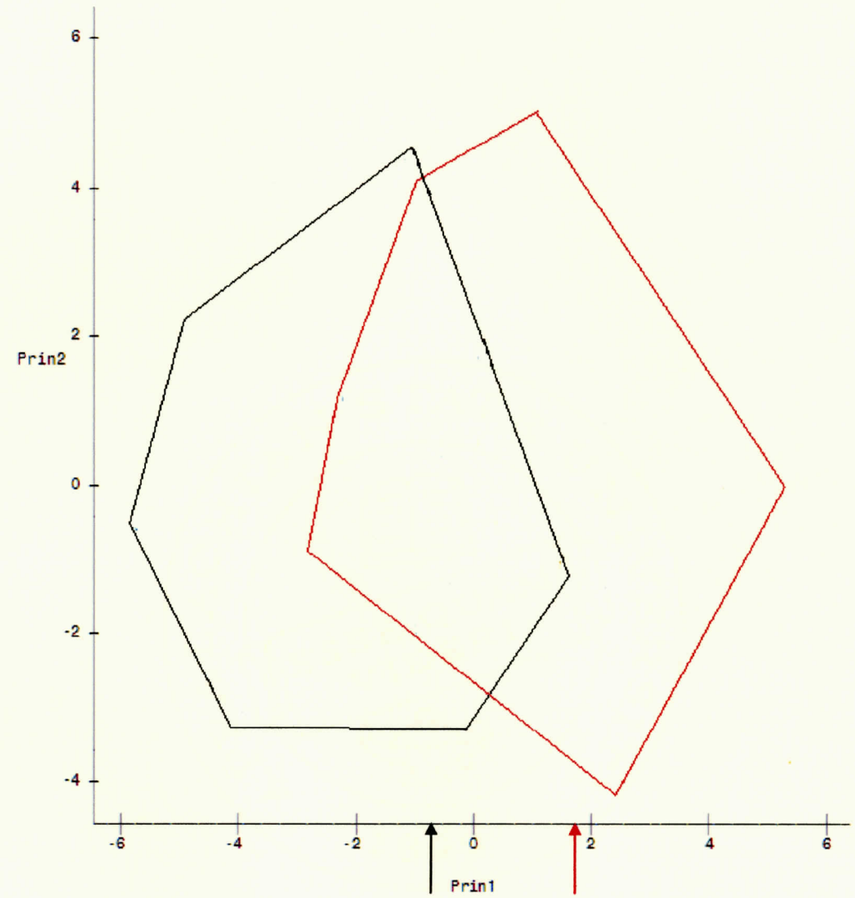

Figure 7. Plot of first and second principal components of PCA of 52 specimens of Bat Cave (Red) and Howard's Knob (Black). A significant site difference was found with respect to PC 1 ($F = 30.20$, $P < 0.0001$). Arrows indicate population means. No significant difference was detected in relation to PC 2.

Table 6. Characters retained by stepwise discriminant function analysis for populations of *Plethodon longicrus* and *P. yonahlossee* (Resid=residuals, Meas=original measurements).

Analysis	Data	Characters
All	Resid	TWB, IO, ESL, HL, RHL, ILD
	Meas	IN, TWB, IO, RHL, ESL, HL
Male	Resid	TWB, IO, ESL, PW, FIL
	Meas	ESL, IO, FIL, TWB
Female	Resid	IN, ILD, TFTL, TDB
	Meas	IN, TWB, IO, ILD, RHL, TFTL, PW
Bat Cave vs. other *P. longicrus*	Resid	PW, HD, FEL, ESL, UL, TWB, ILD
	Meas	PW, HD, UL, ILD, ESL, TWB, FEL
Shumont vs. Bearwallow and Grant Mountain	Resid	IO, HD, PW, ES, TWB, UL, HW, TFTL, EL
	Meas	PW, ES, EL, TWB, HD, IO, HW
Howard's Knob vs. Powderhorn Mountain	Resid	TWB, THB, HW, IO
	Meas	TWB, THB, HW, IO, TFTL
Bat Cave vs. Powderhorn Mountain	Resid	TWB, HD, UL, FIL, ILD
	Meas	UL, TWB, HD, TFTL, RHL, THB
Bat Cave vs. Howard's Knob	Resid	PW, ILD, IO, FTL, UL, ES
	Meas	PW, IO, ILD, FTL, HL, HUL, UL
Shumont, Grant and Bearwallow Mountain vs. Powderhorn Mountain	Resid	TWB, BL, ES, ILD, FEL, EL, HD, TFTL, FTL
	Meas	TWB, ES, TFTL, THB, BL, FTL, ILD
Shumont, Grant and Bearwallow Mountain vs. Howard's Knob	Resid	IO, FTL, THB, ES, HL
	Meas	IO, THB, FTL, ES, HL

matter which data was analyzed. When females were analyzed, 76% and 81% of the individuals were correctly identified when the residuals and original measurements were used, respectively.

Table 7. Discriminant function analyses of morphological variation between *Plethodon longicrus* and *P. yonahlossee*.

Analysis	n	Residuals (% correctly classified)	Measurements (% correctly classified)
All	160	77.50%	81.25%
Male	76	80.26%	86.84%
Female	84	76.19%	80.95%

DFA's examining morphological differences among populations within species utilized variation present in several characteristics (Table 6). The DFA successfully discriminated 77% of specimens when the Bat Cave population was compared to the other *Plethodon longicrus* populations using the residuals (Table 8). Almost 88% of the specimens were correctly identified when the original measurements were analyzed. No significant morphological variation was detected by the DFA examining variation between the Shumont Mountain and Bearwallow plus Grant Mountain populations. The discriminant function was successful in correctly classifying 94% and 98% of Powderhorn Mountain and Howard's Knob specimens depending on whether residuals or original measurements, respectively, were used (Table 8).

Table 8. Discriminant function analyses examining morphological variation among populations within species.

Analysis	n	Residuals (% correctly classified)	Measurements
Bat Cave vs. *P. longicrus* (n=108)	108	77.77%	87.96%
Shumont vs. Bearwallow and Grant Mountain	78	57.69%	71.79%
Howard's Knob vs. Powderhorn Mountain	52	94.23%	98.07%

The morphological variation among populations between species detected by the DFA's included five to nine characters (Table 6). The DFA examining variation between Bat Cave and Powderhorn Mountain populations correctly identified over 82% and 86% of the specimens when the residuals and original measurements, respectively, were analyzed (Table 9). A higher percentage of specimens were correctly identified when the Bat Cave and Howard's Knob populations were analyzed, 89% and 95% (Table 9).

When the southern populations of Shumont, Grant and Bearwallow Mountain were compared to Powderhorn Mountain, over 87% of the specimens were correctly classified in the DFA when either residuals or original measurements were used (Table 9). Although the PCA did not detect significant morphological variation between Shumont, Grant and Bearwallow Mountains and Howard's Knob, the DFA was able to correctly classify 84% of the specimens

belonging to these populations (Table 9), using original measurements, indicating morphological variation does exist between these populations.

Table 9. Discriminant function analyses examining morphological variation among populations within species.

Analysis	n	Residuals (% correctly identified)	Original Measurements
Bat Cave vs. Powderhorn Mountain	56	82.14%	85.71%
Bat Cave vs. Howard's Knob	56	89.29%	94.64%
Shumont, Bearwallow and Grant Mountain vs. Powderhorn Mountain	104	88.46%	87.59%
Shumont, Bearwallow and Grant Mountain vs. Howard's Knob	104	78.85%	84.00%

Genetic Analysis of *Plethodon longicrus* and *P. yonahlossee* Populations

High molecular weight DNA was successfully extracted from 53 *Plethodon* specimens (Table 10) and confirmed by gel electrophoresis (Figure 8A). Successful PCR amplification of the desired 832 base pair fragment of the *cytochrome* b gene was also confirmed for all 48 *Plethodon* specimens using a 1% agarose gel. Fragments in the 800 base pair range were assumed to be the desired fragment (Figure 8B). Successful cloning of PCR product was determined for 41 *Plethodon* specimens after an *Eco*RI digestion (Figure 8C). Fragments in the 800 base pair range signified successful cloning reactions.

Sequences were obtained for 30 *Plethodon* specimens but only 24 of these could be fully aligned and analyzed.

Table 10. Specimens sampled for genetic analysis.

Species	Population	DNA	PCR	Clone	Sequence
Plethodon longicrus					
	Bat Caves	5	3	3	3
	Bearwallow Mountain	7	6	5	3
	Grant Mountain	6	6	4	3
	Shumont Mountain	7	6	5	2
	Rock Creek Road	5	4	4	3
	Rock outcrop on U.S. 74-A	2	2	2	2
Plethodon yonahlossee					
	Old Yonahlossee Road	5	5	4	3
	Deck Hill Road	4	4	4	3
	Hampton's Creek Road	5	5	4	2
	Old Turnpike Road	1	1	1	1
	Hebron Road	4	4	3	3
	Beech Mountain	1	1	1	0
Plethodon cylindraceus					
	Bearwallow Mountain	1	1	1	1
	Sand Branch Road	1	1	1	1

The phylogenetic analysis using PAUP* 4.0 was performed using 24 individuals including, 13 assigned to *Plethodon longicrus,* 9 assigned to *P. yonahlossee*, and 2 *P. cylindraceus*. The length of the *cytochrome* b gene fragment analyzed was shortened to 700 base pairs to ensure equal length for each sample. The maximum parsimony heuristic search on 654 of these characters found 89 to be parsimony informative. A total of 117 equally parsimonious trees were constructed each with a consistency index of 0.9462

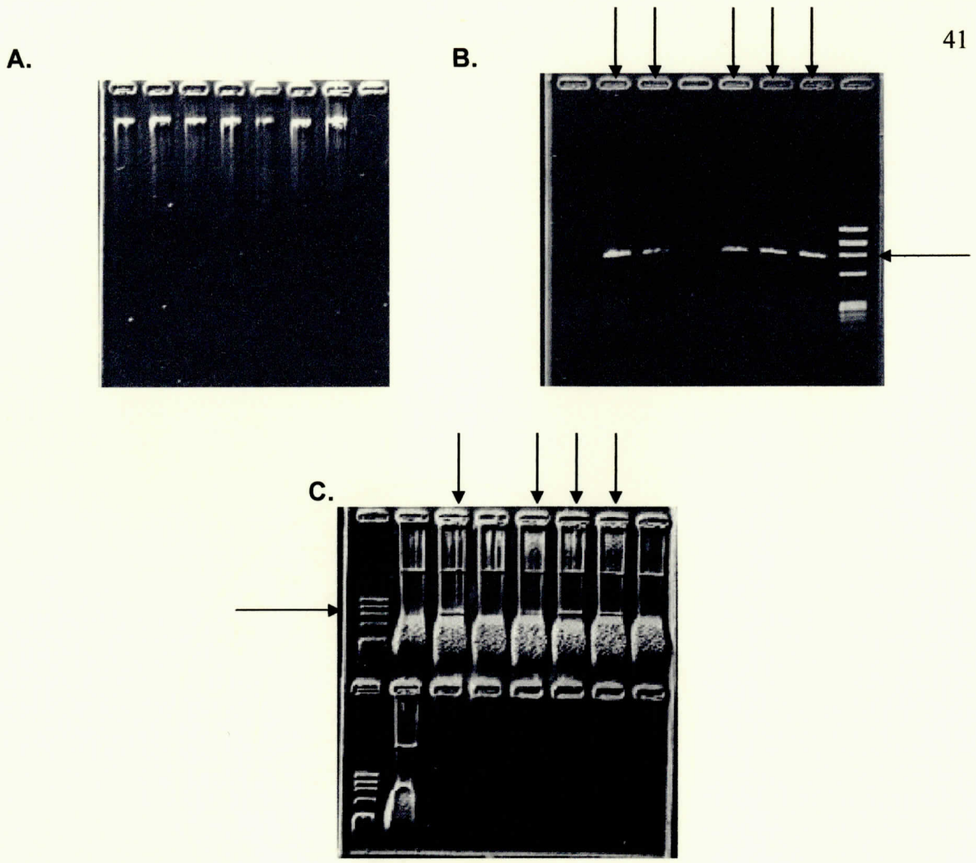

Figure 8. A. Seven successful high molecular weight DNA extractions in lanes 1-7.B. PCR product confirmation of 832 base pair amplification product of *cytochrome* b gene and *Phi*X174/*Hae*III size markers (Lane 8). Any band associated with the third band of the size markers was approximately 800 base pairs in length and the desired PCR product. Five successful products were observed in lanes 2, 3, 5, 6 and 7. C. Restriction digests confirming successful cloning of 832 base pair *cytochrome* b product and *Phi*X174/*Hae*III size markers (lane 1 and 9). Any band associated with the third band of the size markers was understood to signify successful cloning reactions. The results of successful cloning reactions were observed in lanes 3, 5, 6 and 7. All gels were 1% agarose stained with ethidium bromide.

and a retention index of 0.9466. The 50% majority-rule consensus tree (Figure 9) had several important features. The clade including all *P. longicrus* and *P. yonahlossee* specimens was present in all (100%) of the most parsimonious trees. The three Bat Cave individuals clustered together in all (100%) parsimonious trees and were a sister group to all other *P. longicrus* and *P. yonahlossee* populations, but that particular sister group was seen in only 67% of most parsimonious trees. Two separate branches of the larger clade were found in all (100%) most parsimonious trees. One of these clades included one animal from Shumont Mountain and two from Rock Creek and was the sister group to the clade including all remaining individuals. These remaining individuals formed an unresolved trichotomy, with the Old Turnpike Road specimen representing one unsupported branch. The other two branches of the trichotomy include all other northern specimens in one group (Old Yonahlossee Road, Howard's Creek Road and Deck Hill Road) and all southern specimens in the other (Bearwallow Mountain, Grant Mountain, rock outrcrop on U.S. 74-A and Hebron Road).

The bootstrap analysis (Figure 10) shared many of the same characteristics with the consensus tree. The *Plethodon yonahlossee* plus *P.* longicrus clade was supported in all trees. This clade included a trichotomy of branches with modest (53%), good (74%) or high (100%) support. The well supported clade included only the three Bat Cave animals. The moderately well supported clade included animals from Shumont Mountain and the nearby Rock Creek Road. The modestly supported clade included all remaining northern and

southern populations. The southern populations fell within a subclade separate from all northern populations with bootstrap support of 56%.

The phylogram constructed in PAUP* 4.0 (Figure 11) projected the changes of the 89 parsimony informative characters onto a tree showing degree of divergence. The Bat Cave specimens were tightly clustered and they were separated from all other populations by a distance of 82 changes. The remaining populations shared a very long branch (74 changes/units) with a small number of changes/units between individuals within the clade. The Rock Creek Road plus Shumont clade was again present as the sister to the *Plethodon yonahlossee* plus southern populations clade, but was supported by a distance of only three units. Southern and northern populations were segregated on the phylogram.

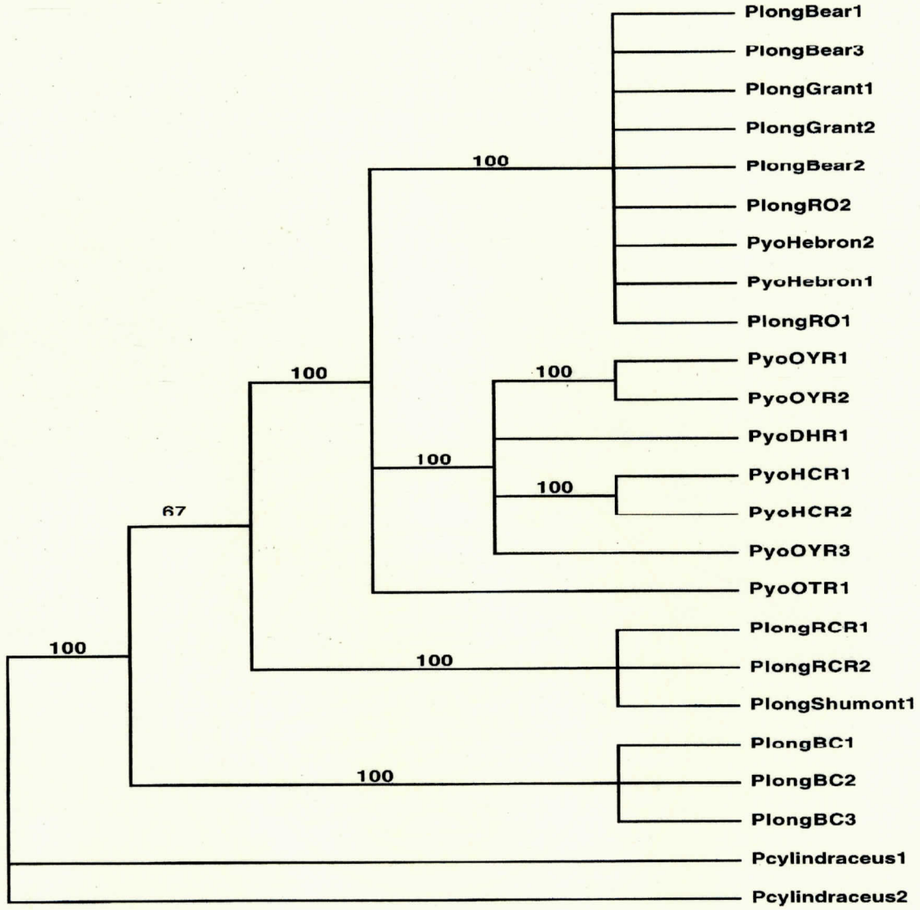

Figure 9. The 50% majority rule consensus tree produced from 117 equally parsimonious trees using a maximum parsimony heuristic search and an aligned 654 base pair fragment of the *cytochrome* b gene from 13 *Plethodon longicrus* (Plong) and 9 *P. yonahlossee* (Pyo), using *P. cylindraceus* (Pcylindraceus) as an outgroup. Populations are designated in the tree as follows: Bear=Bearwallow Mountain, Grant=Grant Mountain, RO=Rock Outcrop on U.S. 74-A, Hebron=Hebron Road, OYR=Old Yonahlossee Road, DHR=Deck Hill Road, HCR=Hampton's Creek Road, OTR=Old Turnpike Road, RCR=Rock Creek Road, Shumont=Shumont Mountain, BC=The Bat Caves. Numbers indicate individual samples.

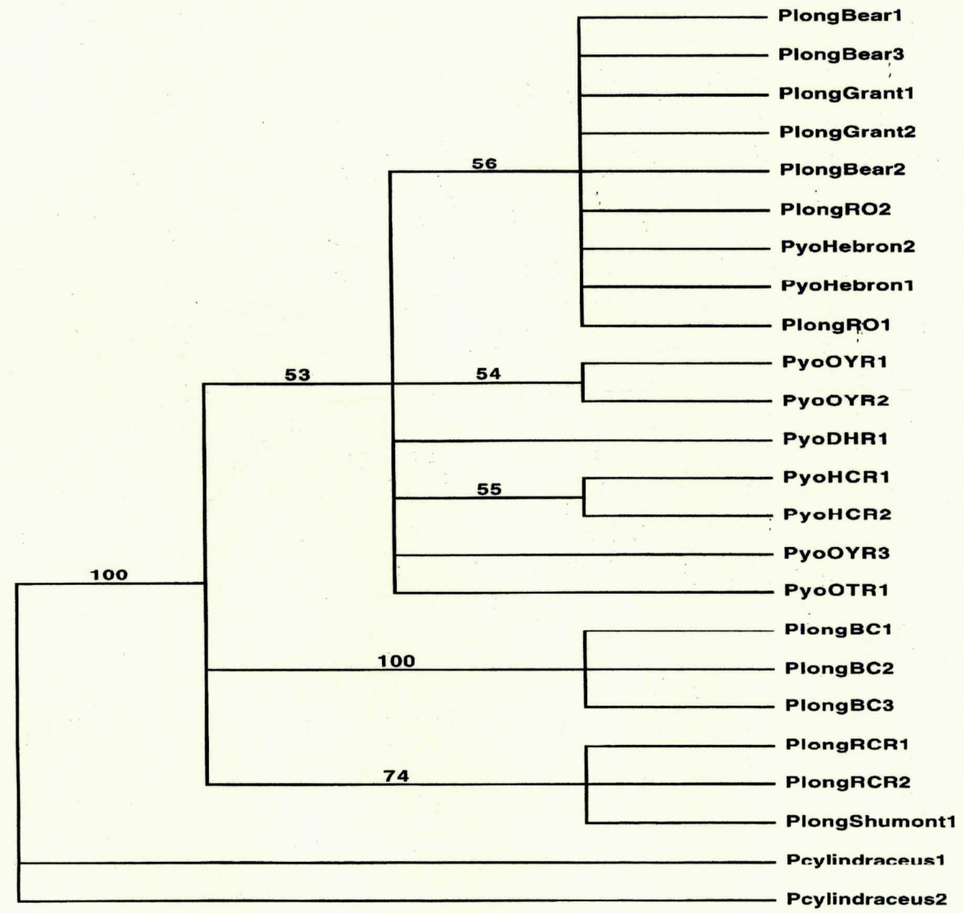

Figure 10. Cladogram generated by bootstrap analysis using a maximum parsimony heuristic search and an aligned 654 base pair fragment of *cytochrome* b gene from 13 *Plethodon longicrus* (Plong), 9 *P. yonahlossee* (Pyo), using *P. cylindraceus* (Pcylindraceus) as an outgroup. Populations are designated in the tree as follows: Bear=Bearwallow Mountain, Grant=Grant Mountain, RO=Rock Outcrop on U.S. 74-A, Hebron=Hebron Road, OYR=Old Yonahlossee Road, DHR=Deck Hill Road, HCR=Hampton's Creek Road, OTR=Old Turnpike Road, RCR=Rock Creek Road, Shumont=Shumont Mountain, BC=The Bat Caves. Numbers indicate individual samples. Maxtrees were set at 50,000 and 100 replicates were run. Bootstrap values above 50% are included along the tree's topology.

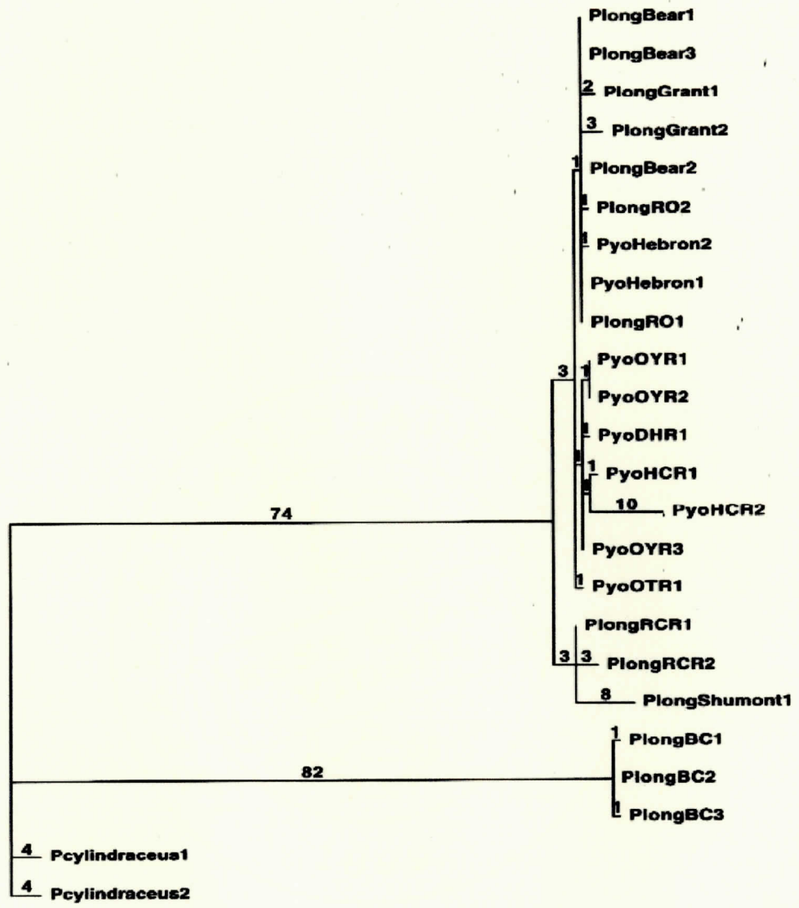

Figure 11. Phylogram produced by using a maximum parsimony heuristic search and an aligned 654 base pair fragment of the *cytochrome* b gene from 13 *Plethodon longicrus* (Plong) and 9 *P. yonahlossee* (Pyo), using *P. cylindraceus* (Pcylindraceus) as an outgroup. Populations are designated in the tree as follows: Bear=Bearwallow Mountain, Grant=Grant Mountain, RO=Rock Outcrop on U.S. 74-A, Hebron=Hebron Road, OYR=Old Yonahlossee Road, DHR=Deck Hill Road, HCR=Hampton's Creek Road, OTR=Old Turnpike Road, RCR=Rock Creek Road, Shumont=Shumont Mountain, BC=The Bat Caves. Numbers indicate individual samples. Values on phylogram are related to genetic divergence.

Discussion

The taxonomic status of *Plethodon longicrus* and its systematic relationship with *P. yonahlossee* has remained an open question since shortly after it was described in 1962. Presence of salamanders of intermediate appearance between the Bat Cave type locality for *P. longicrus* and the southernmost locality for and *P. yonahlossee* at Swannanoa Mountains led Highton (1972) to suggest that the two were merely regional variants of a single species, *P. yonahlossee*. Each of three subsequent studies (Guttman *et al.*, 1978; Blaesing and Hagen, 1983; Justice, 1983) added additional sites and types of information, but nothing definitive has been published to date. The problems in interpreting this group have been related to three distinct but interrelated questions. Is *P. longicrus* distinct from *P. yonahlossee*? If so, what is it geographic range? At what systematic level should it be recognized?

The present study has provided important new information relevant to each of these questions. Significant differences were present in several morphological features, including right hind limb length, between northern *Plethodon yonahlossee* and southern *P. longicrus* populations. This character was one of the primary characters used by Adler and Dennis (1962) in the description of *Plethodon longicrus*. Morphological differences allowed correct assignment of a specimen to "species" between 76% and 86% of the time and supported the distinctiveness of *P. longicrus.* However, the Bat Cave population

of *P. longicrus* was also distinguishable from all other populations that had been previously assigned to *P. longicrus*. This result strongly suggested that *P. longicrus* is restricted to its type locality. Morphological data further suggest that the Shumont, Grant and Bearwallow Mountain populations, traditionally assigned to *P. longicrus*, may represent an additional distinct species. Significant morphological differences also exist within *P. yonahlossee* populations, specifically Howard's Knob and Powderhorn Mountain. Overall, the significant morphological variation is consistent with the presence of more than one species.

In addition to the separate categorization of the Bat Cave population as well as other *Plethodon longicrus* populations based on morphological measurements, this study has revealed genetic variation in the *Plethodon* populations studied. The consistently demonstrated divergence in the phylogenetic analysis between Bat Cave population from all others indicated a more recent separation of the "northern" and remaining "southern" populations. These genetic differences were consistent with the morphological variation between Bat Cave and all other populations, and the combination of results of both analyses suggests the distinctiveness of the Bat Cave population. Additionally, the results suggest the presence of one or two more salamander species, similar in appearance to the Bat Cave population, which are also distinct from northern populations of *P. yonahlossee*. Interpretation of these results depends on considerations of how similar data are being evaluated in study of systematic relationships of the Family Plethodontidae.

One such plethodontid whose systematic status remains in question is *Ensatina eschscholtzii*. This species is composed of several geographically contiguous populations distributed around the Central Valley of California and was described to be a polytypic ring-species by Stebbins (1949). Highton (1998) used allozyme and *cytochrome* b sequence variation to argue for the recognition of eleven distinct species within *E. eschscholtzii*. Levels of genetic distance (Nei distances) between subspecies of *Ensatina* were similar to levels observed between species of eastern *Plethodon* (Highton, 1998).

However, Wake and Schneider (1998) claimed that not enough was known about the evolutionary relationships among the subspecies of *Ensatina eschscholtzii* to distinguish any of them as species. The authors argued that large genetic distances should not be the major factor in elevating subspecies to species (Wake and Schneider, 1998). They reported populations of numerous other species separated by genetic distances equal to or exceeding those observed in *Ensatina*. Wake and Schneider (1998) argued for the use of more factors such as fixed allozyme differences, mitochondrial DNA variation, morphological and ecological differences. Collectively, these would provide more conclusive evidence of independent evolutionary lineages and recognition of specific status (Wake and Schneider, 1998).

Moritz *et al.* (1992) had cautioned against using mitochondrial DNA variation as a basis for distinguishing populations in the genus *Ensatina* because mitochondrial genes showed different variations than the nuclear genes due their clonal and maternal inheritance. Males disperse more than females in this genus

and as a consequence species boundaries can be different depending on whether mitochondrial or nuclear genes are used in the analysis. The discrepancy in dispersion of males and females varies between species of *Plethodon* (Merchant, 1972; Wells and Wells, 1976), but no data is available for either *P. longicrus* or *P. yonahlossee*.

Several other studies have combined molecular and morphometric evidence in describing new species of salamanders (Tilley, 1981; Chippindale *et al.*, 1993; Camp *et al.*, 2002). However, appropriate weighting of each type of data set were unclear. Highton and Peabody (2000) observed that use of morphological data alone led to an underestimate of number of species in the *Plethodon glutinosus* species complex. In addition, Carr (1996) found greater morphological variation within some species in the *P. glutinosus* complex than that seen between other groups of species. All of these show that morphology alone can lead to confusion on the relationships of some populations. Speciation in Plethodontidae has occurred at times without morphological differentiation (Highton, 1995; Highton and Peabody, 2000). Two salamanders, *P. dorsalis* and *P. websteri*, are separated by genetic differences, but are indistinguishable using morphology. These two species represent one of the best examples of morphological stasis in vertebrates (Highton, 1997).

Wake *et al.* (1983) suggest that Plethodontidae contains morphological specialists and generalists. The generalists have sometimes persisted for very long periods in dramatically changing environments, with little or no change in morphology (Wake *et al.*, 1983). Highton (1989, 1999) argued that presence of

morphological stasis in some *Plethodon* force scientists to rely on genetic data as the main tool in systematic studies involving allopatric populations. Allopatric speciation is understood to be a general model in salamanders due to their low vagility. Allopatric speciation produces independent lineages that diverge according to time since common ancestry. This divergence may not be reflected in morphology, but it should be evident as progressively more genetic differences between populations accumulate through time (Highton, 1989).

Genetic differences observed in this study indicated evolutionary relationships among populations in the *Plethodon yonahlossee* group share a similar pattern of geography with the *Eurycea* study by Bonett and Chippindale (2004). Several populations clustered with those closest to them, while others did not. The Rock Creek Road and Shumont populations, geographically closest to each other, always clustered together in the phylogenetic analysis. The Hebron Road population is closer to the southern populations of *P. longicrus*, and it clustered with the Bearwallow and Grant Mountain and rock outcrop on U.S. 74-A populations of *P. longicrus* instead of northern *P. yonahlossee* populations, although it was assigned to *P. yonahlossee* due to coloration. On the other hand, the rock outcrop on U.S. 74-A population of *P. longicrus* clustered separately from the Bat Cave population in the phylogenetic analysis despite the close proximity of the two sites.

Before the conclusions of this study are presented, an introduction to the evolutionary species concept, widely accepted by herpetologists at the present time, is needed. Under this species concept, the species taxon should be “used

to carry names of lineages whose components (if distinguishable) are not incontrovertibly on different phylogenetic trajectories (i.e., sublineages, if distinguishable, are reproductively compatible), as long as these sublineages do not form a paraphyletic group in recovered history" (Frost and Hillis, 1990). The authors go on to argue that allopatric populations that are not shown to be a part of a monophyletic group should be considered distinct evolutionary species (Frost and Hillis, 1990).

Several scenarios can be proposed to explain the systematic relationship between *Plethodon longicrus* and *P. yonahlossee* (Figure 12). The first scenario (Fig. 12.A.) follows the conclusions of Highton (1972) and Guttman *et al.* (1978) that call for *P. longicrus* and *P. yonahlossee* to be considered conspecific. However, the significant morphological and genetic variation observed in the present study between populations is not consistent with recognition of only one species. The second scenario (Fig. 12.B.) follows the conclusions of Blaesing and Hagen (1983) and Justice (1983) and contends the southern populations constitute *P. longicrus* and are distinct from the northern populations of *P. yonahlossee*. This conclusion would ignore the significant genetic and morphological differences between the Bat Cave population at the type locality of *P. longicrus* and all other southern populations. The third and fourth scenarios (Fig. 12.C., D.) both recognize the Bat Cave population and the northern populations as distinct but differ in their recognition of the other southern populations. No significant variation was detected between Shumont Mountain and Bearwallow and Grant Mountain in the morphological analysis, but these

populations were morphologically distinct from the Bat Cave and *P. yonahlossee* populations. This suggests the presence of one distinct species in the hypothesized contact zone between *P. longicrus* and *P. yonahlossee* (Fig. 12.C.). Although no morphological variation was detected among the southern populations, significant genetic variation was observed between the Shumont Mountain and Rock Creek Road populations and the remaining southern populations. These results indicate the presence of two distinct species within the supposed contact zone (Fig. 12.D.).

It should be noted that individuals from Bearwallow Mountain, which occurs at an elevation comparable to some of the other northern populations of *P. yonahlossee*, resemble *Plethodon yonahlossee* specimens in coloration more closely than any of the other "*P. longicrus*" populations, except Hebron Road population. However, this resemblance was not supported by the morphological or genetic data. Also, Powderhorn Mountain specimens, which resemble typical *P. yonahlossee* specimens more so than Bearwallow Mountain specimens, showed significant morphological variation from the other sampled *P. yonahlossee* population.

Plethodon longicrus is referred to as the Bat Cave variation of *P. yonahlossee* in the most recent field guide (Conant and Collins, 1998). However, the Bat Cave population displayed monophyly and clustered separately from the other southern and northern populations in the cladograms, and a significant amount of genetic divergence was observed in the phylogram. In addition, the Bat Cave population was morphologically distinct from all other sampled

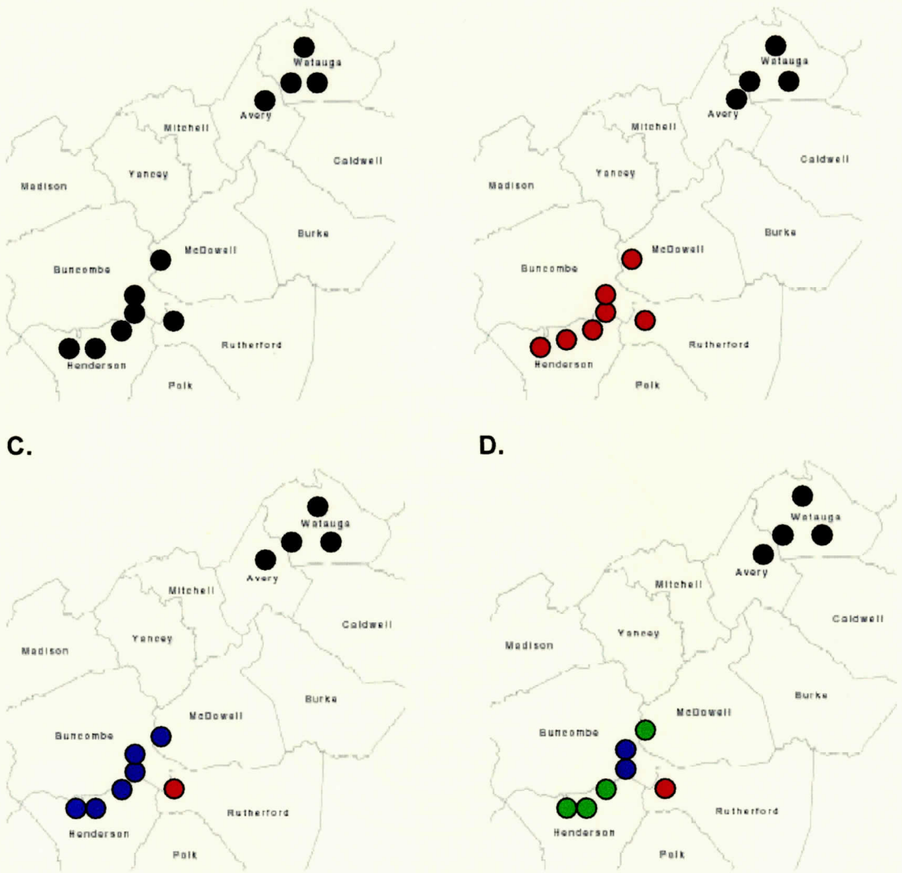

Figure 12. Maps showing possible conclusions based on the results of this study: A. All populations designated as *Plethodon yonahlossee*. B. Distinct separation of the northern populations, *P. yonahlossee* (black), and southern populations, *P. longicrus* (red). C. Designation of the Bat Cave population as *P. longicrus* (red), the northern populations as *P. yonahlossee*, and the other southern populations as another distinct species (blue). D. Same as C., except for the separation of the Shumont Mountain and Rock Creek Road populations from the other southern populations.

populations. Since morphological stasis has been observed on numerous occasions in *Plethodon* (Highton, 1995), the presence of morphological divergence in the Bat Cave population further supports the distinctiveness of this population. The divergence of the Bat Cave population observed in both thegenetic and morphological analyses argues strongly for recognition of this salamander as a valid species.

One might argue that the Bat Cave population represents a unique peripheral population of *Plethodon longicrus* much as the Water Rock Knob population of *Desmognathus imitator* did in Tilley's (2000) study. If that situation were true, recognition of the Bat Cave population as a separate species would render the remaining populations paraphyletic. However, removal of Bat Cave clade did not render any other groups paraphyletic. Therefore, based on the genetic and morphological variation, the Bat Cave population should be considered a distinct species. Because this is the type locality of *P. longicrus*, this population should retain the title.

Although no morphological variation existed between Shumont Mountain and Bearwallow plus Grant Moutain, the genetic divergence displayed by the Shumont Mountain and Rock Creek Road clade suggests the presence of two distinct forms of *Plethodon* within the hypothesized contact zone of *P. longicrus* and *P. yonahlossee*. These two new species represent another example of plethodontids with significant genetic variation but no morphological differences.

Even the monophyly of traditional "*P. yonahlossee*" populations has been questioned by the distinct morphological variation between Powderhorn Mountain

and Howard's Knob populations. The uniqueness of Powderhorn Mountain population will remain questionable until molecular data are available for this population. Also, Hebron Road, a southern population assigned to *P. yonahlossee* because of its appearance, consistently clustered with the southern populations of Bearwallow plus Grant Mountain plus the rock outcrop on U.S. 74-A. In actuality, *P. yonahlossee* forms a species complex of four distinct species based on genetic divergence.

Currently, *Plethodon longicrus* is considered by the state of North Carolina as a species of special concern due to restricted habitat and range. More research is needed to determine status of surrounding populations not included in this study, but the discovery of two new species as well as the recognition of the Bat Cave population demands stronger restrictions to protect these animals. These species should gain state and federal status as endangered and add to the already rich salamander biodiversity of the Southern Appalachians.

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APPENDIX A

Site Information and Catalog Numbers for Specimens Sampled for Morphological and Genetic Analysis.

***Plethodon longicrus*:**

Bearwallow Mountain
NC: Buncombe/Henderson County: Fire Tower Road: 35° 27.634'N, 82° 22.0721'W: 1104m: APPSU 1057, 1082, 1083, 1085, 1086, 1087, 1088, 1089, 1090, 1091, 1092, 1093, 3665, 3666, 3667, 3668, 3670, 8486, 8488, 8494, 8500, 8501, 8503, 8505, 8506, 8508, 24564, 24566, 25284

The Bat Caves
NC: Rutherford County: U.S. 74-A: 35° 26.38'N, 82° 16.38'W: 570m: APPSU 806, 1051, 1052, 1053, 1054, 1055, 1056, 1058, 1059, 1060, 1061, 1062 NCMNH 5438, 5439, 5453, 5454, 6190, 6353, 6353, 25131, 25133, 25139, 25147, 25156, 27342, 27343, 29645, 43952, 43959, 43959, 25271, 25272, 25273

Grant Mountain
NC: Henderson County: Grant Mountain Road: 35° 27.829'N, 82° 19.3221'W: 714m: APPSU 1808, 2623, 6631, , 8403, 8405, 8407, 8408, 8409, 8410, 8477, 8478, 8481, 9985, 11927, 11935, 11936, 11939, 11940, 12591, 12592, 12592, 13287, 13288, 13290, 13291, 13707, 24577, 25286

Shumont Mountain
NC: Buncombe/Henderson County: N.C. Highway 9: 35° 28.19'N, 82° 16.31'W: 784m: APPSU 8064, 8065, 8091, 8510, 8513, 8516, 8519, 8520, 8523, 8531, 8541, 8545, 8547, 8549, 8551, 8553, 8554, 8560, 8563, 8564, 8565, 8566, 8568, 8571, 11919, 11925, 25073

Rock Creek Road
NC: Buncombe County: Rock Creek Road: 35° 30.044'N, 82° 14.223'W: 831m: APPSU 25004, 25005

Rock Outcrop on U.S. 74-A
NC: Henderson County: U.S. 74-A: 35° 27.309'N, 82° 17.835'W: 637m: APPSU 25287, 25288

***Plethodon yonahlossee*:**

Howard's Knob
NC: Watauga County: Rainbow Spring Drive: 36° 15.938'N, 81° 43.453'W: 1252m: APPSU 5415, 5417, 5425, 5428, 5432, 5445, 5722, 5726, 5737, 5741, 5743, 5751, 5752, 5769, 5772, 5774, 5781, 5783, 5785, 5795, 5804, 8359, 8363, 8364, 8366, 8367

Powderhorn Mountain
NC: Watauga County: Elk Mountain Road: 36° 09.734'N, 81° 30.234'W: 435m: APPSU 9356, 9357, 9359, 9360, 9685, 9687, 9689, 9690, 9725, 9726, 9727, 9728, 9729, 9730, 9732, 9733, 9734, 9735, 9738, 9766, 9770, 9772, 10181, 10184, 10298, 10301

Hebron Road
NC: McDowell County: Hebron Road: 35° 33.905'N, 82° 15.522'W: 756m: APPSU 25021, 25022

Howard's Creek Road
NC: Watauga County: Howard's Creek Road: 36° 16.13'N, 81° 43.32'W: 1211m: APPSU 25282, 25283

Deck Hill Road
NC: Watauga County: Deck Hill Road: 36° 11.35'N, 82° 40.19'W: 1025m: APPSU 25278

Old Turnpike Road
NC: Watauga County: Old Turnpike Road: 36° 10.03'N, 81° 44.25'W: 931m: APPSU 24550

Old Yonahlossee Road
NC: Avery County: Old Yonahlossee Road: 36° 04.16'N, 81° 31.41'W: 1184m: APPSU 25033, 25035, 25036

Beech Mountain
NC: Watauga County: 36° 11.98'N, 81° 53.78'W: 1380m: APPSU 24516

Plethodon cylindraceus

Bearwallow Mountain
NC: Buncombe/Henderson County: Fire Tower Road: 35° 27.634'N, 82° 22.0721'W: 1104m: APPSU 25285

Sand Branch Road
NC: McDowell County: Sand Branch Road: 35° 31.57'N, 82° 15.60'W: 735m: APPSU 25051

VITA

Andrew Thomas Coleman was born in Montgomery, Alabama, on July 26, 1980. He and his family moved from Fort Deposit, Alabama, to Greenville, Alabama, in 1985, where he graduated from Greenville High School in 1998. He attended the University of Alabama, Tuscaloosa, and graduated cum laude with a Bachelor's of Science degree in biology in 2002. He was accepted by Appalachian State University and started working on his Master's of Science degree in biology in the fall of 2002.

After the M.S. is awarded in the summer of 2005, he will begin work on his doctorate studying sea turtle physiology at University of Alabama, Birmingham. He plans to pursue a career in environmental policy once he finishes school. His current residence is 233 Sherling Lake Road, Greenville, Alabama, 36037. His parents are Mr. and Mrs. Mark Coleman of Greenville, Alabama.