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Ecological associations and genetic divergence in Black-bellied Salamanders (*Desmognathus quadramaculatus*) of the Southern Appalachian Mountains

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Abstract. The discovery and subsequent description of cryptic biodiversity is often challenging, especially for groups that have undergone rapid lineage accumulation in the relatively recent past. Even without formal descriptions, understanding genetic diversity patterns as they relate to underlying ecological or historical processes can be important for conservation. The dusky salamanders of the genus *Desmognathus,* with 20 described species, comprise the second largest genus of plethodontid salamanders in the eastern United States. However, due to the presence of high genetic diversity and relatively few morphological synapomorphies, the number of species is likely to increase. For the three nominal species within the *D. quadramaculatus* species complex, including *D. quadramaculatus, D. folkertsi*, and *D. marmoratus*, we used a portion of the mitochondrial genome and nuclear markers in the form of amplified fragment length polymorphisms (AFLP) to uncover spatial patterns of genetic diversity. Within *D. quadramaculatus* and *D. marmoratus*, we uncovered four well-supported lineages with the mitochondrial sequences; phylogeographic patterns were not congruent with the AFLP data. Both sets of markers identified a clear isolation by stream distance. Using multiple regressions, we found that historical river drainages and terrestrial ecoregions explained the phylogeographic patterning we observed for *D. quadramaculatus.*

Keywords. Salamanders, *Desmognathus quadramaculatus*, *Desmognathus marmoratus*, AFLP, streams, *Desmognathus folkertsi*, mtDNA, ecoregions.

INTRODUCTION

In light of the constant threat of anthropogenic habitat alteration (Davis et al., 2008) and the ubiquity of cryptic biodiversity (Bickford et al., 2006), the discovery and subsequent descriptions of unique evolutionary lineages are central to understanding the full

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extent of biodiversity on Earth. However, the discovery and subsequent description of cryptic species is challenging, especially for organismal groups that have experienced rapid lineage accumulation in the recent past (Kozak et al., 2006; Wiens et al., 2006) and that lack obvious morphological differentiation. This is certainly true in lungless salamanders (Plethodontidae) where many species boundaries and evolutionary relationships have been concealed by the absence of obvious morphological synapomorphies (Wake, 1966; Larson et al., 1981; Wake et al., 1983; Carr, 1996; Adams and Rohlf, 2000; Highton and Peabody, 2000; Chippindale et al., 2004; Mueller et al., 2004; Wiens et al., 2006; Tilley et al., 2008).

Salamanders are a challenging group for which to delimit species boundaries and uncover cryptic biodiversity. Many salamander taxa exhibit extensive intraspecific variation in traits, such as color and pattern. At the same time, they exhibit extreme interspecific morphological similarity, which is usually coupled with high levels of genetic diversity (Wake, 1966, 1991; Wake et al., 1983; Highton and Peabody, 2000; Wake and Jockusch, 2000; Gao and Shubin, 2003; Mueller et al., 2004; Kozak et al., 2006; Wiens et al., 2006; Beamer and Lamb, 2008; Tilley et al., 2008). The Plethodontidae is the most species-rich family of salamanders containing approximately 68% of all known species (amphibiaweb.org), with new species being described regularly because of extreme morphological similarities and widespread homoplasy (e.g., Tilley and Mahoney, 1996; García-París and Wake, 2000; Jockusch et al., 2001; Camp et al., 2002; McCranie et al., 2005; Hanken et al., 2007).

The dusky salamanders (*Desmognathus*) comprise one of the most species-rich genera within Plethodontidae (Vieites et al., 2007), with the hotspot of biodiversity in the Appalachian Mountains (Kozak et al., 2006; Wiens et al., 2006; Vieites et al., 2007). Most desmognathan species are restricted to, or are associated with, swift-flowing mountain streams in the Appalachian Mountains of eastern North America. Many recent studies agree that the 20 currently recognized species may be a significant underestimation of the full extent of the biodiversity within the genus (Kozak et al., 2005; Beamer and Lamb, 2008), and the number of recognized species will likely increase due to the presence of extensive morphological stasis, rampant homoplasy, and high levels of genetic diversity among and within groups. To complicate modern-day taxonomy, studies have revealed a more complex and intertwined evolutionary history for many species within the genus *Desmognathus* (Titus and Larson, 1996; Rissler and Taylor, 2003; Kozak et al., 2005; Jones et al., 2006; Beamer and Lamb, 2008; Tilley et al., 2008), especially for *D. marmoratus* and *D. quadramaculatus* (Titus and Larson, 1996; Rissler and Taylor, 2003; Jackson, 2005; Jones et al., 2006). Interestingly, these studies have suggested that some populations of *D. quadramaculatus* are more closely related to populations of *D. marmoratus*, a wholly aquatic and relatively rare salamander species (Titus and Larson, 1996; Rissler and Taylor, 2003; Jackson, 2005; Jones et al., 2006) with a limited geographic distribution. However, to date, there have been no published studies focusing exclusively on the phylogeography of *D. quadramaculatus* across its range.

The use of nuclear markers in plethodontid salamander species, with the exception of allozymes, is uncommon for studies of fine-scaled population structure, although sequencing nuclear genes is becoming more common. That said, amplified fragment length polymorphism (AFLP; Vos et al., 1995) is a PCR-based, anonymous dominant nuclear-marker technique that has been extensively used to investigate intraspecific phylogeographic patterns, uncover new species, and delimit species boundaries in many groups (Shaw, 2002; Seman et al., 2003; Wang et al., 2003; Creer et al., 2004; Finn et al., 2006; Mendelson and Simons, 2006; Garoia et al., 2007; Kinkead et al., 2007; Nicolè et al., 2007), especially in

plants, (e.g., Hoarau et al., 2001; Garcia et al., 2004; Albach et al., 2006; Agrimonti et al., 2007; Andrade et al., 2007; Assefa et al., 2007; Nicolè et al., 2007), microbes and fungi (e.g., Vos et al., 1995; Blears et al., 1998; Bensch and Åkesson, 2005), and invertebrates (Wilding et al., 2001; Shaw, 2002; Carisio et al., 2004; Mock et al., 2004; Pizzo et al., 2006). Studies that use AFLP in vertebrate taxa are limited (Bensch and Åkesson, 2005), but this number has increased over the past few years (Ogden and Thorpe, 2002; Wang et al., 2003; Makowsky et al., 2008). At present, there are only a few known studies that involve the use of AFLP in salamander taxa (Voss and Shaffer, 1997; Curtis and Taylor, 2003; Riberon et al., 2004; Lowe et al., 2006; Whitlock et al., 2006; Jehle et al., 2007; Kinkead et al., 2007; Milá et al., 2010), even though AFLP is a useful marker that can be used to investigate population structure and genetic variability at the species level (Mueller and Wolfenbarger, 1999).

The goals of our study were to use both mitochondrial and AFLP data to: 1) address the phylogeographic and genetic patterns across populations of *D. quadramaculatus,* and 2) assess the spatial patterns of divergence, specifically testing the role of drainages (i.e., current versus historical river drainages) as barriers of gene exchange.

MATERIALS AND METHODS

The study taxon

Desmognathus quadramaculatus is a species complex comprised of at least three nominal species, including, *D. quadramaculatus* (Black-bellied Salamander), *D. folkertsi* (Dwarf Black-bellied Salamander), and *D. marmoratus* (Shovel-nosed Salamander). *Desmognathus quadramaculatus* exhibits the largest geographic range of the three and is a semi-aquatic species that can be found in swift-flowing, montane streams extending from southern West Virginia southward through the Great Smoky and Unicoi Mountains, and into the Blue Ridge escarpment of western North Carolina. This species reaches its southern-most geographic limit in the upper Piedmont of northern Georgia (Jensen et al., 2008).

Desmognathus folkertsi and *D. marmoratus* both exhibit relatively small geographic ranges and are often syntopic with that of *D. quadramaculatus*. *Desmognathus folkertsi* is a recently described, semi-aquatic species (Camp et al. 2002) known from northern Georgia and abutting areas of the Carolinas; whereas, *D. marmoratus* is an aquatic species that has a larger geographic distribution extending from southern Virginia, south along the Blue Ridge Mountains to northern Georgia. *Desmognathus folkertsi* appears to represent a cohesive, monophyletic taxon (Jackson, 2005; Wooten 2007; Wooten et al., 2010). However, *D. marmoratus*, as it is currently recognized, consists of several lineages and may be a paraphyletic taxon (Jackson, 2005; Jones et al., 2006; Kozak et al., 2006). Jones et al. (2006) suggested the recognition of southernmost populations of *D. marmoratus* as a separate species (*D. aureatus*).

Sampling locations

We examined 281 individuals from 56 populations *D. quadramaculatus* from the southern Appalachian Mountains in the eastern United States, as well as closely related species, including *D. monticola* (n = 15), *D. folkertsi* (n = 23), and *D. marmoratus* (n = 8; Fig. 1). Detailed information on locality, museum voucher number, Genbank accession number, and river drainage are presented in Appendix 1. Voucher specimens were euthanized in using ethyl 3-aminobenzoate methanesulfonate

(MS 222; Sigma-Aldrich) at a concentration of 2 g / L. An approximately 1 cm piece of tail tissue was removed for genetic analysis and frozen (-80ºC) in the permanent tissue archive at The University of Alabama Herpetology Collection (UAHC). Specimens were then fixed in 10% formalin and preserved in 70% ethanol. All specimens were deposited in the UAHC stored at The University of Alabama, Tuscaloosa or in the Appalachian State Herpetology Collection (APPSU), Boone, North Carolina.

Mitochondrial DNA extraction and sequencing

Whole genomic DNA was extracted from approximately 5 mm of tail tissue using the DNeasy tissue-kit protocol for animal tissues (Qiagen, Valencia, CA). Amplification of the 12S rRNA and a portion of the valine transfer tRNA regions of the mitochondrial genome was completed on all individuals by using primers B and G from Titus and Larson (1996) and following a modified methodology of Rissler and Taylor (2003). This portion of the mitochondrial genome has been shown to be a highly informative marker for discriminating intra- and interspecific relationships in *Desmognathus* (Titus and Larson, 1996; Crespi et al., 2003; Rissler and Taylor, 2003; Rissler et al., 2004). ExoSapIT (U.S. Biochemicals Corp., Cleveland, OH) at 37 ºC for 45 min and 80 ºC for 15 min was used to purify the PCR products.

Cycle-sequencing was completed in forward and reverse directions on the purified PCR products using BigDye Terminator (Applied Biosystems, Foster City, CA), followed by purification with Sephadex G-50 (Sigma Aldrich Corp., St. Louis, MO), and visualized on an ABI 3100 automated DNA sequencer. Additional mtDNA sequences were retrieved from Genbank for *D. wrighti* (outgroup), *D. quadramaculatus, D. marmoratus, D. aeneus,* and *D. monticola,* and these accession numbers are listed in Appendix 2. Collapse 1.2 (http://darwin.uvigo.es/software/collapse.html) was used to remove any identical haplotypes before phylogenetic analyses.

Amplified fragment length polymorphism

Our AFLP procedures were modified from the protocol by Voss et al. (1995) and Blears et al. (1998). Digestion reactions in 20 µl volumes were performed on whole genomic DNA with a concentration of 25 ng / µl using the following cocktail per reaction: 2 µl *Eco*RI 10X restriction buffer, 1.0 µl EcoRI, 0.2 µl *MSE*, and 12 µl H_2O. Following a 5 h incubation period at 37 ºC, 20 µl of ligation mixture consisting of 12 µl H_2O, 4 µl 10X T4 DNA ligase buffer, 1.5 µl of each double-stranded adapter pair (75 pmol), and 1.0 µl of T4 ligase was added to the digestion solution. The double-stranded adaptor pairs were constructed from the following complementary single-stranded oligonucleotides: *Eco*RI adaptor: 5'-CTC GTA GAC TGC GTA CC-3' AND 5'-AAT TGG TAC GCA TAC-3'. Ligation was performed for 10 h at 16 ºC. Following ligation, each solution was diluted with 160 µl H_2O.

Two increasingly selective PCR amplifications of the ligated DNA were performed. For preselective amplification, 10µl of the diluted restriction-ligation product was used as a template and added to 40 µl preselective amplification solution consisting of: 5 µl 10X PCR buffer, 1.3 µl of each preselective primer (15 pmol), 4.0 µl dNTP (10 mM), 1.0 µl formamide, 2.5 µl $MgCl_2$ (25 mM), 24.75 µl H_2O, and 0.5 µl Taq DNA polymerase. Four preselective primer combinations were used including: *Eco*RI + AC / *MSE* + CA; *Eco*RI + CA / *MSE* + CA; *Eco*RI + CA / *MSE* + CG; and *Eco*RI + CA / *MSE* + GC. The PCR cycling conditions were a preliminary 72 ºC extension for 60 sec followed by 20 cycles of 94 ºC for 50 sec, 56 ºC for 60 sec, and 72 ºC for 120 sec. Preselective solutions were diluted with 125 µl of H_2O following PCR cycling.

For selective PCR, 5 µl of diluted preselective solution was added to 20 µl of selective PCR solution which consisted of 0.5 µl formamide, 10 µl H_2O, 2.5 µl 10X PCR buffer, 3 µl $MgCl_2$ (25 mM), 3 µl dNTP (10 mM), 1.5 µl (0.5 pmol) of the flourophore (6-FAM)-labeled *Eco*RI primer, 1.5 µl (25 pmol) of the unlabeled *MSE* selective primer, and 0.5 µl Taq DNA polymerase. The PCR

cycling conditions were: a touchdown cycle of 94 ºC for 50 sec, 57 ºC for 60 sec, and 72 ºC for 120 sec, followed by 20 cycles of 94 ºC for 50 sec, 56 ºC for 60 sec, and 72ºC for 120 sec. The 20 cycles were followed by a final extension at 72 ºC for 10 min.

Three combinations of selective primer combinations were chosen including: *Eco*RI + CAA / *MSE* + CA; *Eco*RI + CAA / *MSE* + CG; and *Eco*RI + CAA / *MSE* + GC from a primer screening procedure in another study (Wooten and Tolley-Jordan, 2009). When combined, these combinations produced between 450 and 555 well-defined bands per sample, distributed widely across the 60-350 scoring window. The selective amplification products were purified using fine Sephadex G-50 (Sigma-Aldrich Corp., St. Louis, MO). We loaded 1.5 µl of purified product per sample along with 0.5 µl GeneScan-500 ROX ladder (Perkin-Elmer) into 96-well plates and samples were analyzed using an automated ABI 3100 with GeneScan software. Electropherograms were imported into GeneMarker v1.6 for further analyses.

Phylogenetic reconstruction – mtDNA and AFLP

Approximately 600 bp of the 12S rRNA and valine transfer tRNA regions were sequenced. Forward and reverse sequences were assembled, and any ambiguous sites were verified using Sequencher 4.6 (Gene Codes, Ann Arbor, MI). Clustal W procedure in BioEdit v7.0.9 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) was used to align all sequences. The alignment was manually corrected. The sequences were sufficiently similar to allow manual correction following alignment without restrictions based upon the gap positions in the hypothesized loop regions of the 12S rRNA sequences. We used *D. wrighti* as the outgroup in all phylogenetic reconstructions; *D. wrighti* is currently accepted as the basal member of the genus *Desmognathus* (Titus and Larson, 1996; Rissler and Taylor, 2003; Jones et al., 2006; Tilley et al., 2008).

Phylogenetic relationships among haplotypes using mtDNA sequences were estimated using maximum likelihood and Bayesian procedures. For the Bayesian procedure, the model of sequence evolution that best fit the mtDNA sequence data was determined using MRMODELTEST v2.2 (http://www.abc.se/~nylander). MRBAYES v3.1 was then used to conduct a Bayesian phylogenetic analysis (Huelsenbeck and Ronquist, 2001) using the TrN + Γ model of evolution. Six heated Markov chains were run for 10×10^6 generations, sampling every 1000 generations for a total of 10,000 samples. Three replicate searches were conducted to verify that the analyses were optimal by producing similar topologies and ln-likelihood scores (Huelsenbeck and Bollback, 2001). The burn-in procedure was used to eliminate topologies generated before the ln-likelihood was stabilized. Posterior probabilities were estimated as the proportion of trees sampled after the burn-in procedure containing each of the observed bipartitions (Larget and Simon, 1999).

For the maximum-likelihood procedure, MODELTEST v3.7 (Posada and Crandall, 1998) was used to estimate the model of sequence evolution. Garli v0.951 (Zwickl, 2006) was used to reconstruct phylogenetic relationships among haplotypes using the maximum-likelihood procedure with the TrN + Γ model of evolution. Statistical support for each branch was assessed using bootstrap analysis with 1000 replications in Garli v0.951 (Zwickl, 2006).

For AFLP analyses, each primer combination was used to create a unique template to normalize the data across the different runs, and this step standardized the calling of peaks across the four different primer combinations using GeneMarker v1.6 (SoftGenetics, State College, Pennsylvania). Peaks were called between 60-350bps, and these data were entered into a binary matrix as discrete variables (1 for presence and 0 for absence). The binary matrix from each primer combination was combined into one data set and analyzed as phenotypes. A Jaccard dissimilarity matrix was generated in R Package v4.0 (Casgrain and Legendre, 2001) from the combined binary matrix. A phylogram was generated in PAUP* (Swofford, 2002) using the minimum evolutionary procedure. Statistical support for each branch was assessed using both neighbor-joining and parsimony analyses with 1000 replications in PAUP* (Swofford, 2002). For analyses, Hardy-Weinberg equilibrium was assumed for the *D. quad-*

ramaculatus, D. folkertsi, and *D. marmoratus* populations, and we assumed that each peak (i.e., fragment) represented a unique sequence (Zhivotovsky, 1999; Kinkead et al., 2007; Measey et al., 2007).

Genetic diversity indices

Patterns of genetic diversity within and across lineages and river drainage basins were investigated using DnaSP v4.0 (Rozas and Rozas, 1999; Rozas et al., 2003) for populations of *D. quadramaculatus.* For the three nominal species in the *D. quadramaculatus* complex, haplotype diversity (Nei, 1987), sequence diversity (k; Tajima, 1983), nucleotide diversity (p; Nei, 1987), the number of mutations per site (θ; Nei, 1987), and the total number of mutations (h; Nei, 1987) were calculated to measure sequence diversity. In order to test predictions made by the neutral theory of molecular evolution (Kimura, 1983) and for evidence of population-expansion events, we calculated Tajima's D (Tajima, 1989), Fu and Li's D* test statistic (Fu and Li, 1993), Fu and Li's F* test statistic (Fu and Li, 1993) and Fu's Fs statistic (Fu, 1997).

For AFLP data, we used Structure v2.2 (Pritchard et al., 2000; Falush et al., 2003; Falush et al., 2007), which is a clustering method that assigns individuals to populations using multilocus genotype data (Pritchard et al., 2000; Falush et al., 2003), to discern between lineages of the *D. quadramaculatus* species complex. We assigned individuals to five groups based upon the Bayesian phylogram to test for congruence between the phylogeny and the group placement using the AFLP fragments, a multiloci, dominant, nuclear markers, using Structure v2.2 (Pritchard et al., 2000; Falush et al., 2003; Falush et al., 2007). In Structure v2.2 (Pritchard et al., 2000; Falush et al., 2003; Falush et al., 2007), we ran 10,000 burn-in with 100,000 iterations for two – 10 (k) groups with the admixture ancestry model and the option of correlated allele frequencies between populations (Falush et al., 2003). We allowed Structure v2.2 to infer the degree of admixture alpha from the AFLP data. When alpha is close to zero, most individuals are from one population; whereas, when alpha is greater than one, individuals are admixed (Evanno et al., 2005). We set lambda, which was a parameter of the distribution of allelic frequencies, to one (Pritchard et al., 2000; Evanno et al., 2005). We completed a pilot run using the AFLP data, and we found that a burn-in of 1000 and MCMC of 10,000, with 10 replications for each value of k, was not sufficient. Because of this, we increased the burn-in to 10,000 and the MCMC to 100,000, with 10 replications for each value of k; we found that the likelihood values and the amount of variation for the likelihood of each k stabilized across replications. Finally, we chose the option of ancestdist, which calculates the 90% probability interval that a particular individual would be assigned to a distinct group.

For AFLP data, we used AFLP-SURV v1.0 (Vekemans, 2002; Vekemans et al., 2002) to estimate genetic diversity with dominant alleles using the Lynch and Milligan (1994) method. Statistical analysis of AFLP fragments was based on the assumption that the fragments act as diploid, dominant markers either being present (scored as 1) or absent (scored as 0). Nei's genetic distance, which is the average, expected heterozygosity of the marker loci was used to estimate genetic diversity from the AFLP fragments. We assumed Hardy-Weinberg equilibrium to examine total gene diversity (*Hj*), mean gene diversity within populations (*Hw*), average gene diversity among populations (*Hb*), and Fst across the landscape (Vekemans, 2002; Vekemans et al., 2002). Because discrete genotypes are not generated from AFLP fragments, each fragment was treated as a single locus with two alleles (Blears et al., 1998). In AFLP-SURV, we chose the Bayesian non-uniform distribution with 1000 permutations for estimates of gene diversity. In addition, we constructed a multidimensional scaling (MDS) plot based on Jaccard's dissimilarity measure (1-similarity; Gower 1971) using Statistica v.6 (StatSoft) and R Package v.4.0 (Casgrain and Legendre, 2001) to see if distinct clusters formed based on dissimilarity among populations or between *D. folkertsi* and *D. quadramaculatus*. In Arlequin v.3.11 (Excoffier et al., 2005), we calculated an AMOVA using the AFLP binary matrix for unique haplotypes and used the same scheme for assigning individuals to populations and groups as in the mtDNA analysis. Finally, we calculated Nei's (1978) unbiased genetic distance (D) between lineages

of *D. folkertsi* and again between *D. folkertsi* and *D. quadramaculatus* using the AFLP binary matrix in GeneAlEx 6.1 (Peakall and Smouse, 2006).

Spatial partitioning of genetic variation

Desmognathus quadramaculatus, like *D. folkertsi*, is a semi-aquatic salamander, and gene flow can occur via aquatic or terrestrial routes (Wooten et al., 2010). Therefore, we evaluated isolation by distance (Wright, 1943; Slatkin, 1993) using both land and aquatic routes of dispersal. We calculated distances among streams using Network Analyst in ArcGIS v9.0. An arbitrary value of 5,000,000 km was assigned to represent streams that were in different river drainages. Straight-line distances (km) via land were calculated for each individual using latitude and longitude between each geographic locality using the R package v4.0. We used Mantel tests in the R package v4.0 (Casgrain and Legendre, 2001) to test these associations.

Analysis of molecular variance (AMOVA; Excoffier et al., 1992) was used to test the partitioning of genetic variation within and across streams and river drainage basins using Arlequin v3.1 (Excoffier et al., 2005). The AMOVA Φ statistics are equivalent to F statistics (Wright, 1931; Wright, 1965) and are determined to be significant based on at least 1000 nonparametric permutations of individuals, populations, or groups of populations. In our analyses, individuals were assigned to a stream and then grouped based on river drainage basin.

We used multiple regression to examine whether patterns of genetic diversity were explained by the following phylogeographic breaks: 1) current taxonomic classification (i.e., *D. folkertsi, D. quadramaculatus*, and *D. marmoratus*), 2) current drainage patterns, 3) historical drainage patterns from the Pliocene (Kozak et al., 2006), 4) freshwater ecoregions (categorized according to sampling location and information available in Abell et al., 1999), and 5) terrestrial ecoregions (categorized according to sampling location and the classification of temperate broad leaf and mixed forests ecoregions in Ricketts et al., 1999). To test these hypotheses, we generated uncorrected genetic distances for each mitochondrial haplotype calculated from PAUP* (Swofford, 2002). These values were considered the dependent variable. For each of the five independent variables, characters were assigned based on geographic locality or taxonomic group, depending on the phylogeographic break, and a matrix of distances between all pairs of haplotypes was generated using R package v4.0 (Casgrain and Legendre, 2001). These matrices were imported into Permute! v3.4 (Casgrain, 2001); multiple-regressions were computed and 1000 permutations were completed (Legendre et al., 1994) to assess the probability that each break explains the patterns of genetic diversity observed. Backwards regression was used to determine the importance of the independent variables.

RESULTS

Overall genetic variation

For the nominal species within the *D. quadramaculatus* complex, 327 sequences representing 800 aligned bases were reported. For *D. quadramaculatus,* 201 unique haplotypes were identified from a total of 281 individuals and 56 sampling localities (Appendix 1). The TrN + Γ model of evolution was chosen from MRMODELTEST v2.2 with nucleotide frequencies of A = 0.2828, C = 0.2201, G = 0.2025, and T = 0.2946; substitution model rate matrix: R(a) [A-C] = 1.0000, R(b) [A-G] = 2.5812, R(c) [A-T] = 1.0000, R(d) [C-G] = 1.0000, R(e) [C-T] = 2.1188, and R(f) [G-T] = 1.0000; Γ distribution shape parameter equaled 0.4069. We reported bootstrap values of $\geq$ 70% (Hillis and Bull, 1993) and pos-

terior probabilities ≥95% (Wilcox et al., 2002) to represent well-supported nodes for the phylogenetic hypothesis.

For *D. quadramaculatus*, the mean number of AFLP fragments generated by each AFLP primer combination was 187.67, and this varied from 167 fragments for primer combination *Eco*RI + CAA / *Mse*I + GC to 212 fragments for primer combination *Eco*RI + CAA / *Mse*I + CG. When all AFLP fragments were considered, a total of 563 fragments were generated from three primer combinations. Of these, 536 were polymorphic for *D. quadramaculatus* from 38 populations. We tested for AFLP fragment-size homoplasy and found that there was no significant correlation between polymorphic AFLP fragment size and frequency of fragment occurrence (r = -0.089, P = 0.348; Vekemans et al., 2002).

Phylogenetic analysis – mtDNA

The Bayesian analysis and the maximum-likelihood phylograms yielded similar topologies for *D. quadramaculatus* species complex and the outgroup. Because of this, we represented the phylogenetic hypothesis of the *D. quadramaculatus* species complex with the Bayesian phylogram with the posterior probabilities and maximum-likelihood bootstrap values for common branches. We generated a 50% majority-rule consensus phylogram from the Bayesian analysis with a mean ln-likelihood = -15898.7 following a burn-in procedure to remove 2600 topologies that were generated before stability of the ln-likelihood was established.

Haplotypes within the *D. quadramaculatus* complex formed a well-supported monophyletic group when all three nominal species were included in the analysis (Figs. 2A and 2B). There were two major lineages recovered (Lineage 1 and 2) with strong statistical support; Lineage 1 was comprised of *D. folkertsi*, which formed a monophyletic group, and Lineage 2 was composed of *D. quadramaculatus* and *D. marmoratus* (Fig. 2A). Within Lineage 2, several well-supported lineages were formed; two lineages were found south of the French Broad River (Fig. 2A; Lineage 2A and 2B) and two lineages were found north of the French Broad River (Fig. 2B; Lineages 2C and 2D). Lineage 2A was comprised of both nominal species *D. quadramaculatus* and *D. marmoratus;* whereas; Lineage 2B consisted only of *D. quadramaculatus*. Both Lineages 2A and 2B consisted of individuals from Georgia, South Carolina, and southwestern North Carolina. The northern lineage (Lineage 2C and 2D) consisted of *D. quadramaculatus* and *D. marmoratus* individuals from West Virginia, Virginia, and northern Tennessee. The population from northern Tennessee (Fig. 1, Map Locality 5) formed a monophyletic, well-supported clade; this population is undergoing further research. Lineage 2D consisted of a single population in North Carolina (Fig. 1, Map Locality 13); this population formed a well-supported and basal lineage to Lineage 2C. However, because Lineage 2D consisted of a single population in an isolated geographic area, surveys for *D. quadramaculatus* populations are underway in surrounding streams.

Phylogenetic analysis – AFLP

Desmognathus folkertsi and *D. quadramaculatus* formed well-supported, distinct lineages when the AFLP data were used, but within the species, no clear geographic patterns were found (data not shown). *Desmognathus marmoratus* individuals were nested within

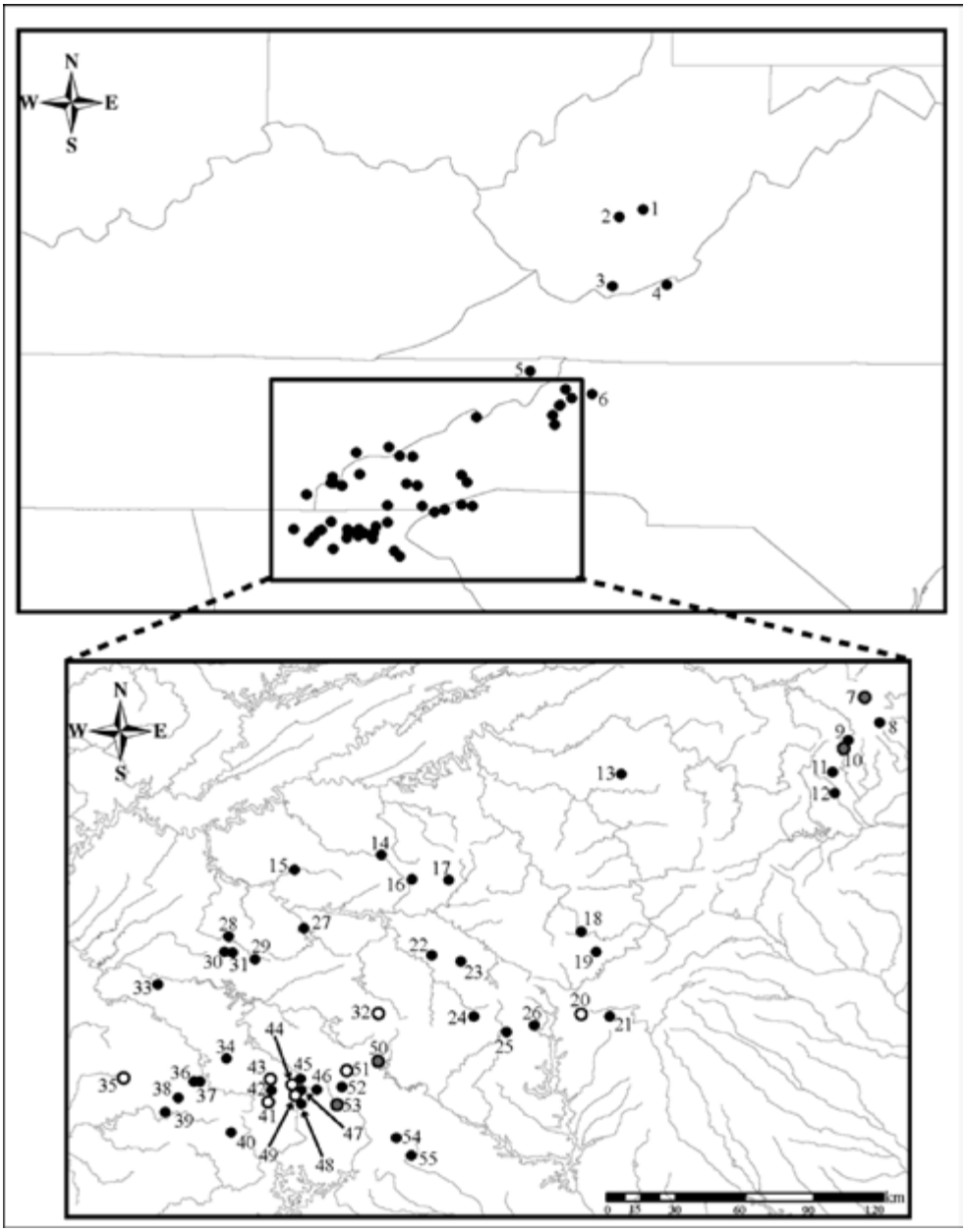

Fig. 1. Study localities in the southern Appalachian Mountians. Sampling localities spanned parts of West Virginia, Virginia, North Carolina, Tennessee, South Carolina, and Georgia.

D. quadramaculatus for the AFLP, but these nodes were not well-supported. Furthermore, the MDS plot did not reveal any distinct groups for *D. quadramaculatus*, *D. folkertsi*, or *D. marmoratus*.

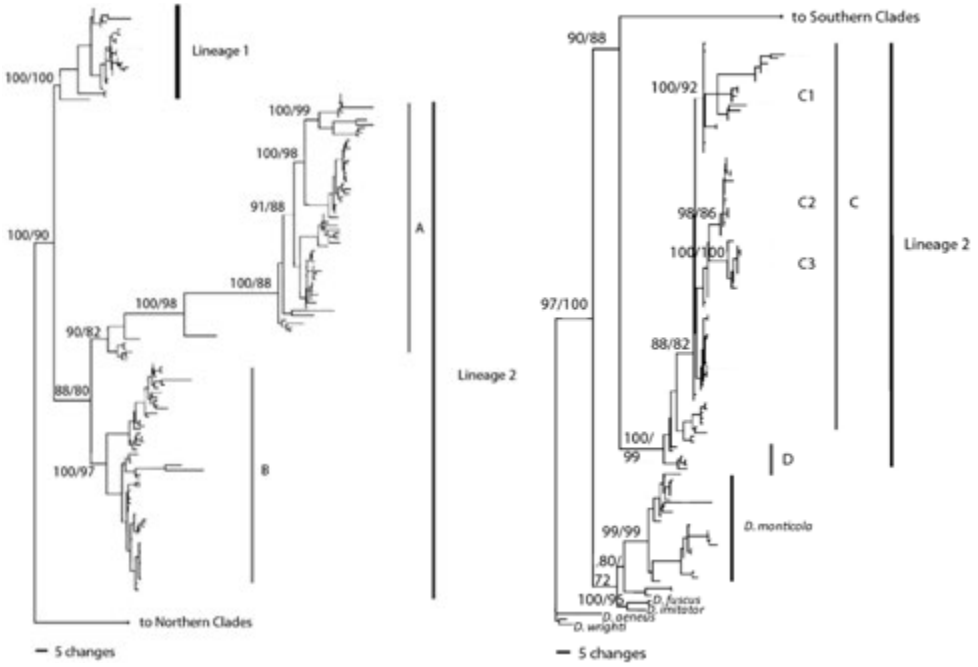

Fig. 2. (part 2a) Bayesian phylogram using the 12S rRNA portion of the mitochondrial genome with the maximum-likelihood model of evolution TrN + G for unique haplotypes of *D. quadramaculatus*, *D. folkertsi*, and *D. marmoratus*. Lineage 1 is *D. folkertsi*. Lineage 2A is basically *D. quadramaculatus* and *D. marmoratus* that occur in southern areas including northern Georgia, North Carolina, and southern Tennessee. Lineage 2B contains *D. quadramaculatus* from populations of Georgia, South Carolina, and North Carolina. (part 2b) Northern populations of *D. quadramaculatus*. Lineage 2C contains populations from Virginia, West Virginia, North Carolina, and Tennessee. Lineage 2C-3 is a clade in Tennessee (see text). Only support values on major nodes are given (bootstrap values from 1000 replicates). See Appendix 1 and 2 for more detail on specific localities and samples.

Statistical analyses of genetic data

Haplotypes within the *D. quadramaculatus* species complex exhibited typical to high levels of genetic diversity. *Desmognathus folkertsi* exhibited the lowest level of genetic diversity among the three nominal species with a k = 1.16%; whereas, *D. quadramaculatus* and *D. marmoratus* exhibited high levels of diversity with a k = 12.02% and k = 20.08%, respectively (Table 1). Both *D. quadramaculatus* and *D. marmoratus* exhibited Tajima' D, Fu and Li's D*, Fu and Li's F*, and Fu's F statistics were not significant; however, these same measures for *D. folkertsi* were significantly negative (Table 1). This suggests that populations of *D. quadramaculatus* and *D. marmoratus* have been established for a long period of time and have not experienced recent range expansions. However, for *D. folkertsi,* the phylogeny had shallower branches than expected under the neutral model of evolution, which is consistent with a recent range expansion.

When the data were partitioned by river drainage for *D. quadramaculatus,* the Catawba River drainage exhibited the highest levels of diversity k = 184.164; whereas, the

Table 1. Comparative summary statistics of the mitochondrial sequence variation in *Desmognathus quadramaculatus*, *D. folkertsi*, and *D. marmoratus*. **π**: nucleotide diversity per site with Jukes and Cantor correction; k: mean number of nucleotide differences; **η**: total number of mutations; **θ**: the amount of variation expected at each nucleotide site if there is neutral evolution.

	D. quadramaculatus	*D. folkertsi*	*D. marmoratus*
Haplotype diversity ± SD	0.950 ± 0.008	0.822 ± 0.083	0.978 ± 0.054
π ± SD	0.230 ± 0.0154	0.012 ± 0.004	0.333 ± 0.039
K	96.184	6.866	160.667
η	439	61	335
θ per site	0.177	0.028	0.245
Tajima's D	0.967 (P > 0.10)	-2.305 (P < 0.01)	1.785 (0.10 > P > 0.05)
Fu and Li's D*	-1.551 (P > 0.10)	-2.956 (P < 0.05)	1.166 (P > 0.10)
Fu and Li's F*	-0.342 (P > 0.10)	-3.227 (P < 0.02)	1.494 (0.05 < P < 0.10)
Fu's Fs statistic	18.63	-2.401	3.569

Table 2. Comparative summary statistics of the 12S rRNA mitochondrial sequence variation in *Desmognathus quadramaculatus* partitioned by river drainage. Symbols as in Table 1.

	Tennessee (n = 64)	Coosa/ Tallapoosa (n = 21)	Chattahoochee (n = 14)	Savannah (n = 10)	New River (n = 10)	Catawba (n = 11)
Haplotype diversity ± SD	0.977 ± 0.010	0.948 ± 0.031	1.000 ± 0.027	1.000 ± 0.045	0.878 ± 0.025	1.000 ± 0.039
π ± SD	0.227 ± 0.022	0.284 ± 0.035	0.128 ± 0.006	0.283 ± 0.069	0.2013 ± 0.031	0.307 ± 0.056
k	140.599	156.990	74.099	163.422	112.131	184.164
η	538	369	407	388	375	401
θ per site	0.224	0.186	0.220	0.238	0.141	0.228

Tennessee, Savannah, New, Chattahoochee, and Coosa/Tallapoosa River drainages exhibited moderate levels of genetic diversity, k = 140.599, k =163.422, k = 112.131, k = 70.099, and k = 156.990, respectively, for mtDNA (Table 2). Only 4.47% of the genetic diversity occurred among drainages but 90.37% of the genetic diversity was partitioned within drainages (Table 3). This pattern is indicative of gene flow across the landscape, either by natural or human-mediated means.

The expected heterozygosity values (*Hj*) calculated using the AFLP data for the two main mitochondrial lineages within the *D. quadramaculatus* species complex, Lineage 1 (*D. folkertsi*) and Lineage 2 (*D. quadramaculatus* plus *D. marmoratus*) were equal to 0.241 and 0.229, respectively (Table 4). To calculate the expected heterozygosity values (*Hj*) in only *D. quadramaculatus*, individuals were divided into two groups using lineages derived from the mitochondrial topology (i.e., Lineages 2A and 2B and Lineages 2C and 2D). Group 1 included *D. quadramaculatus* individuals from Lineages 2A and 2B, and Group 2 included *D. quadramaculatus* individuals from Lineages 2C and 2D. Group 1 and Group 2

Table 3. Partition of genetic variation for mitochondrial sequences in *Desmognathus quadramaculatus* determined by AMOVA.

	d.f.	Sum of squares	Variance component	Percentage of variation
Among groups (lineages*)	1	640.945	3.893	5.160
Among population (drainages**) Within groups	3	440.933	3.376	4.470
Within populations	188	12821.412	68.199	90.370
Total	192	13903.290	75.468	100.000

* Lineages (Fig. 2). ** Five major river drainage basins including New River, Catawba, Tennessee, Chattahoochee, Coosa/Tallapoosa, Savannah were used.

Table 4. Genetic diversity derived from AFLP data partitioned by lineage inferred from the mitochondrial genome for *Desmognathus quadramaculatus*, *D. folkertsi*, and *D. marmoratus*.

	n	Number of loci	% Polymorphic loci	*Hj** ± SE
Lineage 1 (*D. folkertsi*)	10	563	71.4	0.241 ± 0.008
Lineage 2A + 2B (*D. quadramaculatus* and *D. marmoratus*)**	27	563	68.4	0.222 ± 0.007
Lineage 2C + 2D (*D. quadramaculatus* and *D. marmoratus*)***	31	563	71.9	0.237 ± 0.007

*Hj: Nei's gene diversity; expected heterozygosity under Hardy-Weinberg genotypic proportions. ** southern populations. *** northern populations

Table 5. Genetic diversity derived from AFLP data computed over all populations for *Desmognathus quadramaculatus*. *Hw*: mean Nei's gene diversity; mean within-population expected heterozygosity under Hardy-Weinberg genotypic proportions; *Ht*: total gene diversity; *Hb*: Nei's Dst; average gene diversity among populations in excess of that observed within populations; *Fst*: Wright's fixation index; proportion of total gene diversity that occurs among as opposed to within populations.

n	*Hw* ± SE	*Ht* ± SE	*Hb* ± SE	*Fst* ± SE
58	0.2293 ± 0.0074	0.2358 ± 0.0013	0.0066 ± 0.0001	0.0279 ± 0.0312

exhibited expected heterozygosity (*Hj*) values equal to 0.222 and 0.237, respectively. There was little genetic differentiation among study sites of *D. quadramaculatus* with a total gene diversity (*Ht*) equal to 0.236 and an Fst equal to 0.028 (Table 5).

Analysis of molecular variance (AMOVA) for the AFLP data using three populations (Lineage 1, Group 1, and Group 2; see description above) and two groups derived from the maximum-likelihood topology (Fig. 2; Lineage 1 and Lineage 2) supported the mtDNA conclusions that most of the variation (88.44%) is found within the populations (Φ_{CT}).

Table 6. Partition of genetic variation in *Desmognathus quadramaculatus* determined by AMOVA using AFLP fragment data.

	d.f.	Sum of squares	Variance component	Percentage of variation
Among groups (species*)	1	231.951	5.264	5.940
Among populations (lineages**) within groups	1	222.043	4.978	5.620
Within populations	65	5094.329	78.374	88.440
Total	67	5548.323	88.616	100.000

* *D. folkertsi* and *D. quadramaculatus* only

Table 7. Multiple regression tests of genetic distance, historical and modern river drainage connections, freshwater and terrestrial ecoregions assignment based on geographic locality, and species group based upon uncorrected genetic distance and current taxonomic assignment.

Matrix	b	P
Species-level group*	0.101	0.003
Current drainage basin	-0.049	0.085
Historical drainage basin	-0.108	0.037
Freshwater ecoregions	0.002	0.474
Terrestrial ecoregions	0.116	0.043

* *D. quadramaculatus*, *D. folkertsi*, or *D. marmoratus*

The proportion of the variation explained by among groups (Φ_{SC}) and among populations within groups (Φ_{ST}) was small, comprising only 5.94% and 5.62%, respectively (Table 6). Nei's D value between the Lineage 1 and Lineage 2 (Fig. 2) in the *D. quadramaculatus* species complex was 0.028, and that between *D. quadramaculatus* and *D. folkersi* was 0.0542.

The optimal number of populations (k) computed in Structure v2.2 (Pritchard et al., 2000; Falush et al., 2003; Falush et al., 2007) using the AFLP fragment data was three; however, those three groups were not congruent with the three studied taxa. The estimated probability of three populations (ln P(D)) was equal to –16557.7, with a variance of ln P(D) equal to 1048.5, $\alpha = 0.2552$, and Fst = 0.3317. *Desmognathus folkertsi* was placed into the first inferred cluster 43.3% of the time, more often than in either the second or third inferred cluster. *Desmognathus quadramaculatus* and *D. marmoratus* were grouped into the first inferred cluster 64.0% of the time, and 10.9% and 25.0% for the second and third inferred clusters, respectively.

Testing phylogeographic breaks

We examined the impact of river drainages and ecoregions on phylogeographic patterns in *D. quadramaculatus*. We found individuals of *D. quadramaculatus* to be isolated

by stream (r = 0.335, P < 0.0001) and straight-line (r = 259.526, P = 0.001) distance using the mitochondrial sequences. However, when using the AFLP fragment data, *D. quadramaculatus* populations were only isolated by stream distance (r = 0.135, P = 0.024). We found that current taxonomic species, Pliocene river drainage basin, and terrestrial ecoregions explained the observed phylogeographic pattern in *D. quadramaculatus* (R^2 = 0.017; P = 0.013; Table 7). Neither current river drainage basin nor freshwater ecoregions helped to explain the phylogenetic patterns in *D. quadramaculatus* (Table 7).

DISCUSSION

Lineages and population structure – mitochondrial sequences

The phylogenetic analysis within the *D. quadramaculatus* species complex generated from the mitochondrial sequences revealed two major lineages, Lineage 1 and Lineage 2 (Fig. 2). Lineage 1 consisted of only *D. folkertsi*; Lineage 2 consisted of both northern and southern populations of *D. quadramaculatus* and *D. marmoratus*. The lineages of *D. marmoratus* formed well-supported branches within *D. quadramaculatus*.

Within Lineage 2, which contained both *D. quadramaculatus* and *D. marmoratus*, four well-supported lineages were uncovered (Fig. 2). Overall, Lineage 2 was separated geographically, with distinct northern (Lineage 2C and 2D) and southern (Lineage 2A and 2B) lineages. One lineage of particular interest is a single population of *D. quadramaculatus* found in North Carolina (Lineage 2D) which formed a well-supported and basal relationship to *D. quadramaculatus* in Lineage 2C (Fig. 2). Because this basal relationship was formed using individuals only from an isolated, single population located north of the French Broad River (Fig. 1, Map Locality 13), future research is underway to investigate surrounding geographic areas to reveal the phylogenetic patterning in this area. Overall, Lineage 2C appears to be a polytomy with few well-supported branches, which may be indicative of a recent population expansion. Within Lineage 2C, a single population from Tennessee (Fig. 1, Map Locality 5) appears to be a unique evolutionary lineage that is well nested within Lineage 2C and is of future research interest (Fig. 2).

Although our sample size was limited for *D. marmoratus* due to drought conditions during sampling years, the inclusion of these samples in our analyses allowed us to make some comparisons with *D. quadramaculatus*. Our mitochondrial sequence data suggests that southern *D. marmoratus* populations are nested within southern *D. quadramaculatus* lineages (See Lineage 2A) with strong statistical support (Fig. 2). However, due to a small sample size for southern *D. marmoratus* and the fact that we only used one portion of the mitochondrial genome to discern these fine-scale genetic patterns, our results for the relationship between southern *D. quadramaculatus* and southern *D. marmoratus* do not corroborate the published phylogenetic relationships that suggest a sister relationship between southern populations of *D. marmoratus* with *D. folkertsi* (Jackson, 2005; Jones et al., 2006).

For northern populations of *D. marmoratus* (Lineage 2C), we uncovered a complex, intertwined relationship between *D. marmoratus* and *D. quadramaculatus*, where popu-

lations of *D. marmoratus* are more closely related to populations of *D. quadramaculatus*, rather than to populations of itself. These results corroborate the findings of other authors, and indicate that *D. marmoratus*, especially northern populations, may not be an exclusive species in relation to *D. quadramaculatus* (Titus and Larson, 1996; Rissler and Taylor, 2003; Jackson, 2005; Jones et al., 2006).

Past research using mitochondrial sequence evidence revealed that *D. marmoratus* and *D. quadramaculatus* are not monophyletic (Titus and Larson, 1996; Rissler and Taylor, 2003; Jones et al., 2006); our data revealed similar phylogenetic patterns. Voss et al. (1995) found that of 16 allozyme loci, eight were fixed between populations of *D. marmoratus* north and south of the Eastern Continental Divide. In an unpublished master's thesis, Jackson (2005) revealed that *D. quadramaculatus* and *D. marmoratus* from populations in the northern part of the geographic range were nested together when phylogenetic analysis was completed using three portions of the mitochondrial genome (cytochrome b, ND4, and 12S) and a nuclear gene (GAPDH). More research that includes additional and strategic population sampling is needed to tease apart these complex evolutionary relationships among *D. folkertsi, D. quadramaculatus*, and *D. marmoratus*.

Lineages and population structure – AFLP fragments

Our study is one of only a few that used AFLPs to investigate population genetics in plethodontid salamanders (Lowe et al., 2006; Wooten, 2007; Wooten et al., 2010). Our AFLP fragment data were sufficient to differentiate between *D. folkertsi* and *D. quadramaculatus,* but were not able to distinguish between *D. marmoratus* and *D. quadramaculatus*. The results from the AFLP data and the mitochondrial data were not congruent, indicating that these markers may not be suitable for fine-scale phylogenetic analysis in desmognathan taxa. Regardless, future taxonomic revisions will likely be necessary, because nether *D. marmoratus* or *D. quadramaculatus* do not appear to be monophyletic lineages.

Genetic diversity among populations

Patterns of genetic diversity can often be explained by historical drainage connections for many freshwater species, including fishes and amphibians (Mayden, 1988; Burridge et al., 2006; Jones et al., 2006; Kozak et al., 2006). For *D. quadramaculatus* populations, we found that there was as much genetic variance explained within a single stream as there was between streams or drainages. Semi-aquatic salamander species, including *D. folkertsi, D. quadramaculatus*, and *D. monticola*, are typically used as fish bait, particularly for fishing for black bass (genus *Micropterus*), and, therefore, bait-bucket release has been used to explain phylogenetic and disjunct geographic range patterns in some taxa (Martof, 1953; Jensen and Waters, 1999; Bonett et al., 2007). Bait-bucket release may explain some of the genetic partitioning among populations of these and other salamander species (Wooten et al., 2010); however, fine-scale population studies and phylogenetic analysis may reveal that some of these populations are disjunct, relict populations that are isolated and not associated with bait-bucket release (Camp and Wooten, ms under review).

Phylogeographic breaks and phylogeography

Current river drainages do not significantly explain the phylogenetic patterns that are observed in the *D. quadramaculatus* species complex. In fact, our data suggest that historical drainages have played a more important role in shaping these patterns. This is not surprising considering that modern drainages are composites of historical drainages and there is a strong association between historic drainage patterns and phylogenetic relationships (Kozak et al., 2006; Jones et al., 2006). In our analyses, individuals from the upper Savannah River drainage form sister relationships with individuals from the lower Tennessee River drainage (Fig. 2; *quadramaculatus* 78-81). In addition, individuals from the Catawba River drainage form relationships with individuals from the New River drainage; it has been hypothesized that the Catawba River was once part of the New River drainage system (Map Localities 8-10; Jones et al., 2006) and this would explain the observed phylogenetic patterning. It is also possible that the observed phylogeographic patterns are a result of movement among and between populations, across both aquatic and terrestrial routes. However, it is unknown how far individuals of the *D. quadramaculatus* species complex move across either route; it is difficult to estimate dispersal rates and distances (Milá et al., 2010) and these data are absent for most species (Grant et al., 2010). That said, we do know, however, it is not unlikely that during times of flooding that these salamanders can use terrestrial routes for dispersal, augmenting gene flow among populations (Camp and Wooten, ms under review). We also found that the phylogenetic patterns can be explained by breaks in terrestrial, but not freshwater ecoregions. This may help support the idea that salamanders in the *D. quadramaculatus* complex exhibit phylogenetic patterns due to limited dispersal abilities across terrestrial terrain without the aid of flooding, but not freshwater ecoregions, where the salamanders can move along the waterways for dispersal, which may help to stabilize their populations (Grant et al., 2010).

In *D. quadramaculatus*, the patterns of genetic diversity across the landscape have been shaped by historical drainage patterns that have occurred throughout the history of the Appalachian Mountains. Because of the complex topology, including the presence of large rivers and valleys and steep topological relief, many barriers to gene flow exist; phylogeographic patterns of *D. quadramaculatus* reflect this isolation. We found that paleodrainage patterns influence the genetic diversity of *D. quadramaculatus* more than current drainage patterns. These patterns are similar to those reported in *Eurycea bislineata* (Kozak et al. 2006), and support the idea that paleodrainages directly influence phylogenetic patterns in many fish and salamander that are restricted or may rely on water as a means of dispersal. Results presented here further corroborate and extend previous mitochondrial studies that suggest that historical river drainage patterns influence the phylogenetic patterns of desmognathan species (Voss et al., 1995; Rissler and Taylor, 2003; Jackson, 2005; Jones et al., 2006).

CONCLUSIONS

Undescribed species, parallel evolution, and morphological conservatism are factors that contribute to the complexity of desmognathan taxonomy (Jackson, 2005). *Desmog-*

nathus quadramaculatus and *D. marmoratus* exhibited high levels of genetic diversity for desmognathan salamanders; however, this may be due to small sample size for *D. marmoratus* and the presence of undescribed species in *D. quadramaculatus*. The genetic diversity of *D. quadramaculatus* was not partitioned by current river drainage, but the phylogeographic patterns may be explained, at least in part, by historical river drainage connections. In addition, terrestrial ecoregions, rather than freshwater ecoregions, explained the genetic distances between individuals. This provides additional evidence that *D. quadramaculatus* likely disperses via both terrestrial and aquatic pathways, with gene flow being enhanced through terrestrial routes, but constrained within drainages. Thus, future conservation programs that may be developed to protect these salamanders should consider the terrestrial environment in addition to stream quality. In summary, more research is needed to tease out the complexly interwoven nature of the evolutionary history shared by these three salamander species.

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Appendix 1. Samples used in the genetic analyses.

Lineage	Species	Map Locality	River Drainage	County	State	Locality	Latitude	Longitude	Accession Number
1	Folkertsi	51	Tennessee	Towns	GA	Hiawassee River	34.824	83.733	EU144133
1	Folkertsi	51	Tennessee	Towns	GA	Hiawassee River	34.824	83.733	EU144134
1	Folkertsi	44	Tennessee	Union	GA	West Fork Wolf Creek	34.768	83.946	EU144163
1	Folkertsi	51	Tennessee	Towns	GA	Hiawassee River	34.824	83.733	EU144136
1	Folkertsi	49	Tennessee	Union	GA	Helton Creek	34.749	83.926	EU144148
1	Folkertsi	51	Tennessee	Towns	GA	Hiawassee River	34.824	83.733	EU144135
1	Folkertsi	35	Coosa/Tallapoosa	Gilmer	GA	Holly Creek	34.795	84.603	EU144146
1	Folkertsi	44	Tennessee	Union	GA	West Fork Wolf Creek	34.768	83.946	EU144203
1	Folkertsi	44	Tennessee	Union	GA	West Fork Wolf Creek	34.768	83.946	EU144204
1	Folkertsi	43	Tennessee	Union	GA	Cooper's Creek	34.790	84.031	EU144139
1	Folkertsi	43	Tennessee	Union	GA	Cooper's Creek	34.790	84.031	EU144141
1	Folkertsi	32	Tennessee	Clay	NC	Muskrat Branch	35.047	83.609	EU144210
1	Folkertsi	49	Tennessee	Union	GA	Cooper's Creek	34.749	83.926	EU144140
1	Folkertsi	49	Tennessee	Union	GA	Helton Creek	34.749	83.926	EU144150
1	Folkertsi	49	Tennessee	Union	GA	Helton Creek	34.749	83.926	EU144152
1	Folkertsi	49	Tennessee	Union	GA	Helton Creek	34.749	83.926	EU144157
1	Folkertsi	49	Tennessee	Union	GA	Helton Creek	34.749	83.926	EU144151
1	Folkertsi	43	Tennessee	Union	GA	Cooper's Creek	34.790	84.031	EU144138
1	Folkertsi	51	Tennessee	Towns	GA	Hiawassee River	34.824	83.733	EU144186
1	Folkertsi	56	Savannah	Clay	NC	Tributary of Hiawassee River	34.760	84.004	EU144145
1	Folkertsi	49	Tennessee	Union	GA	Helton Creek	34.749	83.926	EU144153
1	Folkertsi	49	Tennessee	Union	GA	Helton Creek	34.749	83.926	EU144154
1	Folkertsi	20	Savannah	Pickens	SC	Eastatoe Creek	35.051	82.819	EU144221
2-A1	marmoratus	50	Savannah	Rabun	GA	Moccasin Creek	34.844	83.587	EU552279
2-A1	marmoratus	50	Savannah	Rabun	GA	Moccasin Creek	34.844	83.587	EU599125
2-A1	marmoratus	53	Chattahoochee	White	GA	Smithgall Woods	34.690	83.770	EU599126

Lineage	Species	Map Locality	River Drainage	County	State	Locality	Latitude	Longitude	Accession Number
2-A2	marmoratus	10	Catawba	Caldwell	NC	Dixon Creek	36.107	81.782	EU552322
2-A2	marmoratus	10	Catawba	Caldwell	NC	Dixon Creek	36.107	81.782	EU552321
2-C1	marmoratus	10	Catawba	Caldwell	NC	Dixon Creek	36.107	81.782	EU552320
	marmoratus	7	New River	Watauga	NC	Howard Creek	36.281	81.721	EU144212
	monticola	39	Coosa/Tallapoosa	Gilmer	GA	Holly Creek	34.663	84.441	EU552229
	monticola	39	Coosa/Tallapoosa	Gilmer	GA	Owltown Creek	34.663	84.441	EU552244
	monticola	38	Coosa/Tallapoosa	Gilmer	GA	Big Turniptown Creek	34.717	84.390	EU552272
	monticola	38	Coosa/Tallapoosa	Gilmer	GA	Big Turniptown Creek	34.717	84.390	EU552342
	monticola	38	Coosa/Tallapoosa	Gilmer	GA	Big Turniptown Creek	34.717	84.390	EU552273
	monticola	39	Coosa/Tallapoosa	Gilmer	GA	Owltown Creek	34.663	84.441	EU552245
	monticola	40	Coosa/Tallapoosa	Lumpkin	GA	Nimbelwill Creek	34.582	84.184	EU552247
	monticola	54	Tennessee	Habersham	GA	Demerest	34.560	83.539	EU552251
	monticola	49	Tennessee	Union	GA	Helton Creek	34.749	83.926	EU552250
	monticola	40	Coosa/Tallapoosa	Lumpkin	GA	Nimbelwill Creek	34.582	84.184	EU552248
	monticola	51	Tennessee	Towns	GA	Hiawassee River	34.824	83.733	EU552274
	monticola	32	Tennessee	Clay	NC	Muskrat Branch	35.047	83.609	EU144207
	monticola	32	Tennessee	Clay	NC	Muskrat Branch	35.047	83.609	EU144208
	monticola	53	Chattahoochee	White	GA	Smithgall Woods	34.690	83.770	EU552280
	monticola	39	Coosa/Tallapoosa	Gilmer	GA	Holly Creek	34.663	84.441	EU144175
2-A1	quadramaculatus	35	Coosa/Tallapoosa	Murray	GA	Laurel Creek	34.795	84.603	EU552230
2-A1	quadramaculatus	39	Coosa/Tallapoosa	Murray	GA	Owltown Creek	34.663	84.441	EU552232
2-A1	quadramaculatus	35	Coosa/Tallapoosa	Murray	GA	Laurel Creek	34.795	84.603	EU552231
2-A1	quadramaculatus	40	Coosa/Tallapoosa	Lumpkin	GA	Nimbelwill Creek	34.582	84.184	EU552234
2-A1	quadramaculatus	38	Coosa/Tallapoosa	Gilmer	GA	Big Turniptown Creek	34.717	84.390	EU552233
2-A1	quadramaculatus	10	Catawba	Caldwell	NC	Dixon Creek	36.107	81.782	EU552316
2-A2	quadramaculatus	56	Savannah	Clay	NC	Tributary of Hiawassee River	34.760	84.004	EU144147
2-A2	quadramaculatus	7	New River	Watauga	NC	Howard Creek	36.281	81.721	EU144215
2-A2	quadramaculatus	7	New River	Watauga	NC	Howard Creek	36.281	81.721	EU552313

Lineage	Species	Map Locality	River Drainage	County	State	Locality	Latitude	Longitude	Accession Number
2-A2	quadramaculatus	7	New River	Watauga	NC	Howard Creek	36.281	81.721	EU552311
2-A2	quadramaculatus	12	New River	Burke	NC	Steele's Creek	35.906	81.829	EU552327
2-A2	quadramaculatus	7	New River	Watauga	NC	Howard Creek	36.281	81.721	EU552309
2-A2	quadramaculatus	7	New River	Watauga	NC	Howard Creek	36.281	81.721	EU552312
2-A2	quadramaculatus	7	New River	Watauga	NC	Howard Creek	36.281	81.721	EU552314
2-A2	quadramaculatus	12	New River	Burke	NC	Steele's Creek	35.906	81.829	EU552331
2-A2	quadramaculatus	11	New River	Avery	NC	North Harper Creek	36.007	81.855	EU552337
2-A2	quadramaculatus	11	New River	Avery	NC	North Harper Creek	36.007	81.855	EU552333
2-A2	quadramaculatus	10	Catawba	Caldwell	NC	Green Mountain Creek	36.107	81.782	EU552323
2-A2	quadramaculatus	11	New River	Avery	NC	North Harper Creek	36.007	81.855	EU552335
2-A2	quadramaculatus	11	New River	Avery	NC	North Harper Creek	36.007	81.855	EU552336
2-A2	quadramaculatus	12	New River	Burke	NC	Steele's Creek	35.906	81.829	EU552328
2-A2	quadramaculatus	12	New River	Burke	NC	Steele's Creek	35.906	81.829	EU552329
2-A2	quadramaculatus	11	New River	Avery	NC	North Harper Creek	36.007	81.855	EU552334
2-A2	quadramaculatus	27	Tennessee	Graham	NC	Tapoco	35.381	83.901	EU552300
2-A3	quadramaculatus	21	Savannah	Pickens	SC	Wildcat Creek	35.037	82.708	EU552237
2-A3	quadramaculatus	23	Tennessee	Jackson	NC	Pumpkintown Creek	35.251	83.289	EU552295
2-A3	quadramaculatus	22	Tennessee	Macon	NC	Rickman Creek	35.273	83.401	EU552303
2-A3	quadramaculatus	22	Tennessee	Macon	NC	Rickman Creek	35.273	83.401	EU552302
2-A3	quadramaculatus	26	Savannah	Oconee	SC	Tributary of Chattoga River	34.808	83.121	EU552355
2-A3	quadramaculatus	26	Savannah	Oconee	SC	Tributary of Chattoga River	34.808	83.121	EU552356
2-A3	quadramaculatus	23	Tennessee	Jackson	SC	Pumpkintown Creek	35.251	83.289	EU552296
2-A4	quadramaculatus	36	Coosa/Tallapoosa	Gilmer	NC	Rock Creek	34.781	84.328	EU552260
2-A4	quadramaculatus	36	Coosa/Tallapoosa	Gilmer	GA	Rock Creek	34.781	84.328	EU552261
2-A4	quadramaculatus	37	Chattahoochee	Gilmer	GA	Stanley Creek	34.783	84.303	EU552263
2-A4	quadramaculatus	37	Chattahoochee	Gilmer	GA	Rock Creek	34.783	84.303	EU552262
2-A4	quadramaculatus	35	Coosa/Tallapoosa	Gilmer	GA	Holly Creek	34.795	84.603	EU144176
2-A4	quadramaculatus	51	Tennessee	Towns	GA	Hiawassee River	34.824	83.733	EU552275

Lineage	Species	Map Locality	River Drainage	County	State	Locality	Latitude	Longitude	Accession Number
2-A4	quadramaculatus	47	Tennessee	Union	GA	Helton Creek	34.748	83.909	EU144193
2-A4	quadramaculatus	47	Tennessee	Union	GA	Helton Creek	34.748	83.909	EU144192
2-A4	quadramaculatus	47	Tennessee	Union	GA	Helton Creek	34.748	83.909	EU144189
2-A4	quadramaculatus	43	Tennessee	Union	GA	Cooper's Creek	34.790	84.031	EU144205
2-A4	quadramaculatus	42	Tennessee	Union	GA	Flat Creek	34.748	84.026	EU144197
2-A4	quadramaculatus	43	Tennessee	Union	GA	Cooper's Creek	34.790	84.031	EU144202
2-A4	quadramaculatus	44	Tennessee	Union	GA	Wolf Creek	34.768	83.946	EU552281
2-A4	quadramaculatus	28	Tennessee	Monroe	TN	Turkey Creek	35.347	84.193	EU552372
2-A4	quadramaculatus	28	Tennessee	Monroe	TN	Turkey Creek	35.347	84.193	EU552371
	quadramaculatus	51	Tennessee	Towns	GA	Hiawassee River	34.824	83.733	EU552277
	quadramaculatus	33	Tennessee	Polk	TN	Big Lost Creek	35.160	84.469	EU552369
	quadramaculatus	51	Tennessee	Towns	GA	Hiawassee River	34.824	83.733	EU144187
	quadramaculatus	27	Tennessee	Graham	NC	Tapoco	35.381	83.901	EU552301
	quadramaculatus	29	Tennessee	Monroe	TN	Tributary of Tellico River	35.258	84.090	EU552374
	quadramaculatus	29	Tennessee	Monroe	TN	Tributary of Tellico River	35.258	84.090	EU552375
	quadramaculatus	51	Tennessee	Towns	GA	Hiawassee River	34.824	83.733	EU552276
	quadramaculatus	22	Tennessee	Macon	NC	Rickman Creek	35.273	83.401	EU552303
	quadramaculatus	16	Tennessee	Swain	NC	Noland Creek	36.281	81.721	EU552305
	quadramaculatus	16	Tennessee	Swain	NC	Noland Creek	36.281	81.721	EU552306
	quadramaculatus	15	Tennessee	Blount	TN	Abram's Creek	35.608	83.936	EU552361
	quadramaculatus	14	Tennessee	Sevier	TN	Laurel Falls	35.667	83.597	EU552366
	quadramaculatus	14	Tennessee	Sevier	TN	Laurel Falls	35.667	83.597	EU552367
2-B1	quadramaculatus	55	Chattahoochee	Rabun	GA	Nancytown Creek	34.504	83.481	EU552235
2-B1	quadramaculatus	55	Chattahoochee	Rabun	GA	Nancytown Creek	34.504	83.481	EU552256
2-B1	quadramaculatus	55	Chattahoochee	Rabun	GA	Nancytown Creek	34.504	83.481	EU552257
2-B1	quadramaculatus	55	Chattahoochee	Rabun	GA	Nancytown Creek	34.504	83.481	EU552258
2-B1	quadramaculatus	54	Chattahoochee	Habersham	GA	Demerest	34.560	83.539	EU552252
2-B1	quadramaculatus	54	Chattahoochee	Habersham	GA	Demerest	34.560	83.539	EU552254

Lineage	Species	Map Locality	River Drainage	County	State	Locality	Latitude	Longitude	Accession Number
2-B1	quadramaculatus	54	Chattahoochee	Habersham	GA	Demerest	34.560	83.539	EU552253
2-B1	quadramaculatus	24	Tennessee	Macon	NC	Glenn Falls	35.035	83.237	EU552286
2-B1	quadramaculatus	24	Tennessee	Macon	NC	Glenn Falls	35.035	83.237	EU552287
2-B1	quadramaculatus	22	Tennessee	Macon	NC	Rickman Creek	35.273	83.401	EU552304
2-B1	quadramaculatus	25	Savannah	Oconee	SC	Tributary of Chattoga River	34.974	83.110	EU552351
2-B1	quadramaculatus	25	Savannah	Oconee	SC	Tributary of Chattoga River	34.974	83.110	EU552352
2-B1	quadramaculatus	23	Tennessee	Jackson	NC	Pumpkintown Creek	35.251	83.289	EU552294
	quadramaculatus	26	Savannah	Oconee	SC	Tributary of Chattoga River	34.808	83.121	EU552354
2-B1	quadramaculatus	19	Tennessee	Transylvania	NC	Looking Glass Falls	35.289	82.761	EU552288
2-B1	quadramaculatus	23	Tennessee	Jackson	NC	Pumpkintown Creek	35.251	83.289	EU552297
2-B1	quadramaculatus	21	Savannah	Pickens	SC	Wildcat Creek	35.037	82.708	EU552236
2-B1	quadramaculatus	18	Tennessee	Haywood	NC	Hungry Creek	35.368	82.817	EU552289
2-B1	quadramaculatus	18	Tennessee	Haywood	NC	Hungry Creek	35.368	82.817	EU552290
2-B1	quadramaculatus	21	Savannah	Pickens	SC	Wildcat Creek	35.035	83.237	EU552353
2-B1	quadramaculatus	20	Savannah	Pickens	SC	Eastatoe Creek	35.051	82.819	EU552350
2-B2	quadramaculatus	40	Coosa/Tallapoosa	Lumpkin	GA	Nimbelwill Creek	34.582	84.184	EU552246
2-B2	quadramaculatus	37	Chattahoochee	Gilmer	GA	Stanley Creek	34.783	84.303	EU552264
2-B2	quadramaculatus	41	Tennessee	Union	GA	Suches Creek	34.701	84.040	EU144206
2-B2	quadramaculatus	51	Tennessee	Towns	GA	Hiawassee River	34.824	83.733	EU144188
2-B2	quadramaculatus	51	Tennessee	Towns	GA	Hiawassee River	34.824	83.733	EU552278
2-B2	quadramaculatus	52	Chattahoochee	Habersham	GA	Spoilcane Creek	34.760	83.752	EU552282
2-B2	quadramaculatus	32	Tennessee	Clay	NC	Muskrat Branch	35.047	83.609	EU144209
2-B3	quadramaculatus	40	Coosa/Tallapoosa	Lumpkin	GA	Nimbelwill Creek	34.582	84.184	EU552249
2-B3	quadramaculatus	44	Tennessee	Union	GA	West Fork of Wolf Creek	34.768	83.946	EU144171
2-B3	quadramaculatus	54	Chattahoochee	Habersham	GA	Demerest	34.560	83.539	EU552255
2-B3	quadramaculatus	34	Coosa/Tallapoosa	Fannin	GA	Star Creek	34.871	84.203	EU552270
2-B3	quadramaculatus	43	Tennessee	Union	GA	Cooper's Creek	34.790	84.031	EU144200
2-B3	quadramaculatus	43	Tennessee	Union	GA	Cooper's Creek	34.790	84.031	EU144199

Lineage	Species	Map Locality	River Drainage	County	State	Locality	Latitude	Longitude	Accession Number
2-B3	quadramaculatus	34	Coosa/Tallapoosa	Fannin	GA	Star Creek	34.871	84.203	EU552269
2-B3	quadramaculatus	34	Coosa/Tallapoosa	Fannin	GA	Star Creek	34.871	84.203	EU552271
2-B3	quadramaculatus	44	Tennessee	Union	GA	West Fork of Wolf Creek	34.768	83.946	EU144167
2-B3	quadramaculatus	44	Tennessee	Union	GA	West Fork of Wolf Creek	34.768	83.946	EU144168
2-B3	quadramaculatus	44	Tennessee	Union	GA	West Fork of Wolf Creek	34.768	83.946	EU144170
2-B3	quadramaculatus	47	Tennessee	Union	GA	Helton Creek	34.748	83.909	EU144194
2-B3	quadramaculatus	42	Tennessee	Union	GA	Flat Creek	34.748	84.026	EU144196
2-B3	quadramaculatus	44	Tennessee	Union	GA	West Fork of Wolf Creek	34.768	83.946	EU144169
2-B3	quadramaculatus	43	Tennessee	Union	GA	Cooper's Creek	34.790	84.031	EU144198
2-B3	quadramaculatus	36	Coosa/Tallapoosa	Gilmer	GA	Rock Creek	34.781	84.328	EU552259
2-B3	quadramaculatus	38	Coosa/Tallapoosa	Gilmer	GA	Big Turniptown Creek	34.717	84.390	EU552348
2-B3	quadramaculatus	38	Coosa/Tallapoosa	Gilmer	GA	Big Turniptown Creek	34.717	84.390	EU552344
	quadramaculatus	38	Coosa/Tallapoosa	Gilmer	GA	Big Turniptown Creek	34.717	84.390	EU552349
	quadramaculatus	38	Coosa/Tallapoosa	Gilmer	GA	Big Turniptown Creek	34.717	84.390	EU552343
	quadramaculatus	38	Coosa/Tallapoosa	Gilmer	GA	Big Turniptown Creek	34.717	84.390	EU552345
	quadramaculatus	38	Coosa/Tallapoosa	Gilmer	GA	Big Turniptown Creek	34.717	84.390	EU552347
	quadramaculatus	38	Coosa/Tallapoosa	Gilmer	GA	Big Turniptown Creek	34.717	84.390	EU552346
2-B3	quadramaculatus	37	Chattahoochee	Gilmer	GA	Stanley Creek	34.783	84.303	EU552265
2-B3	quadramaculatus	37	Chattahoochee	Gilmer	GA	Stanley Creek	34.783	84.303	EU552266
2-B3	quadramaculatus	37	Chattahoochee	Gilmer	GA	Stanley Creek	34.783	84.303	EU552267
	quadramaculatus	2	New River	Nicholas	WV	Collison Creek	38.113	81.144	EU144142
	quadramaculatus	1	New River	Fayette	WV	Glade Creek	38.191	80.897	EU552396
2-C1	quadramaculatus	46	Tennessee	Union	GA	Unnamed tributary of Nottely River	34.750	83.850	EU552268
2-C1	quadramaculatus	6	New River	Henderson	NC	West Fork of French Broad River	36.227	81.435	EU552307
2-C1	quadramaculatus	9	Catawba	Watauga	NC	Green Mountain Creek	36.114	81.778	EU552318
2-C1	quadramaculatus	9	Catawba	Watauga	NC	Middle Fork at Payne's Branch	36.114	81.778	EU552324

Lineage	Species	Map Locality	River Drainage	County	State	Locality	Latitude	Longitude	Accession Number
	quadramaculatus	6	New River	Henderson	NC	West Fork of French Broad River	36.227	81.435	EU552283
2-C2	quadramaculatus	2	New River	Nicholas	WV	Collison Creek	38.113	81.144	EU144144
2-C2	quadramaculatus	4	New River	Giles	VA	Laurel Branch	37.392	80.642	EU552382
2-C2	quadramaculatus	2	New River	Nicholas	WV	Collison Creek	38.113	81.144	EU552241
2-C2	quadramaculatus	6	New River	Henderson	NC	West Fork of French Broad River	36.227	81.435	EU552315
2-C2	quadramaculatus	6	New River	Henderson	NC	West Fork of French Broad River	36.227	81.435	EU552332
2-C2	quadramaculatus	3	New River	Mercer	WV	Unnamed tributary of Bluestone River	37.377	81.224	EU552406
2-C2	quadramaculatus	4	New River	Giles	VA	Laurel Branch	37.392	80.642	EU552381
2-C2	quadramaculatus	4	New River	Giles	VA	Laurel Branch	37.392	80.642	EU552379
2-C2	quadramaculatus	6	New River	Henderson	NC	West Fork of French Broad River	36.227	81.435	EU552284
2-C2	quadramaculatus	1	New River	Fayette	WV	Glade Creek	38.191	80.897	EU552402
2-C2	quadramaculatus	3	New River	Mercer	WV	Unnamed tributary of Bluestone River	37.377	81.224	EU552409
2-C2	quadramaculatus	2	New River	Nicholas	WV	Collison Creek	38.113	81.144	EU552238
2-C2	quadramaculatus	4	New River	Giles	VA	Laurel Branch	37.392	80.642	EU552383
2-C2	quadramaculatus	1	New River	Fayette	WV	Glade Creek	38.191	80.897	EU552403
2-C2	quadramaculatus	6	New River	Henderson	NC	West Fork of French Broad River	36.227	81.435	EU552298
2-C2	quadramaculatus	4	New River	Giles	VA	Laurel Branch	37.392	80.642	EU552380
2-C2	quadramaculatus	6	New River	Henderson	NC	West Fork of French Broad River	36.227	81.435	EU552308
2-C2	quadramaculatus	3	New River	Mercer	WV	Unnamed tributary of Bluestone River	37.377	81.224	EU552413
2-C3	quadramaculatus	5	Tennessee	Sullivan	TN	Harper's Creek	36.475	82.090	EU552357

Lineage	Species	Map Locality	River Drainage	County	State	Locality	Latitude	Longitude	Accession Number
2-C3	quadramaculatus	5	Tennessee	Sullivan	TN	Harper's Creek	36.475	82.090	EU552360
2-C3	quadramaculatus	5	Tennessee	Sullivan	TN	Harper's Creek	36.475	82.090	EU552368
2-C3	quadramaculatus	5	Tennessee	Sullivan	TN	Harper's Creek	36.475	82.090	EU552365
2-C3	quadramaculatus	5	Tennessee	Sullivan	TN	Harper's Creek	36.475	82.090	EU552373
2-C3	quadramaculatus	5	Tennessee	Sullivan	TN	Harper's Creek	36.475	82.090	EU552364
2-C3	quadramaculatus	5	Tennessee	Sullivan	TN	Harper's Creek	36.475	82.090	EU552358
2-C3	quadramaculatus	5	Tennessee	Sullivan	TN	Harper's Creek	36.475	82.090	EU552359
2-C3	quadramaculatus	5	Tennessee	Sullivan	TN	Harper's Creek	36.475	82.090	EU552363
2-C3	quadramaculatus	5	Tennessee	Sullivan	TN	Harper's Creek	36.475	82.090	EU552370
	quadramaculatus	1	New River	Fayette	WV	Glade Creek	38.191	80.897	EU552394
	quadramaculatus	1	New River	Fayette	WV	Glade Creek	38.191	80.897	EU552401
	quadramaculatus	3	New River	Mercer	WV	Unnamed tributary of Bluestone River	37.377	81.224	EU552410
	quadramaculatus	1	New River	Fayette	WV	Glade Creek	38.191	80.897	EU552400
	quadramaculatus	1	New River	Fayette	WV	Glade Creek	38.191	80.897	EU552404
	quadramaculatus	2	New River	Nicholas	WV	Collison Creek	38.113	81.114	EU552240
	quadramaculatus	1	New River	Fayette	WV	Glade Creek	38.191	80.897	EU552398
	quadramaculatus	4	New River	Giles	VA	Laurel Branch	37.392	80.642	EU552377
	quadramaculatus	3	New River	Mercer	WV	Unnamed tributary of Bluestone River	37.377	81.224	EU552412
	quadramaculatus	6	New River	Henderson	NC	West Fork of French Broad River	36.227	81.435	EU552285
	quadramaculatus	4	New River	Giles	VA	Laurel Branch	37.392	80.642	EU552384
	quadramaculatus	6	New River	Henderson	NC	West Fork of French Broad River	36.227	81.435	EU552338
	quadramaculatus	1	New River	Fayette	WV	Glade Creek	38.191	80.897	EU552399
	quadramaculatus	3	New River	Mercer	WV	Unnamed tributary of Bluestone River	37.377	81.224	EU552411

Lineage	Species	Map Locality	River Drainage	County	State	Locality	Latitude	Longitude	Accession Number
	quadramaculatus	6	New River	Henderson	NC	West Fork of French Broad River	36.227	81.435	EU552339
	quadramaculatus	3	New River	Mercer	WV	Unnamed tributary of Bluestone River	37.377	81.224	EU552406
	quadramaculatus	1	New River	Fayette	WV	Glade Creek	38.191	80.897	EU552395
	quadramaculatus	2	New River	Nicholas	WV	Collison Creek	38.113	81.144	EU552243
	quadramaculatus	4	New River	Giles	VA	Laurel Branch	37.392	80.642	EU552376
	quadramaculatus	1	New River	Fayette	WV	Glade Creek	38.191	80.897	EU552397
	quadramaculatus	2	New River	Nicholas	WV	Collison Creek	38.113	81.144	EU552239
	quadramaculatus	3	New River	Mercer	WV	Unnamed tributary of Bluestone River	37.377	81.224	EU552414
	quadramaculatus	3	New River	Mercer	WV	Unnamed tributary of Bluestone River	37.377	81.224	EU552414
	quadramaculatus	9	Catawba	Watauga	NC	Green Mountain Creek	36.114	81.778	EU552319
	quadramaculatus	7	New River	Watauga	NC	Howard Creek	36.281	81.721	EU144214
	quadramaculatus	2	New River	Nicholas	WV	Collison Creek	38.113	81.144	EU552242
	quadramaculatus	7	New River	Watauga	NC	Howard Creek	36.281	81.721	EU144213
	quadramaculatus	9	Catawba	Watauga	NC	Green Mountain Creek	36.114	81.778	EU552317
	quadramaculatus	8	Catawba	Watauga	NC	Middle Fork at Payne's Branch	36.185	81.654	EU552325
	quadramaculatus	8	Catawba	Watauga	NC	Middle Fork at Payne's Branch	36.185	81.654	EU552326
	quadramaculatus	12	New River	Burke	NC	Steele's Creek	35.906	81.829	EU552330
2-D	quadramaculatus	13	Tennessee	Madison	NC	Tributary of Big Laural Creek	35.983	82.662	EU552291
2-D	quadramaculatus	13	Tennessee	Madison	NC	Tributary of Big Laural Creek	35.983	82.662	EU55292
2-D	quadramaculatus	13	Tennessee	Madison	NC	Tributary of Big Laural Creek	35.983	82.662	EU55293

*Individuals from localities 17, 30, 31, and 48 were deleted when identical haplotypes were removed.
Voucher numbers: [1] UAHC 15824, [2] UAHC 15825, [3] UAHC 15827, [4] UAHC 15826 , [5] UAHC 15829, [6] UAHC 15830, [7] UAHC 15832, [8] UAHC 15831, [9] UAHC 15819, [10] UAHC 15821, [11] UAHC 15834, [12] UAHC 15845, [13] APPSU 25402, [14] APPSU 25403, [15] UAHC 15838, [16] UAHC 15833, [17] UAHC 15842, [18] UAHC 15844, [19] APPSU 24504, [20] UAHC 15843, [21] UAHC 15652, [22] UAHC 15653, [23] APPSU 24509, [24] APPSU 24507, [25] APPSU 24510, [26] APPSU 24505, [27] APPSU 24506

Appendix 2. Individuals from Genbank used in the genetic analyses.

Lineage	Species	Accession Number	County	State	Citation
	monticola	AF437369	Giles	VA	Rissler and Taylor 2003
	monticola	AF437362	Craig	VA	Rissler and Taylor 2003
	monticola	AF437353	Giles	VA	Rissler and Taylor 2003
	monticola	AY549660	Giles	VA	Rissler et. al. 2004
	monticola	AY549650	Craig	VA	Rissler et. al. 2004
	monticola	AF437351	Giles	VA	Rissler and Taylor 2003
	monticola	AY549666	Giles	VA	Rissler et. al. 2004
	monticola	AY549644	Craig	VA	Rissler et. al. 2004
	monticola	AY549657	Giles	VA	Rissler et. al. 2004
	monticola	AY549679	Unknown	NC	Rissler et. al. 2004
	monticola	AY549673	Montgomery	VA	Rissler et. al. 2004
	monticola	AF437369	Giles	VA	Rissler and Taylor 2003
2-C1	marmoratus	AF437329	Caldwell	NC	Rissler and Taylor 2003
2-C3	marmoratus	AF437336	Smyth	VA	Rissler and Taylor 2003
2-B1	quadramaculatus	AF437409	Henderson	NC	Rissler and Taylor 2003
2-C1	quadramaculatus	AF437331	Giles	VA	Rissler and Taylor 2003
2-C1	quadramaculatus	AF437332	Smyth	VA	Rissler and Taylor 2003
2-C1	quadramaculatus	AF437320	Giles	VA	Rissler and Taylor 2003
2-C1	quadramaculatus	AF437325	Giles	VA	Rissler and Taylor 2003
2-C1	quadramaculatus	AF437321	Giles	VA	Rissler and Taylor 2003
2-C1	quadramaculatus	AF437322	Giles	VA	Rissler and Taylor 2003
2-C1	quadramaculatus	AF437326	Giles	VA	Rissler and Taylor 2003
2-C1	quadramaculatus	AF437324	Giles	VA	Rissler and Taylor 2003
2-C1	quadramaculatus	AF437333	Giles	VA	Rissler and Taylor 2003
	quadramaculatus	AF437323	Giles	VA	Rissler and Taylor 2003
	quadramaculatus	AF437319	Giles	VA	Rissler and Taylor 2003
	quadramaculatus	AF437330	Giles	VA	Rissler and Taylor 2003
	quadramaculatus	AF437335	Giles	VA	Rissler and Taylor 2003
	quadramaculatus	AF437328	Giles	VA	Rissler and Taylor 2003
	quadramaculatus	AF437327	Giles	VA	Rissler and Taylor 2003
	quadramaculatus	AF437334	Giles	VA	Rissler and Taylor 2003
2-D	quadramaculatus	AF437337	Giles	VA	Rissler and Taylor 2003
	aeneus	AF437410	Graham	NC	Rissler and Taylor 2003
	fuscus	AF437405	Giles	VA	Rissler and Taylor 2003
	fuscus	AF437406	Giles	VA	Rissler and Taylor 2003
	imitator	AF437408	Unknown	TN	Rissler and Taylor 2003
	wrighti	AF437317	Macon	NC	Rissler and Taylor 2003
	wrighti	AY135559	Sevier	TN	Crespi et al. 2003