



Short communication

River drainages and phylogeography: An evolutionary significant lineage of shovel-nosed salamander (*Desmognathus marmoratus*) in the southern Appalachians

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1. Introduction

All models of speciation involve some mechanism for the separation of biological populations that eventually leads to the formation of new species. In the case of aquatic species, biological evolution is often coincident with geological evolution, because populations can become geographically isolated among river systems as drainage morphologies evolve. Thus, for species that are completely restricted to aquatic habitats (e.g., fishes), river drainages provide a powerful framework for reconstructing phylogeny and historical biogeography (Avice, 1994).

The southern region of the Appalachian mountains is a model setting for investigating the historical differentiation of populations in aquatic environments. The region's communities and topography are very old as they were well south of the maximum extent of Pleistocene glaciation. The result has been the persistence of biota in the region and the opportunity for diversification with time. For example, the Tennessee River system, which drains the western slopes and interior of the southern Appalachians, has the highest species richness and greatest number of endemic species of fishes in North America (Jenkins et al., 1972; Starnes and Etnier,

1986), presumably due to the isolation of lineages in drainages separated by long river distances.

Salamanders of the genus *Desmognathus* (Plethodontidae) include a continuum of species that inhabit fully aquatic to fully terrestrial niches. The shovel-nosed salamander, *D. marmoratus* Moore, a relatively rare and patchily distributed endemic species of the southern Appalachians, is the only member of this genus adapted to an entirely aquatic existence. Both larvae and adults spend their entire life in "trout streams" that contain cool, oxygen-rich water (Martof, 1962). As a totally aquatic species, the historical biogeography of *D. marmoratus* has likely been influenced by geologic events that have isolated populations among different river drainages. Indeed, populations of *D. marmoratus* are highly divergent between river drainages on either side of the Eastern Continental Divide (Voss et al., 1995). However, two studies suggest a more complex history of diversification for *D. marmoratus* (Rissler and Taylor, 2003; Titus and Larson, 1996). Specifically, some populations of *D. marmoratus* are genetically more similar to some populations of *Desmognathus quadramaculatus*, a closely related, semi-aquatic species with a presumptively greater potential for terrestrial dispersal. Although, relatively few populations of either species have been examined to date, these results call into question the phylogenetic distinctiveness of *D. marmoratus* and *D. quadramaculatus*, and suggest the possibility that lineages of both species may have traversed, actively or passively, major river drainages during evolution.

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Here, we report on a study of mitochondrial DNA variation among populations of *D. marmoratus*. Our primary objective was to further examine genetic structure across the range of *D. marmoratus*, to test for the presence of well-differentiated lineages that are currently isolated among river drainages. However, because samples of *D. quadramaculatus* were obtained from *D. marmoratus* localities, we also report on genetic relationships between these closely related species. Specifically, we addressed whether there are well-differentiated lineages of special concern within *D. marmoratus* that should be recognized taxonomically. Voss et al. (1995) found fixed allelic differences at eight of sixteen allozyme loci between populations of *D. marmoratus* north and south of the Eastern Continental Divide, indicating extremely high genetic divergence that typically differentiates species. Because southern *D. marmoratus* populations are isolated among relatively few, high-elevation river drainages, corroborating evidence from a second type of molecular marker would justify their recognition as separate evolutionary units of special concern. It is likely, however, that additional isolating events have shaped the historical biogeography of this totally aquatic species across its entire distribution, and such knowledge is important for interpreting regional patterns of biodiversity in the southern Appalachians. In addition, data from very few samples of *D. marmoratus*

(Rissler and Taylor, 2003; Titus and Larson, 1996) suggest that some *D. marmoratus* populations are more closely related to populations of *D. quadramaculatus* than to conspecifics. If this pattern holds across most of the range of *D. marmoratus*, it would suggest a more complex evolutionary history for these species, involving perhaps terrestrial dispersal in addition to river drainage vicariance.

2. Materials and methods

Individuals of *D. marmoratus* and *D. quadramaculatus* were collected throughout the range of *D. marmoratus* in Georgia, North Carolina, South Carolina, and Virginia (Fig. 1). Locality descriptions and sample sizes are presented in Table 1. As described originally by Martof (1962), we used pigment pattern and the morphology of the internal nares to differentiate individuals between these two species. Genomic DNA was extracted from a small sample of tail tissue (Animal Use Protocol 99-063, Clemson University) using the Qbiogene GNOME DNA isolation kit (Carlsbad, CA) with modifications from the manufacturer's protocol (see Jones, 2003). The polymerase chain reaction (PCR) was used to amplify the mitochondrial NADH dehydrogenase subunit 2 (ND2) gene from the extracted genomic DNA. PCR was

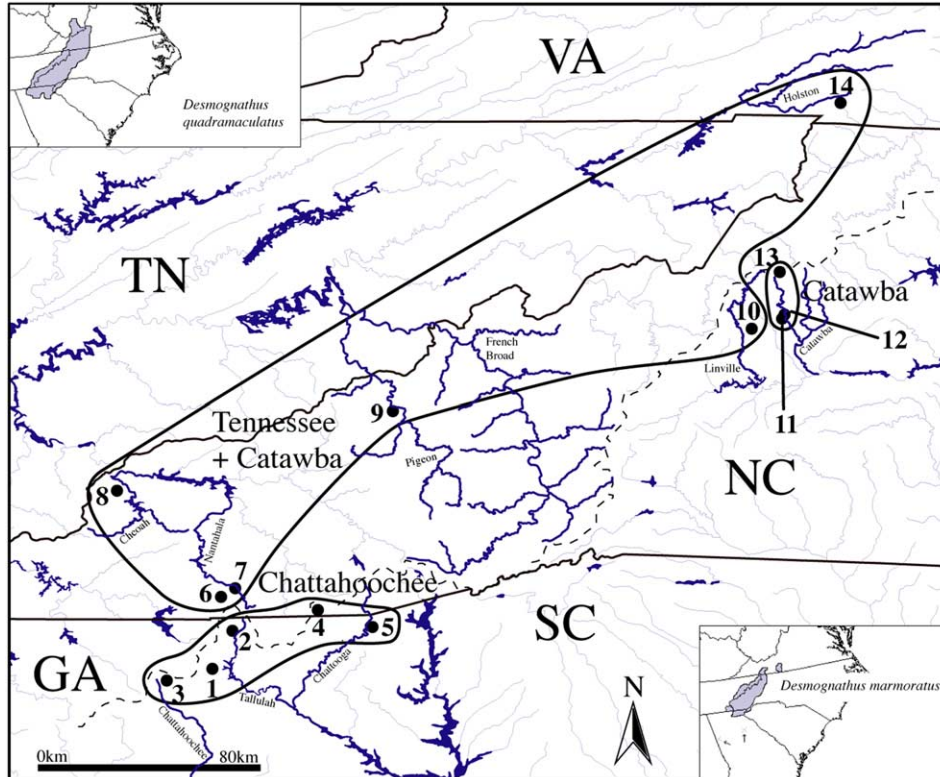


Fig. 1. Sample localities and major rivers of the southern Appalachians. Numbers correspond to localities listed in Table 1. Dashed line indicates the Eastern Continental (Tennessee) Divide. Inset maps provide the approximate ranges of *D. marmoratus* and *D. quadramaculatus* (modified from Petranka, 1998). The three main phylogeographic groupings resolved in this study are outlined with bold lines. By way of comparison, Voss et al. (1995) sampled *D. marmoratus* from the Chattahoochee, Tallulah, Chattooga, and Nantahala river drainages, including sample localities 1–6.

Table 1
Sample collection localities and sample sizes (*n*) for *D. marmoratus* and *D. quadramaculatus* populations used in this study

Species	Population	State	County	Locality name	River	Drainage	GenBank accession numbers
<i>D. marmoratus</i>	1	GA	Rabun	Wildcat Creek (<i>n</i> = 1)	Tallulah	Chattahoochee	AY698025
<i>D. marmoratus</i>	2	GA	Rabun	Tallulah River (<i>n</i> = 2)	Tallulah	Chattahoochee	AY698026 AY698027
<i>D. marmoratus</i>	3	GA	White	Wilks Creek (<i>n</i> = 1)	Chattahoochee	Chattahoochee	AY698030
<i>D. marmoratus</i>	4	NC	Macon	Overflow Creek (<i>n</i> = 1)	Chattooga	Chattahoochee	AY698028
<i>D. marmoratus</i>	5	SC	Oconee	Kings Creek (<i>n</i> = 1)	Chattooga	Chattahoochee	AY698029
<i>D. marmoratus</i>	6	NC	Clay	Buck Creek (<i>n</i> = 2)	Nantahala	Tennessee	AY698034 AY698036
<i>D. marmoratus</i>	7	NC	Macon	Park Creek (<i>n</i> = 1)	Nantahala	Tennessee	AY698033
<i>D. marmoratus</i>	8	NC	Graham	Deep Creek (<i>n</i> = 1)	Cheoah	Tennessee	AY698035
<i>D. marmoratus</i>	9	NC	Haywood	Stevens Creek (<i>n</i> = 1)	Pigeon	Tennessee	AY698037
<i>D. marmoratus</i>	10	NC	Burke	Gingercake Creek (<i>n</i> = 3)	Gingercake Cr.	Catawba	AY698042 AY698043 AY698044
<i>D. marmoratus</i>	11	NC	Caldwell	Craig Creek (<i>n</i> = 4)	Wilson Creek	Catawba	AY698045 AY698047 AY698048 AY698049
<i>D. marmoratus</i>	12	NC	Caldwell	Harper Creek (<i>n</i> = 1)	Wilson Creek	Catawba	AY698046
<i>D. marmoratus</i>	13	NC	Caldwell	Dixon Creek (<i>n</i> = 2)	Dixon Creek	Catawba	AY698052 AY698053
<i>D. marmoratus</i>	14	VA	Smyth	Dave's Branch (<i>n</i> = 4)	Holston	Tennessee	AY698038 AY698039 AY698040 AY698041
<i>D. quadramaculatus</i>	15	GA	Rabun	Wilks Creek (<i>n</i> = 1)	Chattahoochee	Chattahoochee	AY698031
<i>D. quadramaculatus</i>	16	NC	Clay	Buck Creek (<i>n</i> = 1)	Nantahala	Tennessee	AY698032
<i>D. quadramaculatus</i>	17	NC	Burke	Gingercake Creek (<i>n</i> = 1)	Gingercake Cr.	Catawba	AY698051
<i>D. quadramaculatus</i>	18	NC	Caldwell	Craig Creek (<i>n</i> = 1)	Wilson Creek	Catawba	AY698050
<i>D. quadramaculatus</i>	19	VA	Smyth	Dave's Branch (<i>n</i> = 1)	Holston	Tennessee	AY698054

Population numbers 1–14 are indicated in Fig. 1. Populations 15–19 are localities of *D. quadramaculatus* and are sampled from congeneric localities with *D. marmoratus*. Localities listed as part of the Chattahoochee drainage indicate rivers once part of the ancestral Chattahoochee River drainage.

performed with primers that complement sequences encoding the tRNA^{Met} (Macey and Verma, 1997) and a portion of the cytochrome oxidase subunit 1 (COI) gene (Weisrock et al., 2001) using a single initial denaturation step at 95 °C for 60 s, followed by a thermal profile with denaturation at 94 °C for 35 s, annealing at 50 °C for 35 s, and extension at 70 °C for 150 s with 4 s added to the extension per cycle for 30 cycles. Amplification products were purified using the Qiagen QIAquick PCR purification kit. Bi-directional DNA sequencing was accomplished using an Applied Biosystems Big Dye Terminators cycle sequencing kit and primers that complement sequences encoding part of the tRNA^{Met} (from Macey and Verma, 1997), portions of the ND2 gene (5'-TGACAAAACACTYGCACC-3', modified from Macey et al., 2000, and 5'-GCGAAGYTGAGGTTGATTTA-3', this study), and part of the tRNA^{Ala} gene (Macey and Verma, 1997). The cycle-sequencing protocol included a denaturation step at 95 °C for 35 s, an annealing step at 50 °C for 15 s, and an extension step at 70 °C for 35 s (25 cycles total). Unincorporated dye terminators were removed using Qiagen DyeEx spin columns, and reactions were sequenced on an ABI 377 automated DNA sequencer (Clemson University Sequencing Facility).

Forward and reverse sequences of 1039 bp of mitochondrial DNA (mtDNA) encoding the entire ND2 gene from all individuals of *D. marmoratus* and *D. quadramaculatus* were assembled, and ambiguous sites were verified using Sequencher 4.1 software (Genecodes, Ann Arbor, Michigan). These sequences were manually aligned to each other and to four outgroup sequences (*Desmognathus aeneus*, *Desmognathus monticola*, *Desmognathus ocoee*, and *Desmognathus wrighti*; Ken Kozak, unpublished data). These outgroup sequences represent both basal and more derived lineages in *Desmognathine* phylogeny (Rissler and Taylor, 2003; Titus and Larson, 1996). Sequence alignment was straightforward because no length variation was detected among ingroup sequences. There was a small amount of length variation in the outgroup sequences which was alignable after conversion to amino acid sequence. The program Collapse version 1.2, written by D. Posada, was used to parse the total data alignment for unique haplotypes. Haplotypes were assembled into a separate data set and used for all phylogenetic analyses.

Gene trees for sequences of *D. marmoratus* and *D. quadramaculatus* were constructed by parsimony in the program PAUP* Version 4.0b10 (Swofford, 2002) using a

heuristic search with 100 random addition replicates. Bootstrap resampling was performed to analyze parsimony branch support using 1000 bootstrap replicates with 100 random additions per replicate. Decay indices were calculated for each node of the resulting parsimony tree using the program Treerot version 2 (Sorenson, 1999).

After selecting the best fit model of evolution using Modeltest 3.04 (Posada and Crandall, 1998), Bayesian phylogenetic analysis was performed using MrBayes version 3.0b4 (Huelsenbeck and Ronquist, 2001). The HKY model (Hasegawa et al., 1985) with a proportion of sites being invariable (I) and incorporating rate variation (Γ) was selected by hierarchical hypothesis testing of alternative models using Akaike information criterion in Modeltest. One million Markov chain Monte Carlo generations were run with a tree sampled every 1000 generations for a total of 1000 trees. All trees sampled from generations prior to a stable likelihood value were treated as burn in. Three independent runs were performed to increase the probability of convergence on the posterior distribution. Likelihood-corrected pairwise sequence divergences were also generated in PAUP using the Bayesian parameter estimates of the HKY+I+ Γ model.

A number of phylogeographic hypotheses concerning the relationships between populations of *D. marmoratus* and *D. quadramaculatus* were tested using a Wilcoxon signed-ranks test (Templeton, 1983). Alternative hypotheses were constructed by parsimony analysis enforcing a constraint tree generated in MacClade version 4.02 (Maddison and Maddison, 2001). Resulting trees were the minimum-length trees compatible with the alternative hypotheses being tested. Wilcoxon signed-ranks tests with two-tailed probabilities were used to assess whether the data were a significantly poorer fit to a constrained alternative topology relative to the unconstrained topology.

3. Results

The complete ND2 data set of 1039 bp contained 402 variable nucleotide positions of which 250 were parsimony informative. Excluding the four outgroup sequences, there were 275 variable positions containing 208 parsimony informative sites. Parsimony analysis produced three trees of 824 steps in length. Bayesian analysis produced a posterior distribution with a mean ln likelihood of -5028.83 and a variance of 37.46. The Bayesian posterior distribution yielded average parameter estimates for nucleotide frequencies (A = 0.401, C = 0.196, G = 0.078, and T = 0.325), the gamma shape parameter ($\alpha = 0.754$), the proportion of invariant sites ($I = 0.35$), and the transition/transversion ratio (4.949).

Parsimony and Bayesian analyses yielded nearly identical topologies. The Bayesian consensus phylogram is

presented in Fig. 2 with both posterior probabilities and parsimony decay index, and bootstrap values mapped onto branches. Our analyses do not support monophyly of either *D. marmoratus* or *D. quadramaculatus* populations. Three major clades corresponding to geographic barriers were resolved (Figs. 1 and 2):

1. In the southernmost portion of the range of *D. marmoratus* (Ancestral Chattahoochee River drainage), *D. marmoratus*, and *D. quadramaculatus* are phylogenetically distinct sister lineages. Interestingly, *D. quadramaculatus* from the geographically proximal Tennessee drainage on the opposite side of the Eastern Continental Divide is basal to populations sampled from the Chattahoochee River drainage.
2. Tennessee River drainage populations of *D. marmoratus* form part of a well-supported group that included the Gingercake Creek *D. marmoratus* population from the Catawba River drainage, which is on the opposite side of the Eastern Continental Divide. The topology also showed that Tennessee River drainage *D. quadramaculatus* was more closely related to Catawba River populations of *D. marmoratus*, and together these formed a sister group to populations of *D. marmoratus* from the Tennessee River drainage.
3. In the Catawba River drainage, samples of *D. marmoratus* and *D. quadramaculatus* from the same site or river system did not form reciprocally monophyletic groups. Relationships between these populations (Dixon, Gingercake, and Wilson Creeks) were not well resolved, as shown by low measures of branch support.

Between southern populations of *D. marmoratus* in the ancient Chattahoochee system (Chattooga, Chattahoochee, Tallulah) and northern populations (Catawba and Tennessee drainages), likelihood corrected distances (results not shown) and branch lengths approached or exceeded those between *D. marmoratus* and *D. monticola*, and between *D. marmoratus* and *D. ocoee* (Fig. 2). Thus, these two population-level groupings in *D. marmoratus* have genetic distances that are equivalent to species-level differences, suggesting the presence of at least two well-differentiated evolutionary lineages within *D. marmoratus*. The same pattern was evident for some populations of *D. quadramaculatus* inhabiting widely separated drainages (Holston River versus Chattahoochee and Nantahala Rivers) or drainages separated by geographic barriers (Chattahoochee River versus Nantahala River).

Several phylogeographic hypotheses regarding the evolution of *D. marmoratus* were tested. These hypotheses tested alternative topologies constructed with currently recognized taxa as monophyletic or with geographically proximate populations forming monophyletic groups. The hypotheses are as follows:

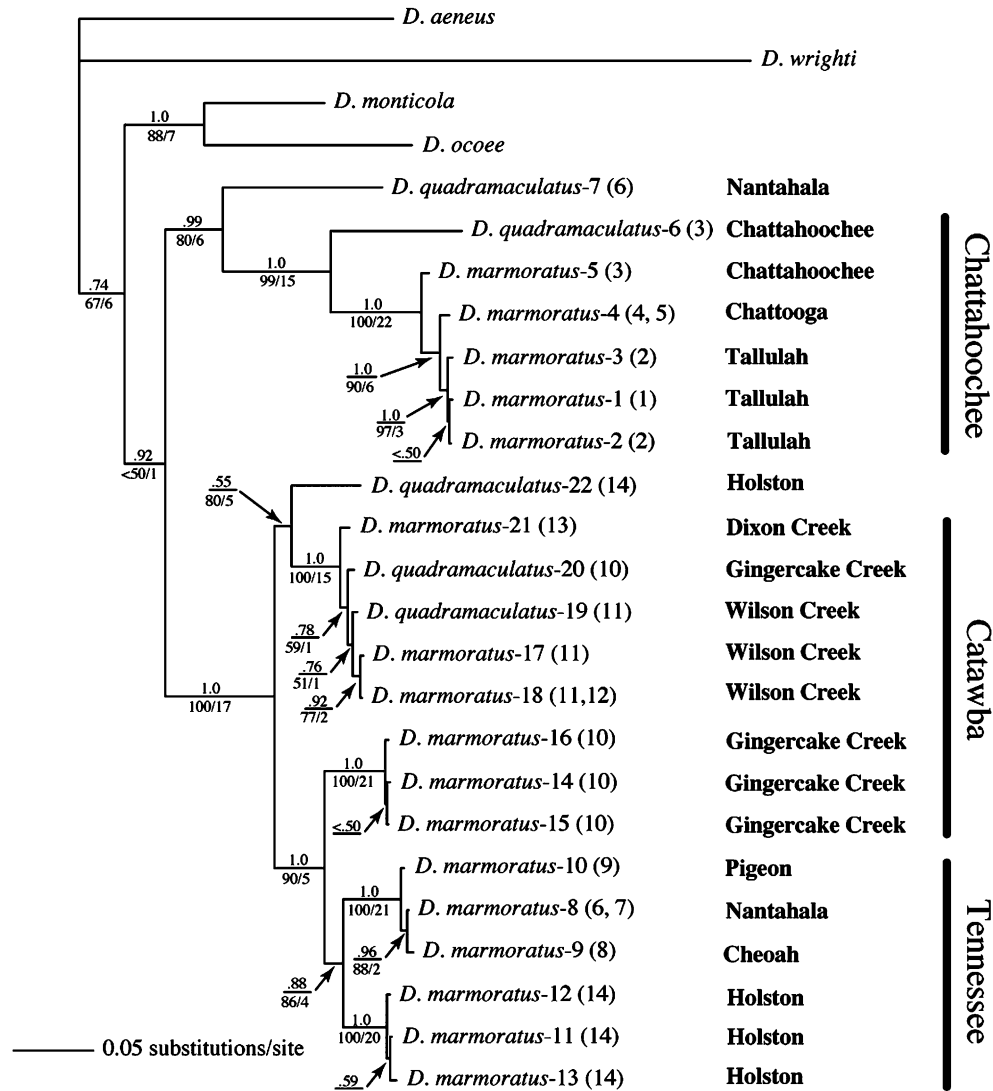


Fig. 2. Bayesian consensus phylogram resulting for all ingroup and outgroup haplotypes. Bayesian posterior probabilities are shown above branches and parsimony bootstrap and decay indices are shown below branches. Numbers in parentheses indicate the locality that a particular haplotype was sampled from. Rivers that haplotypes were sampled from are indicated in bold following the haplotype label. The three main river drainages are denoted by thick vertical bars.

1. Populations of *D. marmoratus* from Dixon and Wilson Creeks (populations 13, 11, and 12, respectively) formed a separate clade with northern populations of *D. quadramaculatus* (populations 10, 11, and 14) in the most parsimonious tree (Fig. 2). When this tree was compared to the shortest-length constraint tree in which populations of *D. marmoratus* from Dixon and Wilson Creek (haplotypes 17, 18, and 21) were the sister group to populations of *D. marmoratus* from the Tennessee River drainage (haplotypes 8–13), the alternative hypothesis was rejected ($p < 0.0001$).
2. The most parsimonious tree showed northern populations of *D. quadramaculatus* (populations 10, 11, and 14) to be paraphyletic (Fig. 2). In comparing this tree to the shortest-length alternative tree constraining northern *D. quadramaculatus* populations (haplotypes 19, 20, and 22) into monophyly, the alternative was rejected ($p < 0.0001$).
3. The overall shortest-length parsimony tree showed populations of *D. quadramaculatus* in the Catawba River watershed (populations 10 and 11) to be paraphyletic (Fig. 2). The alternative tree, where *D. marmoratus* (haplotypes 14–18, and 21) and *D. quadramaculatus* (haplotypes 19–20) populations from Catawba River tributaries were both constrained to be monophyletic, was rejected ($p = 0.0253$).
4. The shortest-length parsimony tree showed the population of *D. marmoratus* from Gingercake Creek (population 10) to be the sister group to populations of *D. marmoratus* in the Tennessee River drainage (populations 6–9 and 14; Fig. 2). When this tree was compared to the shortest-length alternative tree

constraining *D. marmoratus* from Gingercake Creek (haplotypes 14–16) to be the sister group to the clade containing populations of *D. marmoratus* from the Catawba drainage (haplotypes 17, 18, and 21) and northern populations of *D. quadramaculatus* (haplotypes 19, 20, and 22), the alternative hypothesis could not be rejected, but the shorter length of the parsimony topology did approach statistical significance ($p = 0.0578$).

4. Discussion

Patterns of genetic variation among *D. marmoratus* populations suggest an evolutionary history that has been shaped primarily by geological changes of Appalachian River drainages. Phylogenetic analyses identified two major, well-supported monophyletic lineages on either side of the Eastern Continental Divide. Thus, mtDNA data presented here corroborate and extend previous allozyme data in identifying a relatively ancient geologic event that isolated *D. marmoratus* between Tennessee and Chattahoochee River drainages (Voss et al., 1995). These populations appear to have been isolated since the diversion of the ancient Appalachian River, which presumably occurred prior to the Pleistocene (Mayden, 1988; Swift et al., 1986; Thornbury, 1965). The ND2 region sequenced in this study has been shown to evolve at a fairly consistent rate among several vertebrate taxa (0.57–0.69% change per lineage per million years; Bermingham et al., 1997; Macey et al., 1998a,b, 1999; Weisrock et al., 2001). Our data do not reject a molecular clock using a likelihood-ratio test (test statistic, 32.12; degrees of freedom, 24); therefore, it seems reasonable to apply a molecular clock to our data. Applying a molecular evolutionary rate of 0.65% change per lineage per million years derived for salamander ND2 data (Weisrock et al., 2001), Chattahoochee and Tennessee River populations are calculated to have diverged at least 8.9–10.8 million years before present. The divergence time of the populations would have occurred sometime after gene divergence began (Edwards and Beerli, 2000). This timing of divergence is consistent with Swift et al. (1986) and Thornbury's (1965) estimate of a connection between these rivers in the late Tertiary (23.8–1.8 mya) and suggests that populations of *D. marmoratus* on opposite sides of the continental divide have been separated for a very long period of time.

Results from this study also suggest two additional isolating events have influenced the historical biogeography of *D. marmoratus*. First, our results support the idea that the Chattooga, Tallulah, and Chattahoochee River drainages were connected recently, presumably during the Pleistocene (Voss et al., 1995). Consistent with this hypothesis, very little genetic differentiation was observed among *D. marmoratus* populations within

these drainages. Second, our results suggest a historical connection between the Tennessee and Catawba River drainages in the northern part of this species range. We found the Gingercake Creek population of the Catawba River drainage to be genetically more similar to populations of the Tennessee River drainage than to other Catawba River populations. Geological evidence of stream captures in the nearby Linville River (Ross, 1971) and results from several other zoogeographic studies involving stream fishes have suggested a historical connection between populations in the upper Tennessee and upper Catawba systems (Hocutt et al., 1986; Ramsey, 1965; Starnes and Etnier, 1986). Thus, it seems likely that the Gingercake Creek population of *D. marmoratus* arose from dispersing Tennessee River salamanders during a stream capture event, although the possibility of human introductions cannot be discounted (Martof, 1953, 1962).

Desmognathus marmoratus is an endemic habitat specialist completely restricted to high-elevation streams from north Georgia to southern Virginia (Fig. 1), and as part of a single species with a larger range, it is currently of conservation concern (state rank S2 or S3, NatureServe, 2002). Our mtDNA sequence data resolve highly divergent clades found in the southern portion of the range of *D. marmoratus*. These molecular data are in agreement with allozyme data (Voss et al., 1995) in identifying the same geographically isolated lineages on opposite sides of the Eastern Continental Divide. Considered together, these data support an earlier morphological study that recognized populations in South Carolina and Georgia as constituting an independent evolutionary lineage called *D. aureatus* (Martof, 1956), which is restricted to the Chattahoochee, Tallulah, and Chattooga River basins. Efforts to manage and conserve the diversity of shovel-nosed salamanders across their geographic range will require the recognition of the uniqueness of these populations.

The inclusion of five *D. quadramaculatus* samples in our analyses allowed us to make limited comparisons between this species and *D. marmoratus*. In particular, a higher degree of genetic differentiation (>11% pairwise sequence divergence) was observed between populations of the Chattahoochee and Tennessee River systems. It seems likely that the same geological isolating events have influenced the historical biogeography of both *D. marmoratus* and *D. quadramaculatus*. This is somewhat surprising given that *D. quadramaculatus* is presumably capable of terrestrial dispersal. Rissler and Taylor (2003) suggested that differences in sequence diversity for *D. quadramaculatus* and other closely related desmognathines reflect differential arrival times resulting from a northward range expansion, presumably following glaciation. Results presented here suggest that river drainage history may also be important for reconstructing *Desmognathine* phylogeography.

Our results clearly indicate that neither *D. marmoratus* nor *D. quadramaculatus* are monophyletic groups, although conclusions from this study are limited by small sample sizes for each particular stream. Distances between populations of *D. marmoratus* and *D. quadramaculatus* in the Catawba River tributaries (excluding the Gingercake Creek *D. marmoratus*) were very low (<1%); this suggests the possibility of incomplete lineage sorting or prior hybridization between the two species. Specimens from the field, however, were readily distinguishable based on traditionally recognized morphological differences (Martof, 1962), suggesting that recent hybridization between these two species is unlikely. Within this drainage, populations of *D. marmoratus* and *D. quadramaculatus* from streams with closer linear stream connections (Craig and Harper Creeks) were shown to be more closely related than congeners from more distantly connected streams. The use of mtDNA in this study limits our ability to discern fine scale genetic patterns but does reveal broad scale structuring. Studies that utilize nuclear markers would be beneficial in further clarifying the relationships between the Catawba River basin populations of *D. marmoratus* and *D. quadramaculatus*. Nuclear markers may also be able to provide insights into whether or not interbreeding is currently occurring and the direction of such introgression.

Southern populations of either species, south and east of the Eastern Continental Divide, appeared to be more closely related to each other, than to conspecifics in the Tennessee River system or in other northern populations. Similar ecological pressures on either side of the Continental Divide may have selected independently, lineages that exploit totally aquatic, and semi-aquatic niches of streams. Such patterns of speciation are well documented among fishes (e.g., Rundle et al., 2000). Although more complicated explanations can be imagined to explain patterns of genetic variation between *D. marmoratus* and *D. quadramaculatus*, our results suggest the possibility that sympatric populations of *D. marmoratus* and *D. quadramaculatus* arose from separate common ancestors independently on either side of the Eastern Continental Divide. Clearly, the evolutionary histories of these two species are intertwined and a more comprehensive analysis of both species is needed.

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