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Phylogeography and Genetic Diversity of the Seal Salamander (Desmognathus monticola)

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Phylogeography and genetic diversity of the

Seal salamander (Desmognathus monticola)

(TITLE)

BY

Erin D. Casey

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Abstract

Phylogeography is defined as the spatial distribution of taxa with respect to geologic and geographic events. It is well documented that the distributions of many taxa have been affected by glacial events during the Pleistocene Era. The patterns generated can be very complex and result from shifts in climate and/or vegetation.

The Seal salamander, (*Desmognathus monticola*), is one species that still has questions pertaining to its phylogeography. The range of this species extends from southwestern Pennsylvania to northern Alabama and Georgia, with a highly disjunct, state-endangered population in the Red Hills of Alabama. The main goal of this study is to determine the origin of this disjunct population through an extensive field survey. In addition, the utility of a relatively new genetic technique will be tested, with possible conservation implication for this population.

Three hypotheses were proposed to explain the origin of the southern population. First, it is possible that this population may not be disjunct, but instead may have a continuous range extending throughout the state of Alabama. If disjunct, then two additional hypotheses could be proposed. The southern population may represent a recent derivative from the main range, or it may be a relictual population formed through historic glacial events in the Appalachian region.

Based upon a review of topographic maps and an extensive field survey of this intervening region, we concluded that *D. monticola* were not present in this area and that the Red Hills population is truly disjunct. Thus, the first hypothesis could be rejected. To address the final two hypotheses, Intersimple Sequence Repeats were employed, and networks of relatedness were constructed using parsimony and neighbor-joining methods.

These data indicate the Red Hills population (10 bands) and the Tubmill population (8 bands), in the northern extreme of the range, harbor the highest numbers of population-specific bands. Remaining populations had three or fewer population-specific bands, and held only a subset of the bands present in the Red Hills and Tubmill populations. The Tubmill population was sister to the remaining populations; wherever, the Red Hills population was nested within each tree generated. To address this situation, constraint analyses were conducted to place the Red Hills as sister to all other populations. The tree generated was the same length of the unconstrained tree (L=570), which indicates that the Red Hills population could be sister to the remainder of the populations sampled.

Our data thus indicate the potential for two refugial populations, possibly isolated during glacial events of the Pleistocene Era. A bi-directional recolonization from the northern and southern extremes may have occurred. The southern population was probably isolated due to shifts in climate and/or vegetation, while the northern population may be a more traditional glacial refugium.

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Introduction

> In the study of dispersal and distribution of animals, it is important to see that the physical conditions lead, and that in a more or less definite succession the flora and fauna follow; thus the fauna comes to fit the habitat as a flexible material does a mold. The time passed when faunal lists should be the aim of faunal studies. The study must not only be comparative, but *genetic*, and much stress must be laid on the study of the habitat, not in a static, rigid sense, but as a fluctuating or periodical medium.
>
> Charles Adams, 1901

The appearance of the term "phylogeography" has increased steadily since it was first coined by Avise et al. (1987) while determining distribution patterns in marine species. Phylogeography is defined as the spatial distribution of organisms with respect to historic geographic events (Avise, 1998). As a sub-discipline of biogeography, it utilizes dispersal and vicariance events to explain modern distributions of taxa. The perspectives of this field were broadened with the introduction of mitochondrial DNA sequencing techniques in the 1970's by allowing intraspecific networks to be constructed (Avise, 2000). The increased reliance upon these techniques is due in part to the ease and cost effectiveness of PCR as well as the higher genetic variation often seen in these markers. In turn, this increase in genetic variation has permitted finer scale studies of population differentiation, potentially lending insight into past distributional relationships (reviewed by Futuyma and Mayer, 1980, Giddings et al., 1989, Otte and Endler, 1989). Typically, mitochondrial DNA sequences are employed in phylogeographic studies (close to 70 percent of present literature) due to the rapid rate of evolution observed in many mtDNA regions. Despite the high level of variation that mtDNA sequences usually display, it is often necessary to resort to other techniques, such as AFLPs, RAPDs, and

RFLPs, that provide even more variation when examining phylogeographic patterns, estimating genetic diversity and delimiting very closely related species (e.g., Avise et al., 1979; Birt et al., 1995; Angers and Bernatchez, 1998; Beebee and Rowe, 2000; Schmitt and Seitz, 2001).

Phylogeographic patterns have been studied in many taxa (reviewed by Avise, 1998, 2000; Futuyma and Mayer, 1980; Giddings et al., 1989; Otte and Endler, 1989), such as amphibians (e.g., McGuigan et al., 1998; Garcia-Paris et al., 1998), fishes (e.g., Wilson and Hebert, 1996), birds (e.g., Gill et al., 1993), invertebrates (e.g., Juan et al., 1996) and plants (e.g., Soltis et al., 1992, 1997; Mort et al. 2002a). In reptiles, Zamudio et al. (1997) surveyed mtDNA regions, specifically the ND4 and cytochrome B genes, within short horned lizards (*Phrynosoma douglasi*). The overall goal of this study was to determine the phylogeographic pattern of this geographically widespread, ecologically and morphologically variable species that occurs throughout western North America. Nucleotide variation was found at the population level, with fairly deep divergences between clades. A clade of *P. douglasi* sister to remaining populations of the species was recovered in the Pacific Northwest (ID, CA, OR, WA). The network of relationships that was constructed revealed associations between mtDNA patterns, climatic shifts, and geographic events in particular regions.

Geographic barriers, such as mountain ranges, can impact the present distribution of taxa. Aerial insects, such as the tropical butterfly, *Heliconius erato*, of South and Central America, have shown phylogeographic structure as well as related diversification via Müllerian mimicry (Brower, 1994). Two phylogroups were discerned using mtDNA sequences, with the Andes Mountain range in northeastern South America serving as a

long-term barrier to dispersal. Within each group, little sequence divergence was found even between allopatric, morphologically dissimilar populations. The split between the phylogroups separated by the Andes Mountain range is estimated to have occurred approximately 1.5 to 2.0 Mya as a vicariance event, with more recent and rapid evolution in wing coloration.

Outcomes of phylogeographic research can also have conservation implications. Management concerns in the harbour porpoise (*Phocoena phocoena*) due to mortality-related interactions with commercial fisheries have driven studies to assess the inter- and intra-population genetic variation by sequencing portions of the mtDNA control region. High genetic divergence was found between the three basins under consideration (Northeast Pacific, North Atlantic, and Black Sea), with no shared haplotypes among the basins. Some population structuring was found in the Pacific Northwest basin, with high levels of genetic diversity between some of the populations. Three basins were characterized by unique haplotypes with significant divergence among basins, thus indicating the necessity of maintaining populations at the regional level (Rosel et al., 1995).

Five categories were described by Avise et al. (1987) to classify phylogeographic patterns based on data from mtDNA sequences and other techniques (Fig. 1). These patterns range along a continuum from Category I, representing large genetic and geographic gaps detailing deep allopatric lineage separations between populations possibly due to long term extrinsic barriers to Category V, which describes a shallow gene tree, with widespread and common yet closely related and restricted lineages often exhibiting low to medium contemporary gene flow. A classic Category I example has

been noted in studies of the southeastern pocket gopher (*Geomys pinetis*) using RFLP patterns (Avise et al. 1979). A deep genetic east-west partitioning of mtDNA haplotypes was revealed, with specific localization of haplotypes in correspondence to geographic location. In the southeastern United States, the bowfin (*Amia calva*) displays Category V traits (Bermingham and Avise, 1986). The distribution of one mtDNA form is characterized as ancestral due to its common and widespread occurrence, and its center position in a star phylogeny. In addition, it was the closest relative to a distinct group of lineages occurring in the Gulf of Mexico drainages. Between these two categorical extremes are sympatric populations that exhibit deep gene lineages (Category II), lineages that display a relatively recent genetic divergence, while being geographically localized (Category III), and recent geographic splits between populations that still exhibit high gene flow (Category IV).

In total, the phylogeographic patterns described can often be complex and highly variable. It is now well established that past glacial activities and climatic shifts have been major factors affecting the historical distributions of a wide spectrum of taxa. Analyses of chloroplast and mitochondrial DNA haplotypes and the geographic distribution of these haplotypes were used to infer the effect of past glacial events in shaping the genetic architecture of the Pacific Northwest flora and fauna (reviewed by Avise, 2000; Soltis et al., 1991, 1992, 1997). Sequences of cpDNA were conducted in five plant species, each representing diverse life histories. A division of haplotypes into two clades was recovered, with the split occurring in central Oregon. It is hypothesized that several populations among the southern clade may represent refugia from Pleistocene glacial events (Soltis et al., 1991, 1992, 1997). This genetic architecture with respect to

geologic events is typical of taxa of this geographic area. Similar patterns have been documented in rainbow trout (*Onchorynchus mykiss*; Thorgaard, 1983) as well as in song sparrows (*Melospiza melodia*; Zink and Ditmann, 1993).

Likewise, in eastern North America, postglacial recolonization patterns have been examined using molecular data. For example, mtDNA RFLP data were employed to examine the genetic diversity of the lake trout (*Salvelinus namaycush*) in terms of influential glacial events during the Pleistocene Era. RFLP analyses of these regions revealed the origins of modern populations from various refugia in the Atlantic, Mississippian, and Beringian regions of North America. Similar phylogeographic patterns have emerged within freshwater fish native to the southeastern United States (Bermingham and Avise, 1986). Thus, throughout many regions in North America, Pleistocene glacial events have been shown to affect the genetic architecture and geographic distributions of many taxa.

Similarly, the distributions of the *Desmognathus* salamanders (Family: Plethodontidae) may have been affected by glacial events, and thus raise phylogeographic questions pertaining to the range of many species within the genus. Distributions frequently are comprised of disjunct populations, with isolates existing as far as 150 miles from the continuous range (Conant and Collins, 1998). In addition, morphological conservatism and community structure of these salamanders has complicated species delimitation and has led to increased reliance upon genetic data to ascertain species boundaries and to clarify species distributions. Multiple species frequently exist as sympatric populations, with as many as six species being reported in the same location (Southerland, 1986; Tilley and Bernardo, 1993). Under such

circumstances, it is often necessary to rely upon genetic techniques to accurately discern species. Enzyme electrophoresis is commonly employed to study genetic differentiation within and among species of *Desmognathus*, as well as for the identification and recognition of new and/or cryptic species within the genus (Tilley and Schwerdtfeger, 1981; Means and Karlin, 1989; Tilley, 1997; Mead et al., 2001). However, enzyme electrophoresis has shown inconsistencies within *Desmognathus*. For example, populations of *D. ocoee* show variation up to distances of 100m or greater, at which point a genetic "plateau" occurs and differentiation among populations is no longer detected. In other species in the genus (e.g., *D. ochrophaeus*), enzyme electrophoresis has shown little to no variation even between populations separated by distances as great as 1000km (Tilley, 1997).

More recently, DNA-based methods have been employed to reconstruct phylogenies, estimate genetic diversity, and establish networks of relatedness for groups in which enzyme electrophoresis has shown low levels of variation. Several DNA methods are available for this latter application, such as the sequencing of rapidly evolving DNA regions (e.g., Avise, 2000; Soltis and Soltis, 2000; Mort et al., 2001, 2002b) and the use of so-called "hyper-variable" PCR based methods (e.g., RAPDs, AFLPs, microsatellites, and ISSRs; reviewed by Wolfe and Liston, 1998). For phylogeographic studies of animals, sequencing of mtDNA regions has shown utility for determining distribution patterns (e.g., Titus and Frost, 1996; Titus and Larson, 1996; Sullivan et al., 1997; Taberlet et al., 1998; Veith et al., 1998; Durand et al., 1999; Avise, 2000; Hewitt, 2001;). However, within *Desmognathus*, low support for species-level relationships was uncovered (Titus and Larson, 1996). Thus, it is sometimes necessary to

use techniques that yield even higher variation, such as ISSRs, RAPDs, and microsatellites (reviewed by Avise, 1994).

One such technique that has shown utility in detecting variation in even recently-derived taxa is analyses of intersimple sequence repeats (ISSRs). To date, ISSR analyses have primarily been applied to plants, and no studies have been reported on the use of this technique when studying the phylogeography of Plethodontid taxa. Comparisons between allozymes, RAPDs, and ISSRs have shown ISSRs to have considerably more variation than the other techniques (Wolfe and Liston, 1998; Esselman et al., 1999; Li and Ge, 2001; Meng and Chen, 2001; Mort et al., 2002a). ISSRs are PCR-based and employ a single primer that is designed from di- or trinucleotide repeat motifs (i.e. microsatellite regions). Unlike RAPD primers, ISSR primers are not random and are typically 8-10 base pairs longer, thus allowing for higher annealing temperatures. In turn, these higher annealing temperatures yield more repeatable results and an increased confidence in band homology of an individual. The data generated from ISSRs are predominantly dominant/recessive markers that are scored as band present or absent (Wolfe and Liston, 1998); thus, these data are analyzed in the same manner as RAPDs or other similar data (e.g., AFLPs). A key benefit of ISSRs, and other PCR-based methods, is that they allow for non-destructive sampling since PCR can be employed with only a very small tissue sample from each individual. Therefore, these PCR-based techniques are ideal for studies of rare or threatened species.

One species of *Desmognathus* whose phylogeography is still in question is the semi-aquatic salamander *D. monticola*. The present range of *D. monticola* extends from southwestern Pennsylvania through northern Georgia and Alabama (Fig. 2). In addition,

a highly disjunct, state-endangered population is located in the coastal plain region of the Red Hills in Alabama, separated from the main range by approximately 150 miles (Conant and Collins, 1998). However, the origin of this population is still in question. Within *D. monticola*, both enzyme electrophoresis (Tilley et al., 1978; Tilley et al., 1990; Tilley and Bernardo, 1993; Tilley, 1997) and mtDNA sequencing (Titus and Larson, 1996; Mabry and Mort, unpub.) have been unsuccessful in identifying genetic diversity. Thus, in order to address the origin of the Red Hills population as well as other phylogeographic questions, it is necessary to employ a technique that yields a higher degree of genetic variation.

The origin of this highly disjunct southern population raises phylogeographic questions with respect to the glacial history of the Appalachian region. Three explicit hypotheses are advanced to explain the occurrence of the disjunct *D. monticola* population:

- The southern population may not be disjunct, but instead, insufficient fieldwork in this area may have lead to inaccurate range documentation.

However, if this population is truly disjunct, then it is possible that:

- The Red Hills population could be a relatively recent dispersal event, possibly human-mediated.
- The Red Hills population could be relictual having been isolated during the past glacial events.

Thus, the primary goal of my research is to resolve conflicting hypotheses regarding the origin of the Red Hills population of *D. monticola* in Alabama while constructing phylogeographic patterns of this species by sampling numerous populations

throughout the range. The utility of ISSRs in the Plethodontid salamander family and in phylogeographic studies will be determined for use in future research of similar taxa. Additionally, the accurate range of *D. monticola* will be established through extensive field sampling in central Alabama and a survey of existing populations in the Red Hills region will assess the conservation strategies necessary to maintain this region's threatened population.

Methods

Field sampling

Desmognathus monticola is widely distributed from Pennsylvania southward to Georgia and Alabama (Fig. 2), typically inhabiting banks along first or second order streams or seeps containing coarse, rocky substrate (Conant and Collins, 1998; Petranka, 1998). Suitable habitat for populations of *D. monticola* throughout the range were identified by examining topographic maps as well as contacting herpetologists familiar with the genus in specific regions of the country. Southern populations in Alabama and Georgia were located with the assistance of Drs. Carlos Camp (Piedmont College, Georgia) and Craig Guyer (Auburn University, Alabama). Michelle Mabry (Davis and Elkins College, West Virginia) assisted in the location and collection of the North Carolina populations. GPS coordinates and elevation was recorded for each population (Table 1).

At each site, individual *D. monticola* were located by sifting through substrate along stream banks and searching under cover objects (i.e. rocks and logs). Once captured, specimens were sexed by identifying maxillary teeth on the chin of males or by the presence of eggs in females. Tail tissue was collected from *D. monticola* at all sites using a sterile razor blade or scissors to remove approximately 0.5 cm of the tail tip. Tissue samples were placed in sterile 1.5 ml microcentrifuge tubes and transported to the laboratory on ice. Samples were then stored at -20°C in the laboratories at Highlands Biological Station until being shipped on ice to Eastern Illinois University. In addition, *D. monticola* from the Tubmill population in Pennsylvania were provided by Michelle Mabry (Davis-Elkins College).

Voucher specimens were also collected for populations in Georgia, Alabama, Florida and North Carolina, euthanized using Chloretone (1,1,1-trichloro-2-methyl-2-propanol) and preserved in 70% ethanol. Alabama specimens will be housed in the Herpetological collections at Auburn University (AUM 35506-35510/Haines Island Park, Monroe County, Alabama). Florida specimens are the property of Paul Moler of the Florida Fish and Wildlife Commission (Gainesville, Florida) while specimens from Georgia and North Carolina will be housed in the herpetological collections at Eastern Illinois University. Due to the declining populations in West Virginia, Tennessee, and Kentucky, picture vouchers were taken for specimens in accordance with state permit regulations and housed at Eastern Illinois University.

DNA Extraction and Genetic Analyses

Small amounts (approximately 0.6 grams) of thawed tail tissue were homogenized in 1.5ml microcentrifuge tubes using sterile grinders. DNA was extracted using the Promega Wizard Kit (Promega Corporation, Madison) and Qiagen DNeasy Tissue Kit (Qiagen, Inc., Valencia). Test gels (1% agarose) were run for all samples to check for the presence of high-molecular weight DNA. ISSR reactions were conducted in 25μL volumes using 2.5μl of 10x Promega buffer, 4.0μl of 1.25 mM DNTPs, 2.5μl of 50mM $MgCl_2$, 0.5μl of 50μm primer, 0.5μl of DNA and between 0.5 to 1U of TAQ Polymerase. Two primers were used for this study: MANNY ((CAC)4-RC) and 807-1 ((AG)8-RG). Amplifications were conducted on a Stratagene RoboCycler (Stratagene, La Jolla) with a program of 2 minutes at 94°C; 35 cycles of 45sec at 94°C, 50sec at 46°C (MANNY) or 48°C (807-1), and 3min at 72°C.

ISSR reactions were electrophoresed on horizontal 1% agarose gels (1:1 ratio of Sequem Gold Agarose and Sigma Low EEO Agarose) until a migration distance of 10 cm was reached (approximately 3.5 hours). Gels were stained in ethidium bromide for 30 minutes, and destained in distilled water for 30 minutes. Bands were visualized under UV-light, and images were captured using a Fotodyne bench top gel documentation system. Kodak 1D Image Analysis Software Package (Eastman Kodak Corporation, Rochester) was used to score the individual band presence and estimate the size of each fragment. ISSR reactions were replicated once to verify the presence or absence of each band.

Only bands that were consistent between replicates were included in the data set, and bands were scored as present (1) or absent (0) for each primer. A data matrix was produced for each individual primer as well as for a combined data set including both primers. All analyses were conducting using PAUP* (Swofford, 1998) running on a PowerMac (G4). Both unrooted and mid-point rooted dendrograms were inferred under parsimony and neighbor-joining criteria. For the latter, analyses were conducted employing both total character and Nei and Li distances. Relative support for the relationships resolved by each of these analyses was assessed under the same optimality criterion using bootstrap analyses (Felsenstein, 1985). These analyses were conducted using 1000 replicates with 10 random replicates each and TBR-branch swapping. Constraint analyses were employed to determine the potential for a sister relationship between the Red Hills population and remaining taxa.

Results

Field Collections

Collections of tail tips took place from May to July of 2001. A total of 111 individuals from 10 populations were sampled across the entire range of *D. monticola* from Pennsylvania to Alabama (Table 1). The number of individuals collected per population ranged from one to twenty-two, although only populations from which at least five individuals was sampled were included in the ISSR survey. Across the range, elevations at which *D. monticola* populations were found ranged from as low as 63 feet above sea level in the Red Hills (AL) to 2382 feet in Cane Brake Creek (TN); however, only one individual was found in the latter site and was not included in subsequent studies. Populations typically occurred in first or second order streams or seeps located within or adjacent to hardwood forests.

To assess the potential for *D. monticola* populations to exist between the Appalachian range and the Red Hills population, topographic maps were examined to locate suitable habitat locations. In addition, local herpetologists (C. Guyer and G. Folkerts, Auburn University) were contacted for guidance to potential sites. An extensive survey of the most suitable habitat for *D. monticola* populations between the Red Hills population in Monroe County, AL and the northern Alabama populations in Clay County was conducted over a period of seven total days, representing approximately 35 hours in the field. Relative to other locations in the range, this sampling effort was much higher. However, these surveys were still unsuccessful in locating suitable habitat. In addition, the vegetation in this area did not resemble that of the Red Hills or of the Appalachian

Mountain range. Because no *D. monticola* individuals were found within this area, it appears as if the Red Hills individuals do represent a truly disjunct population.

ISSR Banding

In total, 23 primers were surveyed, of which 13 displayed banding patterns. Variation suitable to address the goals of this project was detected in six of these primers. Two primers showed high levels of variation and were optimized: MANNY ((CAC)4-RC) and 807-1 ((AG)8-RG). Five individuals from nine populations in Pennsylvania, West Virginia, North Carolina, Alabama, and Georgia were used in the genetic study. The majority of bands for both primers ranged from 350 to 1500 base pairs in length.

MANNY produced a total of 521 scorable bands, with an average of 11.6 bands per individual and 57.9 bands per population. Ten bands were unique at the individual level, with two bands identifying MC3 and one band present in each of the following individuals: NT21, NT22, WA3, MC4, CW2, BS1-1, WA5, and NT9. A total of twenty bands were population-specific, identifying the following populations: Bluestone (2 bands), Red Hills (8 bands), Coweeta (2 bands), Sosebee Cove (3 bands), and Tub Mill (5 bands). Forty-nine percent of the bands were shared among all populations sampled.

807-1 yielded 701 total bands, with an average of 15.6 per individual and 77.9 bands per population. Three bands were individual specific, identifying each of the following individuals by one band: BS2-2, MC3, and BS1-2. A total of nine bands were population-specific, with three bands identifying Tubmill, two bands for Red Hills, and one band for each of the following populations: Bluestone, Nancytown, Highlands Plateau, and Coweeta. Seventy-five percent of the bands were shared among all populations.

Overall, the combined data set comprised 112 bands that were scored as presence/absence. Twenty-nine bands were specific at the population level (Table 2), and thirteen were specific to individuals. Sixty-three percent of all bands were shared by more than one population. The Red Hills population was quite distinct in having ten population-specific bands, highest by comparison to other populations. In addition, the Tubmill population (PA) at the northern extreme of the range also had a high number of population-specific bands, designated by eight unique character states. The remaining seven populations that were included in the survey had three or fewer population-specific bands each.

ISSR Analyses

A network of relatedness based upon neighbor joining and parsimony optimality criterion was constructed for both primers, and all trees produced were mid-point rooted. The combined data set based upon both primers included 112 characters, 99 of which were parsimony informative. Parsimony analyses yielded 12 trees of 605 steps in length. All 12 trees converged on similar topology, and one will be used for further discussion (Fig. 3). Support for relationships as assessed via bootstrap analyses (Felsenstein, 1985) was moderate to low for populations. Likewise, support within populations was generally lower, with most bootstrap values falling below 50 percent. Four clades that designated populations were resolved in this tree. Individuals from Red Hills (73%), Tubmill (<50%), Sosebee Cove (<50%), and Coweeta (<50%) each grouped as populations, with varying amounts of bootstrap support. The Tubmill population was placed sister to a clade containing individuals from populations in North Carolina, Georgia, and northern Alabama. The remaining populations were largely unresolved due

to low phylogenetic signal, i.e. very few population-specific bands. Some relationships within populations were recovered with moderate support. Within Tubmill, two sub-clades of two individuals received 68 and 83 percent support, respectively. The two sub-clades within Tubmill also grouped to form a clade receiving 60 percent support. Sosebee Cove (61%) and Highlands Plateau (52%) each contained a single sub-clade. The Red Hills population had four individuals forming a sub-clade that received 55 percent support. Several groups of individuals within Bluestone, Mt. Cheaha, and Nancytown formed sub-clades that received less than 50 percent support.

Neighbor joining analyses were conducted based upon distance matrices inferred using both Nei and Li and total character distances as implemented in PAUP* (Swofford, 1998). Nei and Li's distance matrix scores only positive matches, whereas total character length assumes homology for negative characters (band absent). Because bands may be absent for many reasons, it is inappropriate to consider band absence as homologous for ISSR data. Therefore, the most appropriate distance matrices to use for neighbor joining are those estimates based on Nei and Li's methodology. All neighbor joining analyses converged on trees yielding similar topologies (Fig. 4). The Red Hills population held together and was strongly supported (98%), while nesting within the remaining populations. The Tubmill population was sister to the majority of the other populations. Coweeta was the third population recovered, forming a clade (53%) nested with most populations. Several sub-clades within populations were moderately resolved. Three individuals from both Nancytown (51%) and Sosebee Cove (>50%) formed sub-clades. Within the Tubmill population, two sub-clades consisting of pairs of individuals had 67 and 81 percent support. Individuals from Wayah formed two sub-clades, containing two

and three individuals, respectively. Two individuals from both Highlands Plateau (<50%), and Mt. Cheaha (68%) also formed sub-clades.

Comparing the results of the neighbor joining and parsimony methods indicate that the topologies are largely congruent. For example, the same three populations were recovered in both analyses: Coweeta, Red Hills, and Tubmill. Similar clades were also recovered within populations such as the grouping of individuals within Coweeta, Nancytown, Red Hills and Tubmill. In both trees, the Tubmill population was sister to a larger clade comprised of the majority of other populations. The inconsistencies noted among taxa within several populations is most likely due to lack of phylogenetic signal in the ISSR data set. This lack of signal may indicate recently derived populations that have not had sufficient time to accumulate mutations (Mort et al., 2002a, 2002b)

Discussion

According to Conant and Collins (1998), the range of *D. monticola* extends throughout the Appalachian Mountains from southwestern Pennsylvania to northern Alabama. In addition, a highly disjunct, state-endangered population has been recognized in southern Alabama (Fig. 2). This population is located in Monroe County and is approximately 150 miles south of the nearest population of the main range. However, it is unknown how extensively the habitat has been surveyed between the main range and the Red Hills population. Without this information, it is unclear as to whether this population represents a true isolate. Therefore, it is necessary to first establish if the Red Hills population is truly disjunct prior to investigating its origin. To address this question, extensive sampling and habitat surveys were conducted throughout this intervening region.

Prior to fieldwork, topographic maps of Alabama were studied to determine if suitable habitat existed between the northern continuous range and the highly disjunct population in southern Alabama. After examination, it did not appear that suitable habitat for *D. monticola* was present in this area of Alabama. However, field sampling was necessary to confirm this lack of habitat as assessed from topographic maps. Therefore, potential habitats between the continuous range and the disjunct population were surveyed extensively in an attempt to locate additional populations. These surveys failed to locate suitable habitat or additional populations of *D. monticola*. The Red Hills population appears to be disjunct. Furthermore, Drs. Craig Guyer and George Folkerts (Auburn University, Alabama), both familiar with the distributions of *Desmognathus* species in Alabama, supported these conclusions. Thus, all available data indicate that

the Red Hills population is truly disjunct and is separated from the main range by approximately 150 miles.

The overall topography of the Red Hills is deeply dissected and distinct from that of the surrounding area. To the north of the Red Hills lies a region of the Piedmont of Alabama referred to as the Black Belt, while the remainder of the coastal plain lies to the south (Diamond, 1987). The biota of the Red Hills is distinct from these surrounding areas and harbors a high degree of endemic taxa (Diamond, 1987), including the Red Hills Salamander (*Phaeognathus hubrichti*). A comparison of vegetation of the Red Hills with that of *D. monticola* sites in the Appalachians seemed to indicate an overall similarity. In fact, many of the dominant species in the Red Hills regions are also found in the Appalachian mountains of northern Alabama (Diamond, 1987; Dodd, 1991, see Carroll et al., 2000). Between these regions, there is a dramatic shift in vegetation from a deciduous hardwood forest to a forest dominated by coniferous trees. This shift in vegetation could be a major factor explaining the lack of populations in this particular area of Alabama.

Isolated populations, such as the southern Alabama population of *D. monticola*, often present phylogeographic questions pertaining to their origin. Changes in climate due to glacial events can lead to circumstances such as shifts in vegetation, or range contraction/expansion events. These events can result in irregular distributional patterns of taxa, such as isolated or refugial populations, as some of the examples noted in earlier in the flora and fauna of the Pacific Northwest (e.g., Thorgaard, 1983; Soltis et al., 1991, 1992, 1997; Zink and Ditmann, 1993) and the Southeastern United States (e.g., Bermingham and Avise, 1986). As in the state-endangered Red Hills population, there

are often conservation concerns associated with these disjunct populations. Clearly, determining the origins and genetic status of these populations is essential and can aid in deciding upon the best management practices and necessary levels of protection.

Since it was determined that the Red Hills population is disjunct, two main hypotheses were proposed to explain the origin of this population. First, this southern population could represent a relatively recent colonization event from the main range. Typically, this type of population would likely have lower genetic diversity due to the reduction in population size and associated genetic bottleneck that characterize derivative populations (Fig. 5). These populations would be expected to exhibit a lower diversity of bands, and a lower proportion of population-specific bands with respect to the larger, source population(s). Furthermore, a derivative population would be expected to hold a nested position within a dendrogram of the remaining populations.

Alternatively, the Red Hills population could represent a refugial population. It is now well documented that glacial events during the Pleistocene affected the distributions of taxa throughout the United States, and many examples have been documented in the southeast region (e.g., Bermingham and Avise, 1986). For example, mtDNA haplotype divergence was examined in chickadees throughout the southeastern United States. A contact zone in central Alabama was potentially identified, with a relatively recent divergence of lineages occurring during the Pleistocene. In addition, taxa sampled from the formerly glaciated areas exhibited low sequence divergence, probably due to the range expansion events (Gill et al., 1993). Many taxa in the southeastern United States exhibit similar phylogeographic patterns. The Red Hills population could likely represent an ancestral population that served as a source for more recent colonizations of *D.*

monticola into the Appalachian Mountains. These refugial populations tend to harbor higher amounts of genetic diversity, and more recently-derived populations would then largely display only a subset of the diversity found in the source population (Fig. 6). Thus, when assessed with a genetic technique, refugial populations would be expected to show a higher number of unique bands compared to the derivative population. In addition, the source population would be expected to form a sister clade with respect to the remaining populations sampled.

In plants, phylogeographic studies often employ cpDNA RFLPs (e.g., Soltis et al., 1991, 1992, 1997). Research focused on animal distributions more often use mtDNA sequences, due to the appropriate rate of evolution for this type of analysis. However, mtDNA sequences, even in the rapidly evolving D-loop region, were unable to detect nucleotide divergence between populations in *D. monticola* (Titus and Larson, 1996; Mabry and Mort, unpub.). Thus, a technique yielding higher levels of genetic variation was necessary to achieve the goals of this study.

Analyses of ISSR data indicate that the Red Hills population harbors a high diversity of unique bands in comparison to other populations. In total, ten bands specific to the Red Hills individuals were noted in the combined ISSR data matrix. Other populations that were sampled had only a subset of those bands that were present in the Red Hills population and also appear to harbor much lower genetic diversity. One exception to this pattern is the Tubmill population in the northern extreme of the range, which possessed the second highest number of population-specific bands (eight) and appeared to harbor an overall higher band diversity.

Both distance and parsimony based bootstrap analyses of the combined data set strongly support the monophyly of the Red Hills clade. Support for the other populations was generally very low and varied. The Coweeta population from the central part of the range and the northern extreme population of Tubmill were the only other populations that formed clades yielding bootstrap support. The least diversity and lowest support occurred within the populations towards the center of the main range, such as Highlands Plateau (1 population-specific band) and Wayah (0 population-specific bands) in southwestern North Carolina. For example, the Highlands Plateau population contained a single population specific band, whereas the Wayah population had only one band that was unique to a single individual.

Considering the criteria for source and recently-derived populations (Figs. 5 and 6), the origin of the southern Alabama population can be addressed. This population fits the expectations of a source population. The Red Hills population possesses a high number of population-specific bands. In addition, the overall genetic diversity of this population compared to other populations appears to be higher than in the main range. The remaining populations displayed only a subset of the bands present in the Red Hills. These conclusions suggest that the Red Hills population may have served as the source for more current populations in the Appalachian Mountains. However, both parsimony and neighbor joining analyses placed the Red Hills population in a nested position relative to the other populations.

To test the position of the Red Hills population, constraint analyses were conducted by placing this population in a sister position with respect to the remaining populations in the parsimony tree. The tree produced from these analyses was the same

length as the unconstrained tree (L=570), representing no increase in tree length. Therefore, strong evidence was not found against the sister placement of the Red Hills population.

Thus, the Red Hills fits all expectations of a source population. This disjunct population displays a high genetic diversity and more population-specific bands when compared to the remaining populations. The other sampled populations appear to harbor only a subset of diversity that is present in the Red Hills. Although this southern population is nested within the dendrogram, the constraint analyses support the status of this population as sister to the remaining range. Based on these criteria, the Red Hills population appears to represent a source population, probably serving as a glacial refugium during the Pleistocene.

A general lack of resolution was found in populations towards the center of the range in North Carolina, Georgia, and West Virginia. With the exception of the Coweeta (NC), the remaining six populations did not form clades with high bootstrap support (<50%). These more central populations also appeared to harbor lower genetic diversity and fewer population-specific bands (0-3 bands), relative to the Red Hills and Tubmill populations, with ten and eight, respectively. Our data set comprised of 112 characters was within the general range of other ISSR studies (e.g., Esselman et al., 1999; Joshi et al., 2000; Culley and Wolfe, 2001; Patzak, 2001; Raina et al., 2001; Wolfe and Randle, 2001; Mort et al., 2002a). This overall lack of resolution within the central portion of the range is likely due to the lower phylogenetic signal noted for these populations. Even though our data set was similar in size to other studies, additional data is required to resolve the relationships among these more recently-derived populations. However, the

present study clearly documents the utility of ISSRs for phylogeographic studies in amphibians.

As noted above, the Tubmill population in southwestern Pennsylvania represents the northern extreme of the range of *D. monticola*. The results of our ISSR analyses deviated from the pattern of low resolution noted in the other populations, but instead displayed higher genetic diversity (Table 2). In fact, this population displayed the second highest number of population-specific bands, with eight unique to the population (Table 1), and held a sister position relative to the remainder of the populations sampled. In addition, the Tubmill population was second to the Red Hills in overall number of bands. This area in southwestern Pennsylvania remained unglaciated during the most recent glacial periods (Wright and Frey, 1965). These results indicate the possibility for a second refugium present in the northern extreme of the range.

Both the Red Hills and Tubmill populations exhibit higher genetic diversity and increased number of population-specific bands relative to the other populations. Furthermore, both parsimony and neighbor joining analyses recover clades comprising individuals from these populations (Figs. 3 and 4). If these populations served historically as sources for subsequent recolonization, they would be expected to form a sister group relationship with the remaining populations (Fig. 6). Tubmill is a sister lineage in both parsimony and neighbor joining analyses, whereas the Red Hills population is nested well within both dendrograms. However, constraining the Red Hills population to hold a sister position in our parsimony analyses yielded a tree with the same overall length (L=570). Thus, the ISSR data are similar for both the Red Hills and Tubmill populations and are consistent with the expectations for a refugial population.

The results of the present study support the possibility for two glacial refugium at the northern and southern extremes of the range, with subsequent bi-directional recolonization events.

Conclusions

The Red Hills population of *D. monticola* is disjunct from the main range by approximately 150 miles. To determine the origin of this population, ISSRs were employed for the first time in amphibian taxa. This technique yielded informative, scorable data, and may prove useful for similar studies in other amphibians. The Red Hills population appears to have served historically as a source for subsequent recolonization events, possibly during glacial events of the Pleistocene. In addition, the Tubmill population in southwestern Pennsylvania may represent a second refugial population and the source of more recent northern populations. The population in Pennsylvania more likely represents a 'true' glacial refugium, in that much of the surrounding area in southwestern Pennsylvania was glaciated. The Red Hills population is more likely an isolate due to shifts in vegetation and climate as a result of glaciation in more northern areas.

The Red Hills population is currently protected at the state level, primarily due to the decline of suitable habitat in this area. The practice of clear-cutting by timber industries throughout Alabama has greatly altered the original landscape of this state, and destroyed much of the suitable habitat for this species. The influences of this practice are extensive, and continue to have negative impacts on many taxa and associated ecosystems. Our results will further support the listing of the Red Hills population at the state level, and possibly indicate a need for a more protected status for both the population and of the habitat of this species in southern Alabama. Inclusion of additional northern populations and more individuals from each population as well as an extensive vegetative survey would be recommended for future research, as it would assist in teasing

apart the phylogeographic patterns of *D. monticola* and lend additional insight into the status of the Red Hills population.

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Table 1. GPS Coordinates, elevations, and collection information of *Desmognathus monticola* populations from across the entire range.

Locality	N	Latitude	Longitude	Elev.(ft)
Bluestone National Scenic River, site one (Summers CO.), WV	2	37° 34.904'	80° 59.045'	1704
Bluestone National Scenic River, site two (Summers CO.), WV	5	37° 35.071'	80° 58.936'	2046
Cane Break Creek, TN (Monroe CO.)	1	35° 20.722'	84° 13.618'	2382
Coweeta Hydrological Laboratory, NC (Macon CO.)	17	35° 03.785'	83° 26.114'	2331
Highlands Plateau, Horse Cove, NC (Macon CO.)	19	35° 02.251'	83° 08.665'	1304
Nancytown, GA (Habersham CO.)	22	34° 29.676'	83° 29.199'	995
Red Hills, site one, AL (Monroe CO.)	1	31° 43.179'	87° 28.678'	63
Red Hills, site three, AL (Monroe CO.)	11	31° 43.318'	87° 27.733'	1422
Sosebee Cove, GA (Rabun CO.)	22	34° 46.071'	83° 56.806'	2872
Talladega National Forest, AL (Clay CO.)	4	33° 28.746'	85° 48.351'	2042
Tub Mill, PA (Indiana CO.)	6	40° 25.03'	79° 15.08'	1968
Turkey Creek, TN (Monroe CO.)	1	35° 21.289'	84° 10.394'	2285
Wayah Bald, NC (Macon CO.)	6	35° 09.591'	83° 31.663'	1408

Table 2. Total population specific bands for primers MANNY and 807-1 for the nine populations of Seal salamanders sampled across the entire range.

Population	# bands
Bluestone (WV)	3
Coweeta (NC)	3
Highlands Plateau (NC)	1
Mt. Cheaha (AL)	0
Nancytown (GA)	1
Red Hills (AL)	10
Sosebee Cove (GA)	3
Tubmill (PA)	8
Wayah (NC)	0

Figure 1. Five phylogeographic categories, as described by Avise et al. (1987).

Figure 2. Range of *D. monticola*, extending from southwestern Pennsylvania to Alabama. Note the disjunct population in southern Alabama (Conant and Collins 1998).

Figure 3. One of 12 minimum-length trees inferred from parsimony analyses of ISSR data for nine populations of *D. monticola*. Branch lengths (above) and bootstrap values (below) are noted by the corresponding branch. Population codes: TM=Tubmill (PA), BS=Bluestone National Scenic River (WV), HP=Highlands Plateau (NC), WA=Wayah Bald (NC), CW=Coweeta Hydrologic Laboratory (NC), NT=Nancytown (GA), SC=Sosebee Cove (GA), MC=Mt. Cheaha (AL), RH=Red Hills (AL).

Figure 4. Neighbor-joining tree with mid-point rooting based upon a Nei and Li's distance matrix. Bootstrap values are shown above branches. Population codes: TM=Tubmill (PA), BS=Bluestone National Scenic River (WV), HP=Highlands Plateau (NC), WA=Wayah Bald (NC), CW=Coweeta Hydrologic Laboratory (NC), NT=Nancytown (GA), SC=Sosebee Cove (GA), MC=Mt. Cheaha (AL), RH=Red Hills (AL).

Figure 5. Schematic for the characteristics of a recent derivative population. These populations typically undergo a genetic bottleneck, decreasing overall diversity relative to the source population.

Figure 6. Characteristics of source population(s) and subsequent colonization events. A range contraction event could lead to a refugial population that contains high levels of genetic diversity. Subsequent recolonization events would result in founder populations that are lower in genetic diversity relative to the refugial population.

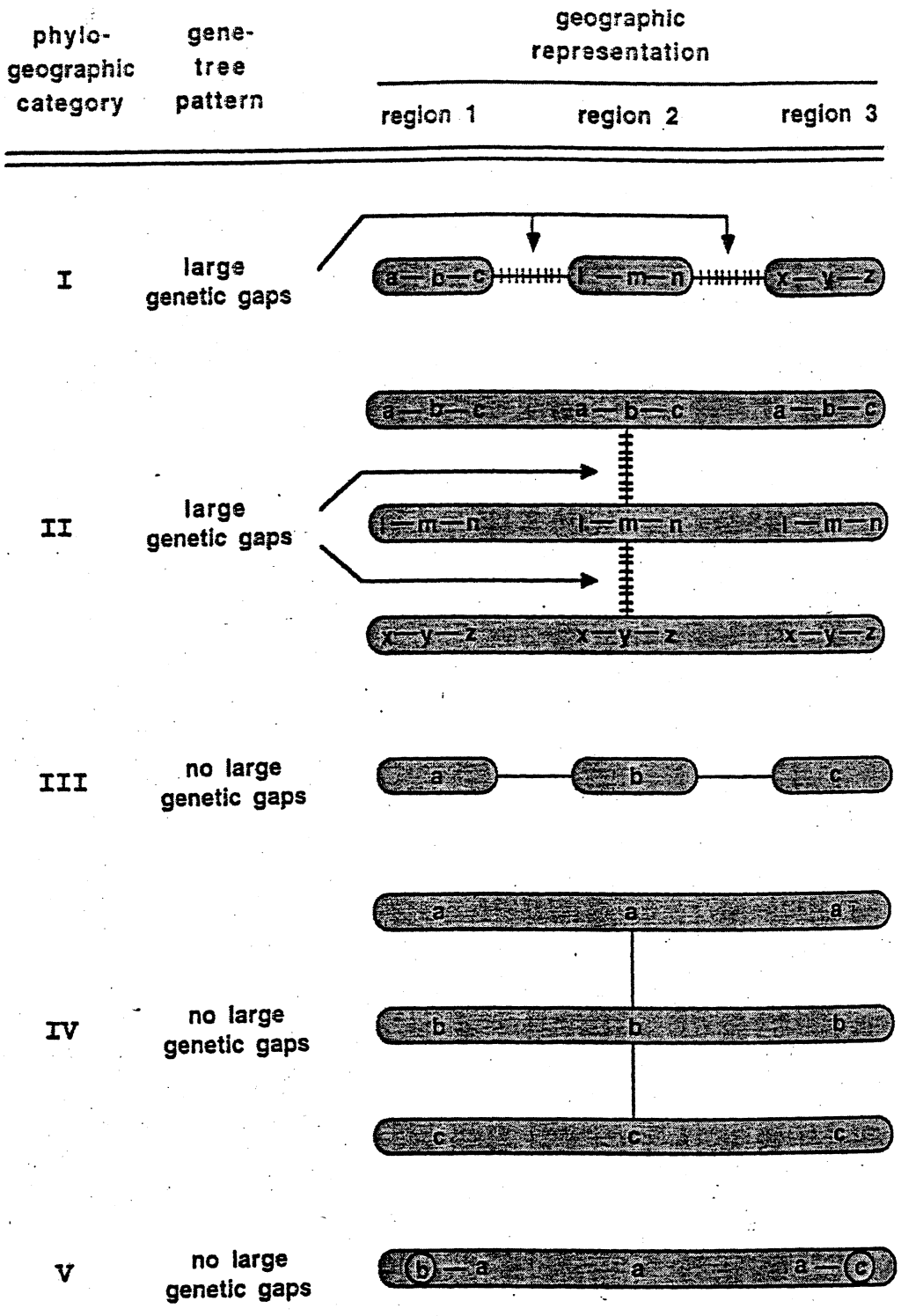
phylo-geographic category
gene-tree pattern
geographic representation
region 1
region 2
region 3
I
large genetic gaps
a—b—c
l—m—n
x—y—z
II
large genetic gaps
III
no large genetic gaps
IV
no large genetic gaps
V
no large genetic gaps

Figure 1.

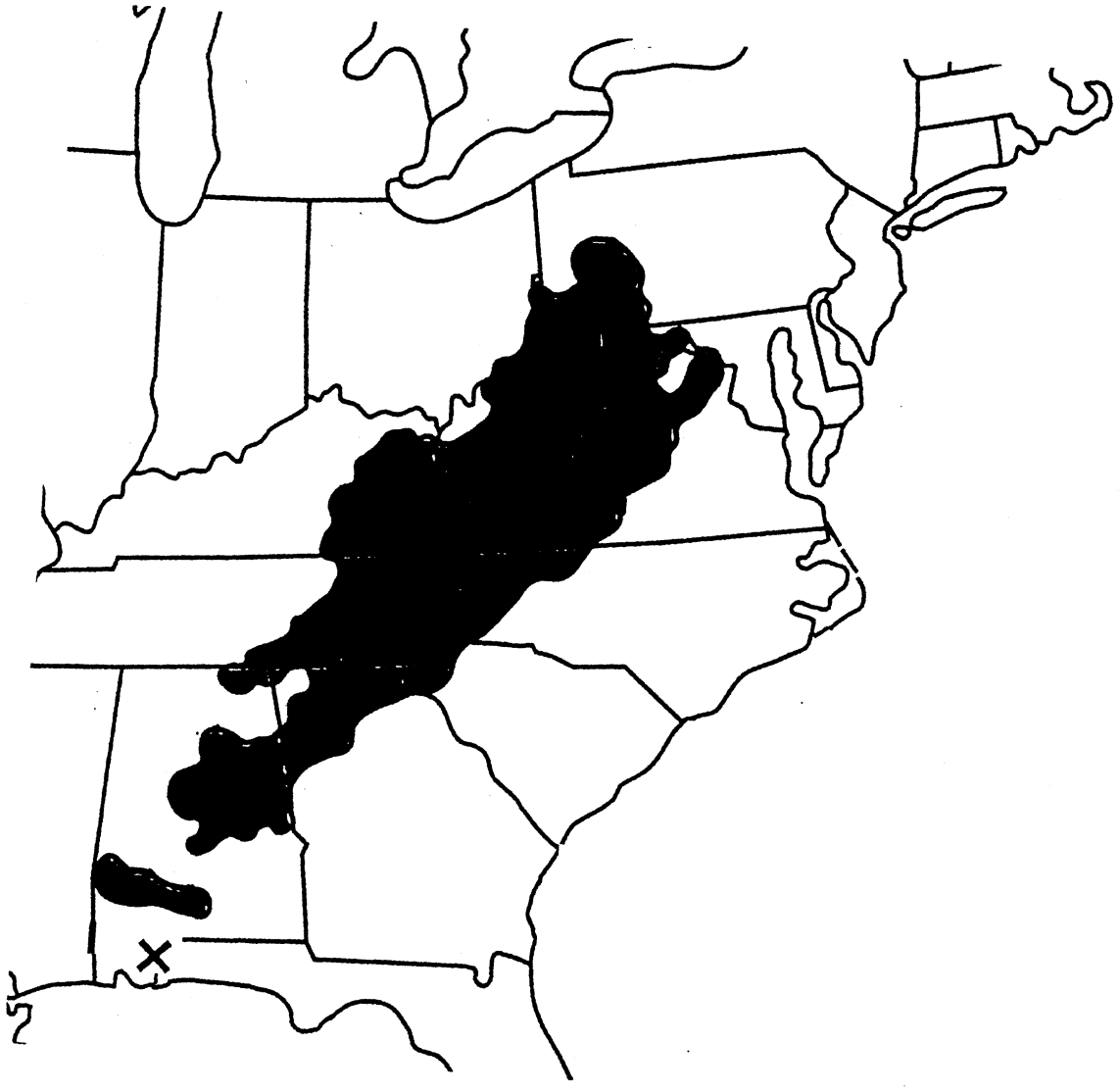

Figure 2.

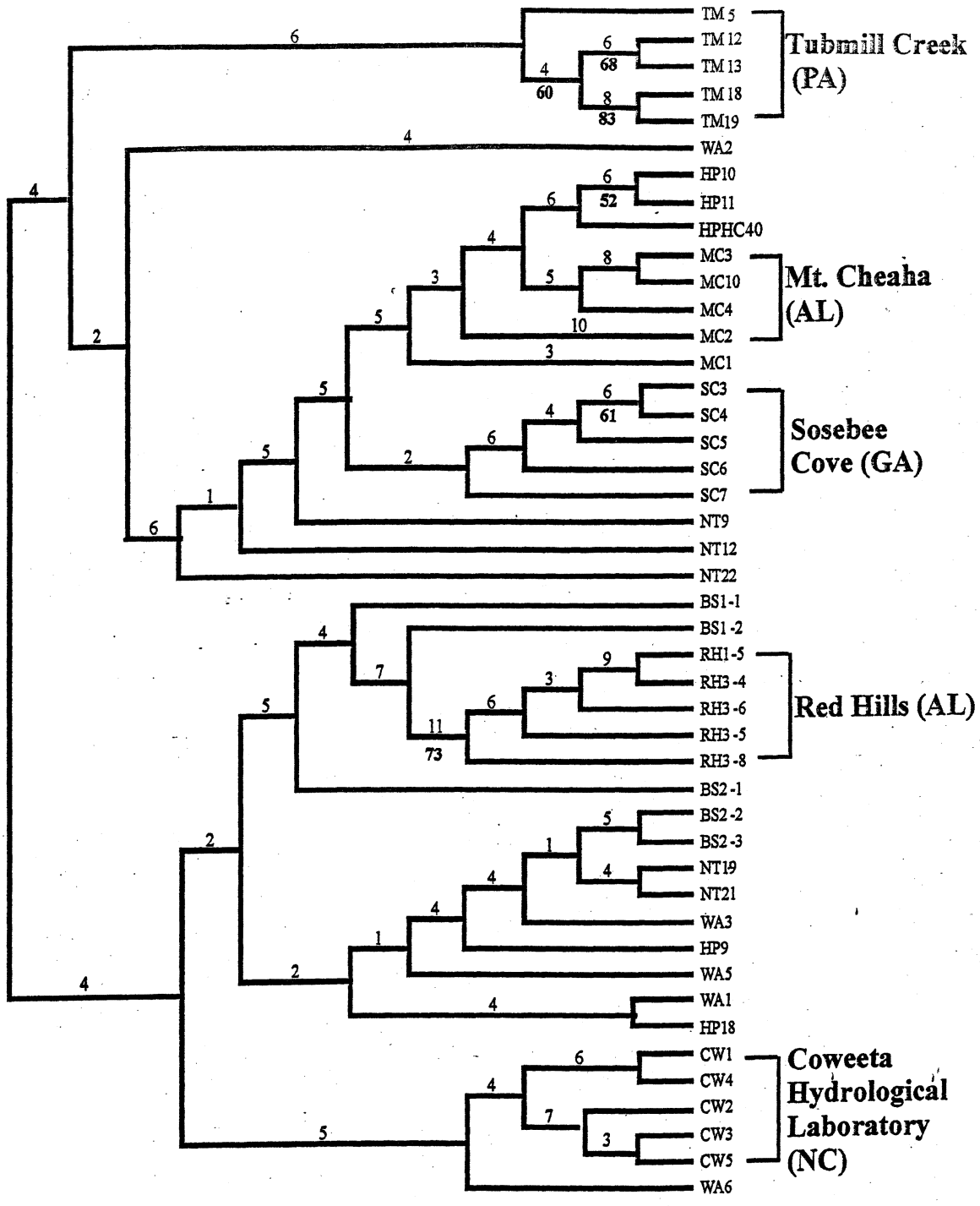
TM 5
TM 12
TM 13
TM 18
TM19
Tubmill Creek (PA)
WA2
HP10
HP11
HPHC40
MC3
MC10
MC4
MC2
Mt. Cheaha (AL)
MC1
SC3
SC4
SC5
SC6
SC7
Sosebee Cove (GA)
NT9
NT12
NT22
BS1-1
BS1-2
RH1-5
RH3-4
RH3-6
RH3-5
RH3-8
Red Hills (AL)
BS2-1
BS2-2
BS2-3
NT19
NT21
WA3
HP9
WA5
WA1
HP18
CW1
CW4
CW2
CW3
CW5
Coweeta Hydrological Laboratory (NC)
WA6

Figure 3.

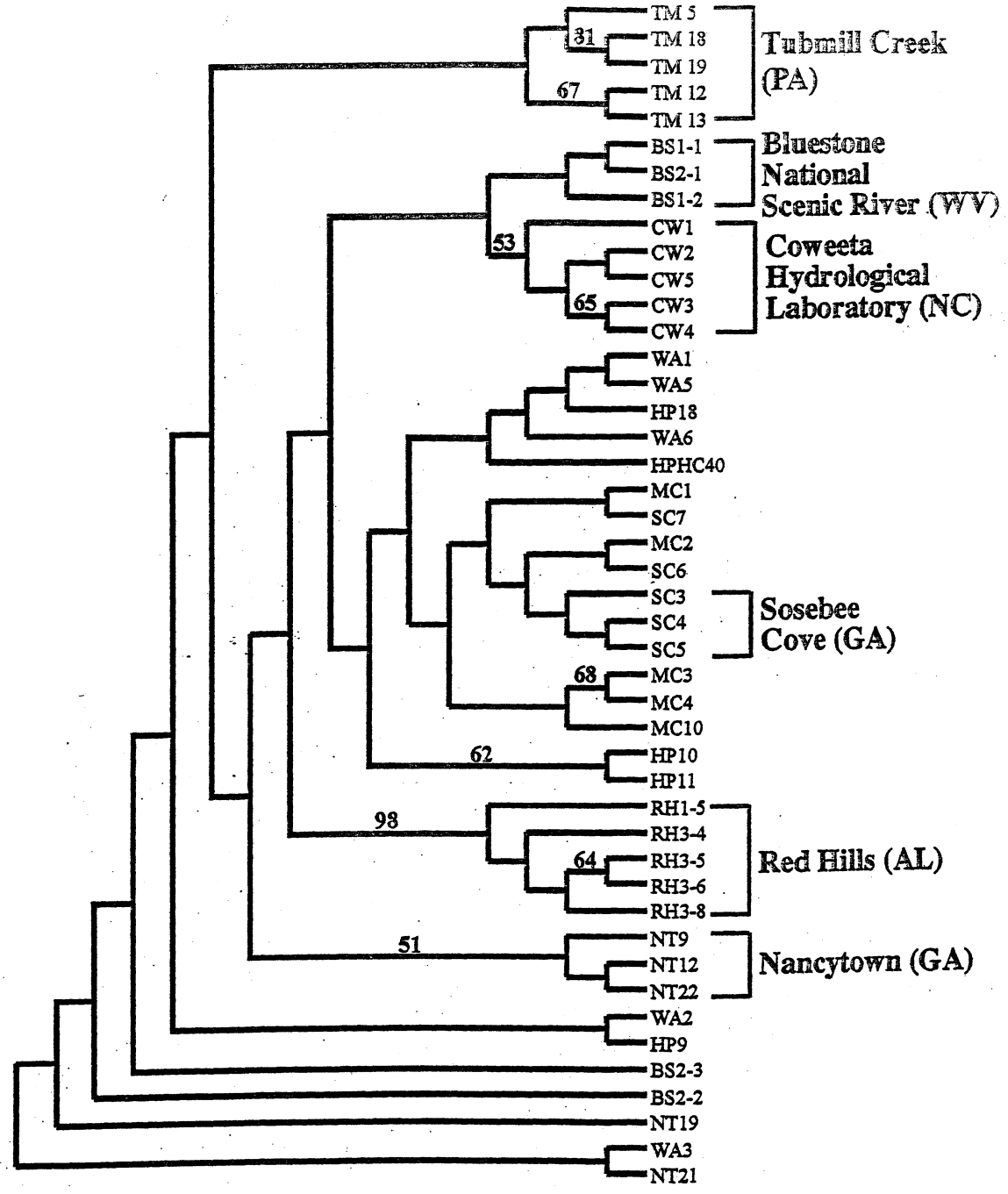
TM 5
TM 18
TM 19
TM 12
TM 13
31
67
Tubmill Creek (PA)
BS1-1
BS2-1
BS1-2
Bluestone National Scenic River (WV)
CW1
CW2
CW5
CW3
CW4
53
65
Coweeta Hydrological Laboratory (NC)
WA1
WA5
HP18
WA6
HPHC40
MC1
SC7
MC2
SC6
SC3
SC4
SC5
Sosebee Cove (GA)
68
MC3
MC4
MC10
62
HP10
HP11
RH1-5
RH3-4
RH3-5
RH3-6
RH3-8
98
64
Red Hills (AL)
NT9
NT12
NT22
51
Nancytown (GA)
WA2
HP9
BS2-3
BS2-2
NT19
WA3
NT21

Figure 4.

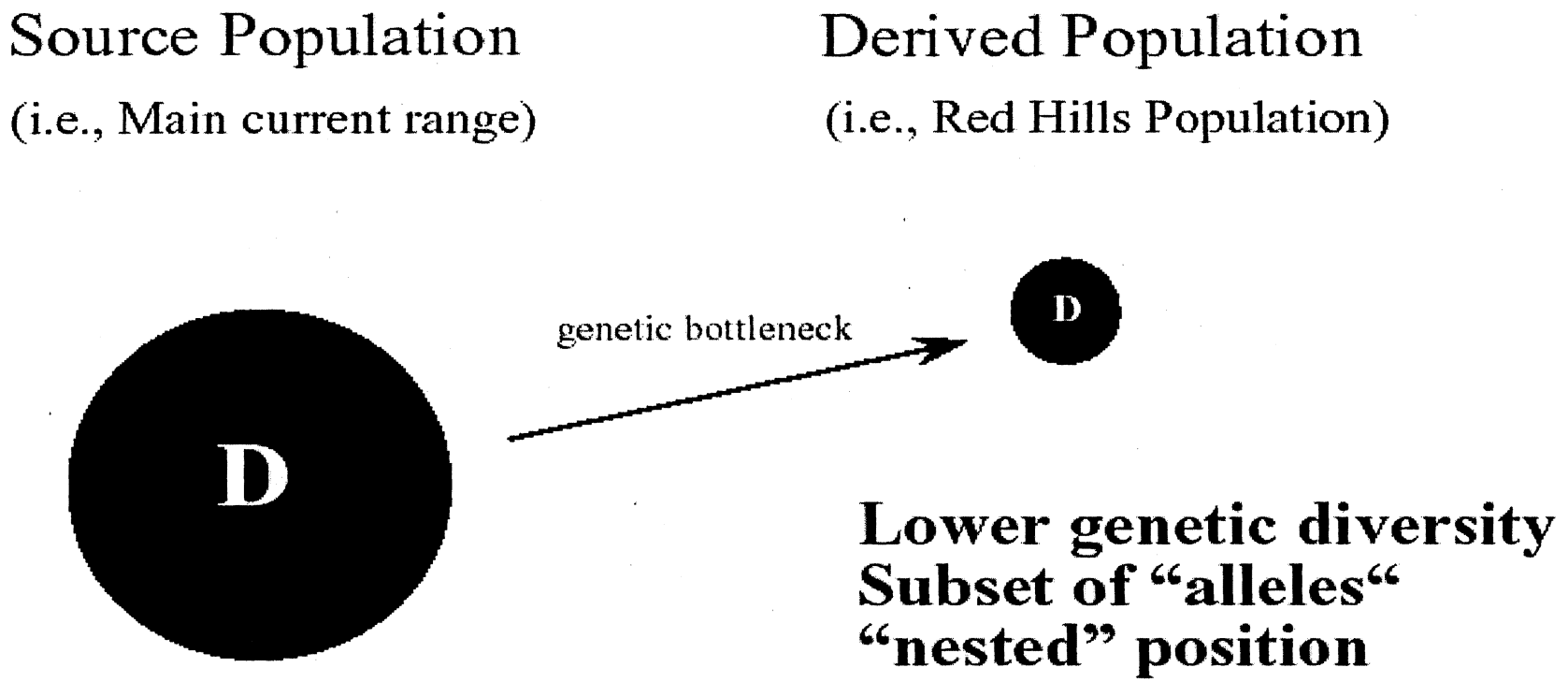
Source Population
(i.e., Main current range)
Derived Population
(i.e., Red Hills Population)
genetic bottleneck
D
D
Lower genetic diversity
Subset of "alleles"
"nested" position

Figure 5.

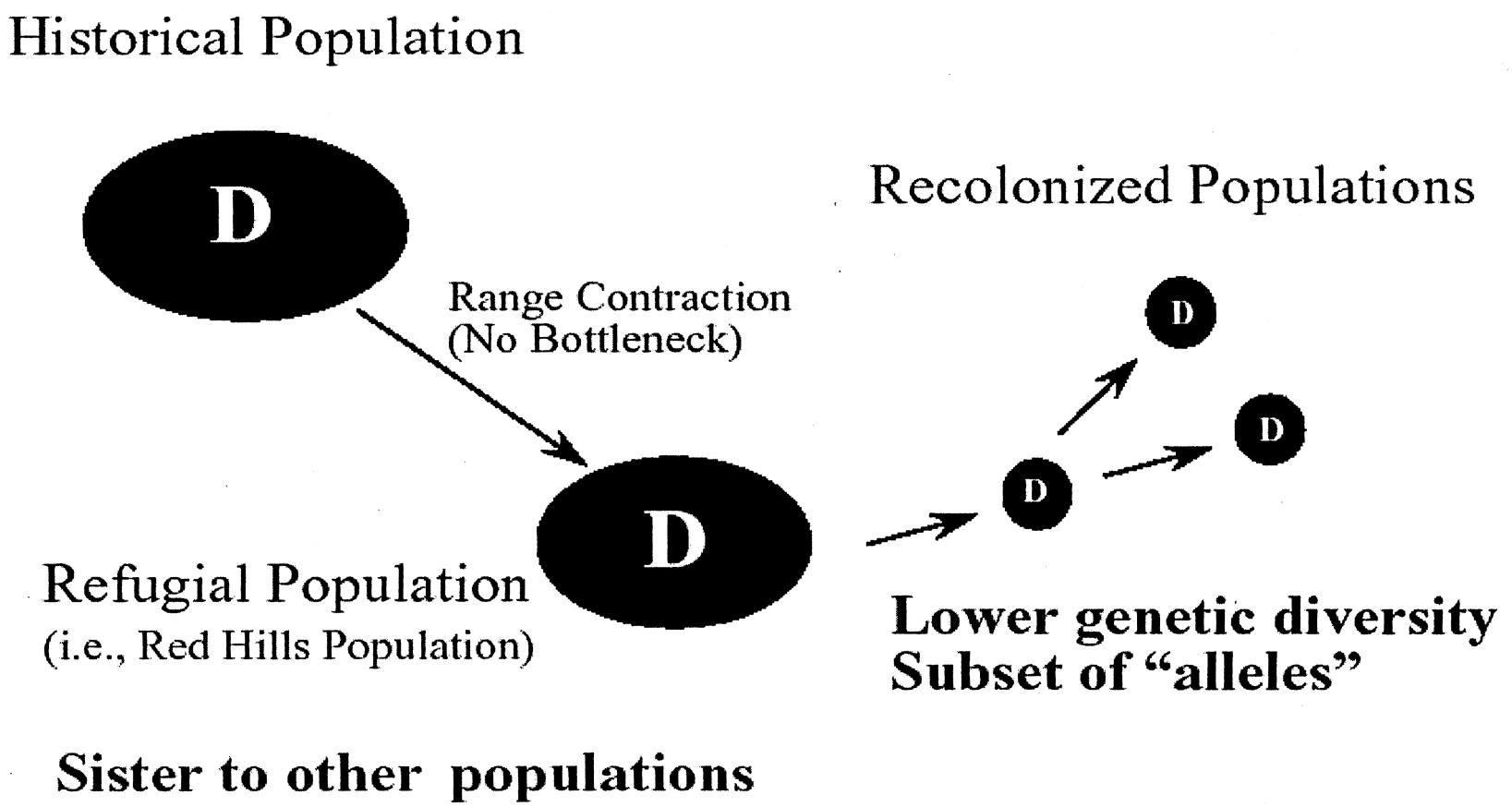
Historical Population
D
Range Contraction
(No Bottleneck)
D
Refugial Population
(i.e., Red Hills Population)
Sister to other populations
Recolonized Populations
D
D
D
Lower genetic diversity
Subset of "alleles"

Figure 6.