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Failed species, innominate forms, and the vain search for species limits: cryptic diversity in dusky salamanders (*Desmognathus*) of eastern Tennessee

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Abstract

Cytochrome B sequences and allozymes reveal complex patterns of molecular variation in dusky salamander (*Desmognathus*) populations in eastern Tennessee. One group of allozymically distinctive populations, which we refer to as the Sinking Creek form (SCF), combines morphological attributes of *Desmognathus fuscus* with *cytB* sequences characteristic of *Desmognathus carolinensis*. This form is abruptly replaced by *D. fuscus* just north of Johnson City, TN with no evidence of either sympatry or gene exchange. To the south, allozymic markers indicate a broad zone of admixture with populations characterized by distinct cytB sequences and that may or may not be ultimately referable to *Desmognathus conanti*. A third distinctive group of populations, which we refer to as the Lemon Gap form (LGF), occurs in the foothills of the Great Smoky and southern Bald Mountains and exchanges genes with *Desmognathus santeetlah* along the escarpment of the Great Smokies, *D. carolinensis* in the southern Bald Mountains, and populations of a different haplotype clade in the Ridge and Valley. We treat all these as innominate forms that may represent "failed species," recognizing that it may never be possible to reconcile species limits with patterns of phylogeny, morphology, and gene exchange in these salamanders.

Introduction

"In short, we shall have to treat species in the same manner as those naturalists treat genera, who admit that genera are merely artificial combinations made for convenience. This may not be a cheering prospect, but we shall at least be free from the vain search for the undiscovered and undiscoverable essence of the term species" (Darwin 1859).

Evolutionary biologists still seek to objectively define species and to operationally delimit them in nature (Sites and Marshall 2003, 2004; Bernardo 2011). Molecular techniques sometimes complicate these pursuits by revealing cryptic diversity and phylogenetic structure within nominate species (Agapow et al. 2004; Pfenniger and Schwenk 2007; Trontelj et al. 2007) and provoking disagreement over which, or even whether, species should be recognized solely on the basis of molecular data (Agapow et al. 2004 vs. Isaac et al. 2004; Highton 1998 vs. Wake and Schneider 1998; Chaitra et al. 2004; Trontelj and Fišer 2009; reviewed by Bernardo 2011). This controversy is complicated by phylogenetic discordance among genes (Shaw 2002; Wiens et al. 2010), between individual genes and evolving lineages

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(i.e., "gene trees" vs. "species trees," Avise 2004), and between molecular and nonmolecular characters and patterns of reproductive isolation (Richmond and Jockusch 2007; Hall and Katz 2011). These issues bear strongly on the systematics of plethodontid salamanders, which combine morphological conservatism, homoplasy, and cryptic diversity to extraordinary degrees (Wake 2009).

Over the past half-century the number of recognized plethodontid species in North America has increased from 56 (Schmidt 1953) to 143 (Tilley et al. 2011), largely because molecular data have revealed morphologically cryptic forms. Highton (1990, 2000) has argued that investigators have actually been overly reluctant to recognize species on the basis of allozymes, particularly in plethodontids, leading to a taxonomy–phylogeny gap (Avise 1989; Bernardo 2011). Others have warned against taxonomic splitting (Chaitra et al. 2004; Isaac et al. 2004), which can actually obscure complex evolutionary patterns (Wake 2009).

The southern Appalachian Mountains have been regarded as a center of plethodontid evolution and diversity since early in the last century (Wilder and Dunn 1920; Dunn 1926). Molecular work has only reinforced that view (Highton 1989; Tilley and Mahoney 1996; Tilley 1997, 2000b; Highton and Peabody 2000; Mead et al. 2001; Camp et al. 2002, 2009; Anderson and Tilley 2003; Crespi et al. 2003, 2010; Tilley et al. 2008). Studies that combine allozyme data with mitochondrial (mtDNA) sequences have been especially effective at revealing unexpected phylogeographic structure and cryptic lineages in that region (Mead et al. 2001; Weisrock and Larson 2005; Tilley et al. 2008). Allozymes provide insight into patterns of differentiation and gene flow at multiple (presumptive) nuclear loci (Mead et al. 2001; Avise 2004), but are less amenable than sequence data to phylogenetic analysis. Rapidly evolving mtDNA sequences may be especially subject to homoplasy (Ballard and Rand 2005; Rubinoff and Holland 2005; Zink and Barrowclough 2008; Fisher-Reid and Wiens 2011) and subject to transfer between lineages via "cytoplasmic capture" (Avise 2004; Chan and Levin 2005). Mitochondrial and nuclear loci can reveal different patterns of exchange via maternal inheritance, gender-biased dispersal, and frequency-dependent mate choice (Irwin 2002; Chan and Levin 2005; Richmond and Jockusch 2007; Barber et al. 2012). Several examples of discordance between mitochondrial and species phylogenies have thus been documented (e.g., Linnen and Farrell 2007; Wiens et al. 2010; Fisher-Reid and Wiens 2011).

Molecular studies of the genus *Desmognathus* (dusky salamanders) have clarified the systematics of several forms (Tilley et al. 1978, 2008; Tilley and Mahoney 1996; Tilley 1997; Camp et al. 2002; Crespi et al. 2010). Our investigations of *Desmognathus* populations along the boundary between the Ridge and Valley and Blue Ridge Physiographic Provinces in Tennessee have revealed enigmatic populations resembling the nominate forms *Desmognathus fuscus*, *Desmognathus conanti*, and *Desmognathus carolinensis*. We here employ cytochrome B sequences and allozymes to clarify the diversity, phylogeography, and evolutionary relationships of these populations. Our sampling is concentrated along a northeast to southwest transect through the Ridge and Valley Province in extreme eastern Tennessee and foothills of the Unaka, Bald, Great Smoky, and Unicoi Mountains. The location of this transect permits us to address three problems: (1) the taxonomic status of Ridge and Valley populations in this region, (2) the genetic interactions between those populations and two geographically adjacent montane forms: *Desmognathus santeetlah* and *D. carolinensis*, and (3) the phylogenetic relationships among all these units. Our results raise the more general problem of reconciling the discordant and "fractal" (Wake 2009) natures of molecular phylogeographic patterns with the necessity of recognizing and naming species (Highton 1990, 2000; Bernardo 2011).

Materials and Methods

Sample localities

Sampling localities, numbered from north to south, are shown in Table S1. For comparative purposes, the allozyme and/or phylogenetic analyses included additional, unnumbered populations of *D. fuscus, D. c.f. fuscus* from the North Carolina Piedmont, and topotypic (Livingston Co., KY) *D. conanti*.

Sequencing methods

Genomic DNA was extracted from tail tips using either the Blood & Tissue DNEasy* Kit (Qiagen Group, Valencia, CA) or standard phenol extraction protocol (Sambrook et al. 1989). A region of the cytochrome B gene was amplified using polymerase chain reaction (PCR) with primers MVZ15 and CytB2 (Moritz et al. 1992) following general methods of Mead et al. (2001). There were 387 positions in the final data set after removal of ambiguous positions for each sequence pair. Reactions were run using one of two proofreading enzymes, Vent (New England Biolabs, Ipswitch, MA) or Phusion (New England Biolabs) following manufacturer protocols. PCR products were purified with Solid-phase Oligo/Protein Elimination resin in Performa Gel Filtration cartridges (Edge Biosystems, Gaithersburg, MD). Each PCR product was sequenced in both directions using BigDye terminator RR Mix (PE Applied Biosystems, Foster City, CA) on an Applied Biosystems 3130xl Genetic Analyzer at Smith College.

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Closely related sequences were compared by eye to confirm all polymorphisms. Uncorrected numbers of substitutions per site (Table S2) were calculated for comparisons among 55 sequences representing the major clades, using Mega 5 (Tamura et al. 2011). GenBank accession numbers for each unique sequence are shown in Table S2.

Phylogenetic analyses

CytB sequences were aligned in SeaView (Galtier et al. 1996; Gouy et al. 2010) with the muscle alignment algorithm (Edgar 2004), generating 387 base pair robust alignments. Genealogies were constructed in RAxML and MrBayes on CIPRES (http://www.phylo.org/). A sequence from *Phaeognathus hubrichti* was designated as the out-group and sequences from *Desmognathus organi* (formerly *Desmognathus wrighti* in part), *Desmognathus aeneus*, and *Desmognathus quadramaculatus*, species which have appeared as basal desmognathans in other phylogenetic studies (Chippindale et al. 2004; Kozak et al. 2005, 2009), were also included. Likelihood analysis was done using RAxML-HPC2 (Stamatakis 2006; Stamatakis et al. 2008) with the GTRCAT model of sequence evolution and nodal support values based on 1000 rapid bootstraps (Felsenstein 1985). Bayesian analyses were performed with the parallel version of MrBayes 3.1.2 using the GTR model of nucleotide substitution (Ronquist and Huelsenbeck 2003). Six simultaneous Markov chain Monte Carlo (MCMC) chains were run for 5000,000 generations sampling every 1000 generations. Burn-in was determined using Tracer (Rambaut and Drummond 2009), and post–burn-in phylogenies were used to estimate posterior probabilities for nodal support in the Bayesian analysis. We restrict the term "clade" to clades in the *cytB* phylogram, identified with Greek letters. We refer to population clusters identified on the basis of other criteria, whether or not they appear coincident with haplotype clades, as "forms," informally named according the localities where we first encountered them. The sequences and files used to construct the phylogenetic trees are available at http://purl.org/phylo/treebase/phylows/study/TB2:S14343.

Allozyme methods

We employed standard methods of horizontal starch gel electrophoresis (Murphy et al. 1996; Tilley and Mahoney 1996). Genotype designations are shown in Table S3. Enzyme abbreviations follow Murphy et al. (1996). Allozyme frequencies and Nei unbiased genetic distances (Nei 1978) were calculated using GenAlEx version 6.3 (Peakall and Smouse 2006) and are based on the same 22 presumptive loci surveyed by Tilley and Mahoney (1996). We report Nei distances only between populations with data for all 22 loci.

STRUCTURE version 2.3 (Pritchard et al. 2000, 2010) was employed to evaluate population clustering and admixture patterns. The program employs an MCMC procedure to assign individual genotypes to K population clusters in a manner that maximizes $\Pr(\text{data}|K)$, the probability of obtaining the genotypic data given that number of clusters. Analyses were performed on the entire set of genotypes from populations with data for all 22 allozyme loci and across marker loci in presumptive zones of admixture. All the runs employed the "admixture" model, under which putatively admixed individuals can be assigned to multiple clusters. In order to establish the most appropriate K value for a particular analysis we performed five runs each for K values of 1–20, using 10^4 burn-in and 10^4 subsequent steps. We then employed HARVESTER version 0.6.93 (Earl and vonHoldt 2012) to identify K values associated with high values of ΔK, a statistic that expresses the second order rate of change in $\Pr(\text{data}|K)$ with respect to K (Evanno et al. 2005). We employed the selected values of K to determine $\ln\Pr(\text{data}|K)$ for each of 10 runs using 10^5 burn-in and 10^6 subsequent steps and examined cluster assignments and admixture patterns for the runs yielding the highest values of $\Pr(\text{data}|K)$.

Population clustering patterns were also visualized with multidimensional scaling analysis (MDS) (Kruskal and Wish 1978; Lessa 1990), employing the ALSCAL procedure in SPSS version 19 (SPSS, Inc., Chicago, IL) operating on a matrix of Nei unbiased genetic distances. For comparative purposes, we included a sample of topotypic *D. conanti* in the MDS analyses.

Permutation tests were employed to evaluate the correlation coefficients for relationships between genetic and geographic distances. Distributions of product-moment correlation coefficients for these relationships were generated for 10,000 permutations in which geographic distances were randomly assigned to genetic distances. P-values were calculated as the proportion of randomly generated values that exceeded the correlation statistics obtained. One-tailed values are reported because the relevant alternative hypothesis is that the variables are positively correlated. Confidence intervals on the y-intercepts were based on 10,000 bootstrapped samples (with replacement).

Results

Major haplotype clades

The maximum likelihood (Figs. 1–2) and Bayesian phylograms have very similar topologies. Both show six geographically exclusive clades (bootstrap percentages ≥65%; posterior probabilities ≥0.90; Figs. 1–2). We refer to these as the α clade (Fig. 1) and, from top to bottom in

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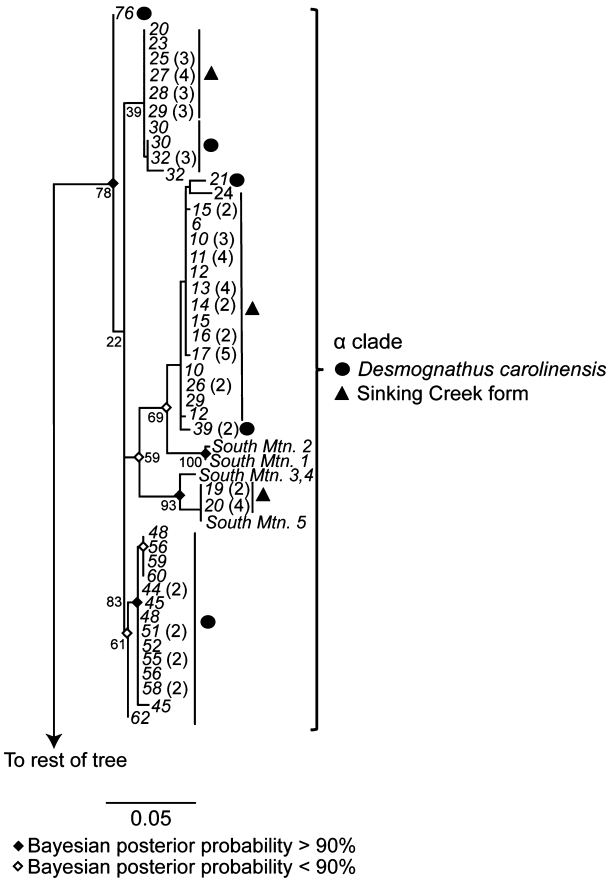

Figure 1. Partial maximum likelihood cladogram of *cytB* sequences comprising Clade α. Locality numbers are italicized. Numbers at nodes indicate bootstrap percentages. Diamonds at nodes indicate clades that were also resolved in the Bayesian analysis, with posterior probabilities >90% (solid diamonds) or <90% (open diamonds).

Figure 2, the *D. fuscus* clade, the β clade, the γ clade, the *D. conanti* clade, and the *D. santeetlah* clade. The *D. fuscus* clade, sister to the α clade on the maximum likelihood tree (bootstrap = 34%), is sister to the remaining clades on the Bayesian phylogram (posterior probability = 0.89).

Levels of sequence divergence (Table S2) are generally much lower among haplotypes of the same clade (0.003–0.080, mean = 0.030 per site) than between haplotypes of different clades (0.059–0.150, mean = 0.104 per site). The only between clade divergences of less than 0.08 per site are for haplotype comparisons involving the β, γ, *D. conanti*, and *D. santeetlah* clades. These four clades form a monophyletic group with low bootstrap support, but a high Bayesian posterior probability (0.91, Fig. 2).

The α clade

Haplotypes of the α clade occur in two morphologically, ecologically, and allozymically distinct forms (Figs. 1, 3, and Fig. 4A and B). Animals of one form are relatively small, gracile, and brightly colored with unkeeled tails (Fig. 4A, black circles in Figs. 1 and 3). They inhabit small streams, seepage areas, wet rock faces, and forest floors, generally at higher elevations in the southern Blue Ridge Physiographic Province. We refer these populations to *D. carolinensis* on the basis of their morphology, ecology, and previous allozyme and sequencing studies. The population at Locality 21 is near populations referred to *D. carolinensis* by Tilley and Mahoney (1996) (their Locality 28) on the basis of allozymes and by Mead et al. (2001) (their "Indian Grave 2" population) on the basis of a *cytB* haplotype. The population at Locality 39 was referred to *D. carolinensis* by Mead and Tilley (2000) (their eastern transect Locality 10) on the basis of allozymes. Localities 30 and 32 are on the southeastern margin of the Ridge and Valley Province at 430 and 511 m, respectively; the other *D. carolinensis* localities sampled in this study are in the Blue Ridge Physiographic Province at higher elevations in the Unaka, Bald, and Black Mountains and on the Blue Ridge Divide.

The remaining α-clade populations morphologically resemble *D. fuscus,* but are (as shown below) allozymically distinct from *D. fuscus* and *D. carolinensis.* We first encountered this form at Localities 12 and 13 along Sinking Creek in and near Johnson City, Washington Co., TN and hereafter refer to it as "the Sinking Creek form" (SCF; Figs. 1, 3, and 4B). SCF individuals are relatively large and robust with weakly keeled tails. While some have bolder dorsal patterns and more speckled ventral surfaces than typical *D. fuscus*, we have been unable to confidently identify other specimens without molecular data. Individuals were collected in saturated mud and gravel, or under cover objects adjacent to water in mucky seepages and small streams at low elevations along the eastern margin of the Ridge and Valley Physiographic Province and in the foothills of the Unaka Mountains. They thus resemble *D. fuscus* and *D. conanti* phenotypically and ecologically and are distinct from the smaller, more gracile, and more terrestrial *D. carolinensis*.

Salamanders that, like SCF, combine morphological features of *D. fuscus* with mitochondrial genomes of *D. carolinensis* have also been reported from several localities in the Piedmont and Coastal Plain (Pittsylvania Co., VA, Guilford Co., NC, and Fairfield Co., SC, Kozak et al. 2005; Clade C4 of Beamer and Lamb 2008; Wilkes Co, NC, Tilley et al. 2008). We therefore included *D. cf. fuscus* haplotypes from each of five localities in the South Mountains of the North Carolina Piedmont (Burke and Wilkes Cos., Table S1) in the phylogenetic analyses. These haplotypes all fall within the α clade (Fig. 1).

Three subclades with high bootstrap support and/or Bayesian posterior probabilities occur within the α clade

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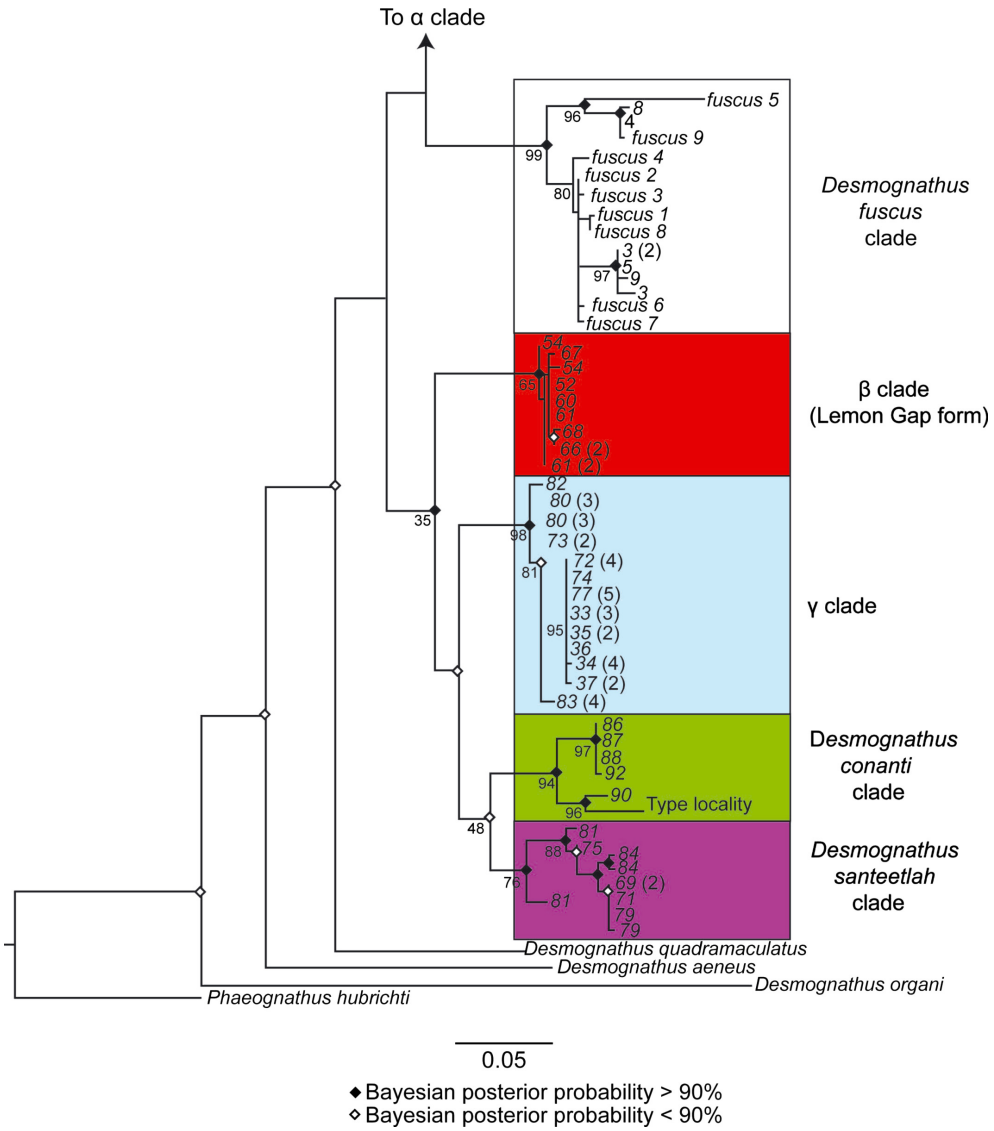

Figure 2. Partial maximum likelihood cladogram of *cytB* sequences exclusive of Clade α. Locality numbers are italicized. Numbers at nodes indicated bootstrap percentages. Diamonds at nodes indicate clades that were also resolved in the Bayesian analysis, with posterior probabilities >90% (solid diamonds) or <90% (open diamonds).

(Fig. 1): (1) a moderately supported clade (bootstrap = 69%; posterior probability = 0.87) including the SCF haplotypes from northeastern (Locs. 6, 10–17, and 24) and two more southern localities (Locs. 26, 29), *D. carolinensis* haplotypes from the Unaka Mtns. (21) and Blue Ridge Divide (39), and South Mountain haplotypes 1 and 2; (2) a strongly supported clade (bootstrap = 93%; posterior probability = 0.98) containing South Mountain haplotypes 3–5 and SCF haplotypes from Localities 19 and 20; and (3) a clade (bootstrap = 83%; posterior probability = 0.99) containing *D. carolinensis* haplotypes from the southern Bald Mountains (44, 45, 48, 51, 52, 55–56, 58–60, and 62). SCF, *D. carolinensis,* and South Mountain haplotypes thus do not form separate subclades within the α clade (Fig. 1).

Maximum levels of sequence divergence within the α clade tend to be high for comparisons between *D. carolinensis* and South Mountain haplotypes (Table S2). The maximum level (0.062 per site) pertains to the comparison between the South Mountain 2 and a SCF haplotype from Locality 19. Levels of divergence are not notably higher for comparisons between SCF and *D. carolinensis* haplotypes. Identical sequences were recovered from a *D. carolinensis* from Locality 30 and an SCF individual from Locality 26, 30 km to the northwest.

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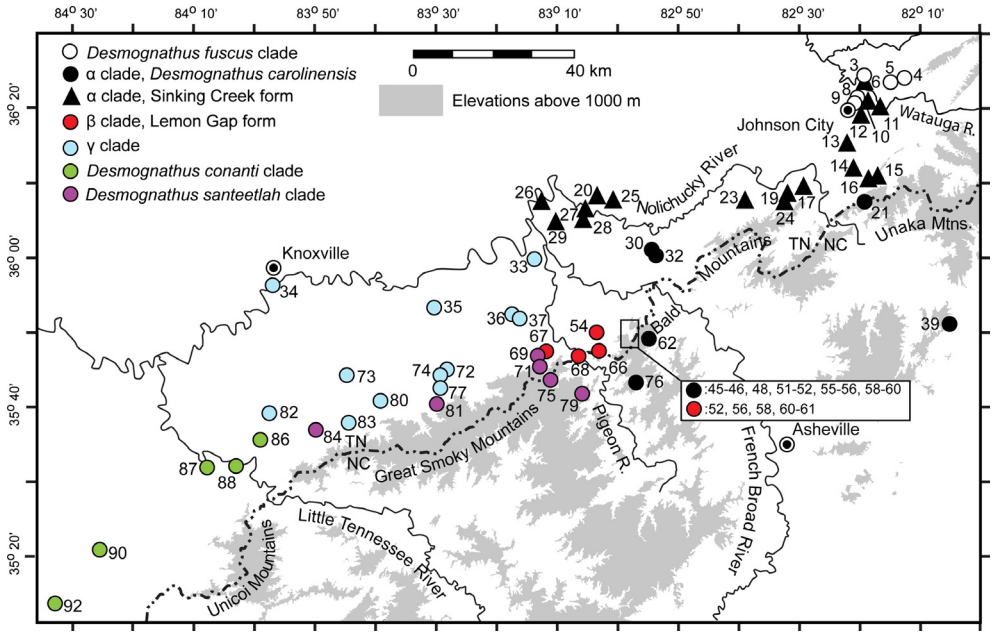

Figure 3. Geographic distributions of *cytB* clades shown in Figures 1 and 2.

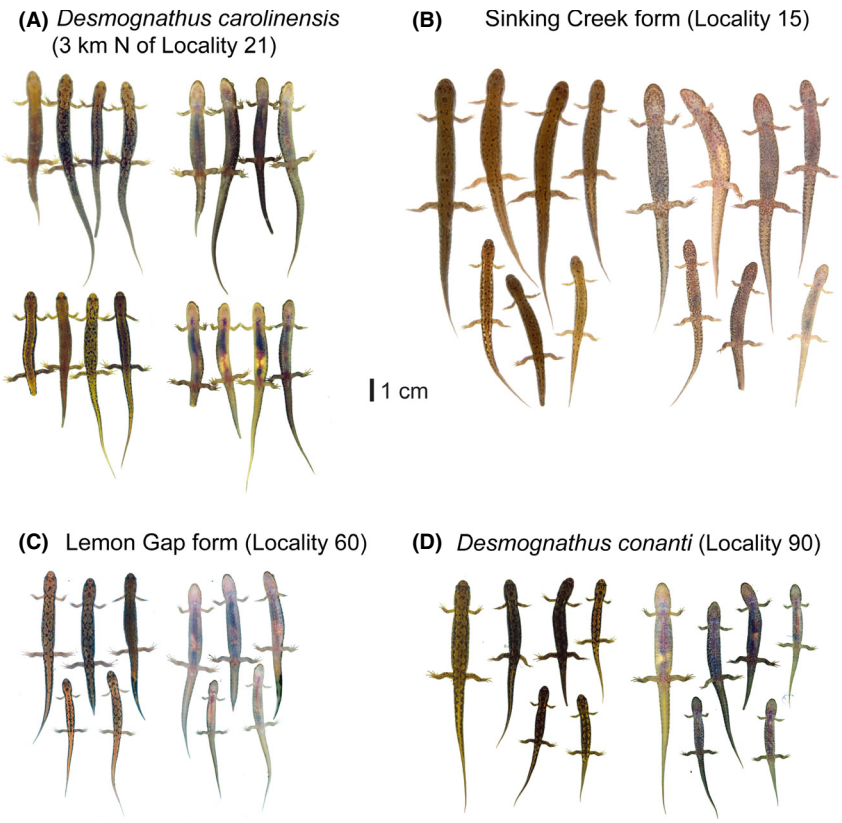

Figure 4. Specimens representing four of the forms treated in this study. (A) *Desmognathus carolinensis* (Locality 21). (B) The Sinking Creek form (Locality 15). (C) The Lemon Gap form (Locality 60). (D). *Desmognathus conanti* (Locality 90).

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The *D. fuscus* clade

Our northernmost clade consists of six haplotypes from Localities 3–5, 8, and 9 in Carter, Sullivan, and Washington Cos., TN, together with *D. fuscus* haplotypes from Massachusetts, Pennsylvania, Ohio, and Virginia (Figs. 2 and 3). We refer this clade as the *D. fuscus* clade on the basis of morphology and the relationships suggested by the haplotypes. Phenotypically, animals of this clade resemble typical *D. fuscus* in having robust bodies, weakly keeled tails, and dorsal patterns consisting of relatively straight dorsal stripes. Levels of sequence divergence between unique haplotypes within this clade range from 0.003 to 0.080 per site (Table S2). The greatest levels of divergence (0.062–0.080) involve the "*fuscus* 5" population near Fancy Gap on the crest of the Blue Ridge in Carroll Co., VA. *D. fuscus* haplotypes are abruptly replaced to the south by those of SCF in the α clade in the vicinity of Johnson City, TN. This contact zone appears to straddle the Watauga River. We have obtained *D. fuscus* haplotypes both north (Locs. 3–5) and south (Locs. 8 and 9) of that stream and a single α clade sequence near the north shore (Loc. 6).

The β clade (the Lemon Gap form)

Sequences of the β clade were obtained from localities straddling the Pigeon River in the foothills of the Bald and Great Smoky Mountains (Fig. 3). Animals from these localities resemble *D. carolinensis* in having bright and highly variable dorsal patterns and we cannot distinguish them from that species without haplotype or allozyme data. To the north and west in the Ridge and Valley, β-clade haplotypes are replaced by those of the γ clade. Our westernmost β clade and easternmost γ-clade localities (67 and 37, respectively) are 11 km apart with no evident physiographic barrier between them. Levels of sequence divergence within this form range from 0.3 to 0.8% (Table S2). The allozyme data (see below) indicate that populations with β-clade haplotypes comprise a genetically distinct group. We first encountered this form at Locality 61 at Lemon Gap on the Cocke Co., TN- Madison Co., NC line in the southern Bald Mountains, and hereafter refer to as "The Lemon Gap form" (LGF). This is the same population that Tilley et al. (1978) (their Loc. 13) referred to as *Desmognathus ochrophaeus*.

The γ clade

Haplotypes of the γ clade occur in the Ridge and Valley Province northwest of the Great Smoky Mountains between the French Broad and Little Tennessee Rivers (Fig. 3). Adult specimens from these populations are relatively robust with variable and sometimes bright dorsal patterns that vary from spotting to wavy or relatively straight dorsal stripes. To the south and west, γ-clade haplotypes are replaced by those of the *D. conanti* clade. Our most southwestern γ clade and most northeastern *D. conanti* clade localities (82 and 86) are 7 km apart on opposite sides of Chilhowee Mountain, Blount Co., TN (Fig. 3). Levels of sequence divergence within the γ clade range from 0.003 to 0.023 substitutions per site (Table S2). We have complete allozyme data for only two populations (Locs. 36 and 73) with γ-clade haplotypes, and will thus refrain from assigning them to a nominate taxon or referring to them as a "form."

The *D. conanti* clade

Our topotypic *D. conanti* sequence falls within a strongly supported (bootstrap = 94%, Bayesian posterior probability = 0.94) clade that also contains haplotypes from Localities 86–88, 90, and 92. The Nei distance between the population at Locality 89, 8.5 km south of haplotype Locality 88, and topotypic *D. conanti* is relatively low ($D = 0.18$) compared to others calculated in this study (Table S4). Specimens from populations in this clade resemble *D. conanti* in having colorful, spotted or striped dorsal patterns, relatively robust morphologies, and moderately keeled tails. We therefore assign populations whose haplotypes fall into this clade, together with the population at allozyme Locality 89, to *D. conanti*. All our eastern Tennessee localities for this clade except 86 lie south of the Little Tennessee River in the foothills of the Unicoi Mountains (Fig. 3). Levels of sequence divergence within the clade average 0.038 substitutions per site (range = 0.003 to 0.053; Table S2). They average 0.047 (range = 0.037–0.053) for comparisons between topotypic *D. conanti* and our eastern Tennessee haplotypes and 0.028 (range = 0.003–0.042) for comparisons among the latter.

The *D. santeetlah* clade

Haplotypes of this clade include one from a population (Loc. 79) that can be referred to *D. santeetlah* on the basis of morphology and allozymes (Tilley 1988; his Area A Population 3). The other localities are located in hybrid zones between *D. santeetlah* and low-elevation populations with β- and γ-clade haplotypes that were referred to as *D. fuscus* by Tilley (1988). Levels of sequence divergence within the *D. santeetlah* clade range from 0.003 to 0.049 substitutions per site (Table S2).

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Patterns of allozymic differentiation

All populations with data for 22 allozyme loci

The HARVESTER analysis indicated a bimodal ΔK distribution with a major peak at $K = 2$ and a minor but distinct one at $K = 9$. Proportional cluster memberships at each locality for those K values are shown in Table 1. For $K = 2$, Cluster 1 corresponds to SCF and reflects its allozymic distinctness from all the other populations in this study. The only other populations with more than 10% of individuals assigned to that cluster are *D. carolinensis* at Locality 21 (29%) and *D. cf. fuscus* at South Mtn. 5 (23%).

The ΔK peak at nine clusters corresponds closely to the number of forms (8) in our a priori classification based on morphology and cytB sequences. There are only two instances in which substantially more than 10% of different forms or cytB clades are assigned to the same cluster. The γ-clade population at Locality 73 shares Cluster 4 with SCF at Locality 31, the only SCF population with a substantial proportion of individuals not assigned to Cluster 1. Sixty-three percent of the γ-clade population at Locality 36 is assigned to Cluster 6, which otherwise corresponds to the LGF. Interestingly, the Locality 36 population does not share a cluster with the other γ-clade population (Locality 73), the only instance in which populations of the same cytB clade are assigned to different clusters. Each of these cases may reflect admixture between the forms involved (see below). All of the remaining forms correspond uniquely to one or, in the case of *D. carolinensis*, two clusters.

Populations of SCF, *D. carolinensis*, LGF, and *D. fuscus* form nonoverlapping clusters in multidimensional scaling (MDS) space (Fig. 5A–C). *D. fuscus*, *D. carolinensis*, and LGF overlap along the first dimension, but are separated along the second and third. Topotypic *D. conanti* does not fall within any of the population groups and is particularly distant from the SCF and *D. fuscus* populations.

Table 1. Proportions of individuals assigned to clusters by the STRUCTURE analysis that yielded the highest probability of obtaining the allozyme data given 2 and 9 clusters.

		$K = 2$ clusters		$K = 9$ clusters									
Locality	cytB clade	1	2	1	2	3	4	5	6	7	8	9	*N*
fuscus 1		0.004	***0.996***	0.002	0.003	0.002	0.005	0.003	0.004	***0.967***	0.003	0.012	13
2	*Desmognathus fuscus*	0.002	***0.998***	0.002	0.003	0.004	0.005	0.002	0.002	***0.978***	0.002	0.002	6
4		0.002	***0.998***	0.001	0.002	0.003	0.004	0.002	0.002	***0.982***	0.003	0.002	14
11		***0.992***	0.008	***0.929***	0.003	0.018	0.003	0.003	0.029	0.003	0.003	0.009	9
12		***0.991***	0.009	***0.960***	0.005	0.006	0.006	0.005	0.005	0.002	0.004	0.007	27
13		***0.987***	0.013	***0.927***	0.006	0.007	0.008	0.006	0.007	0.006	0.012	0.021	49
14	Clade α, Sinking Creek form	***0.995***	0.005	***0.978***	0.002	0.002	0.002	0.004	0.002	0.002	0.003	0.007	13
15		***0.997***	0.003	***0.981***	0.002	0.002	0.003	0.004	0.003	0.001	0.002	0.003	11
16		***0.917***	0.083	***0.892***	0.012	0.011	0.028	0.009	0.007	0.005	0.009	0.026	25
23		***0.982***	0.018	***0.913***	0.011	0.015	0.011	0.004	0.018	0.004	0.009	0.015	9
31		***0.807***	***0.193***	***0.640***	0.003	0.007	***0.314***	0.007	0.004	0.008	0.014	0.003	5
31		0.006	***0.994***	0.003	0.017	0.003	0.037	***0.901***	0.017	0.007	0.009	0.006	11
21	Clade α, *Desmognathus carolinensis*	***0.29***	***0.71***	0.066	0.007	0.033	0.070	0.018	0.009	0.065	***0.686***	0.046	21
38		0.054	***0.946***	0.006	0.006	0.007	0.005	***0.955***	0.004	0.005	0.006	0.006	23
39		0.021	***0.979***	0.003	0.004	0.003	0.005	0.006	0.004	0.007	***0.965***	0.004	20
42		0.009	***0.991***	0.004	0.004	0.002	0.005	***0.967***	0.004	0.003	0.008	0.003	19
78		0.089	***0.911***	0.035	0.013	0.037	0.018	***0.103***	0.008	0.012	***0.707***	0.066	20
S. Mtn. 5	Clade α, *Desmognathus cf. fuscus*	***0.23***	***0.77***	0.003	0.004	0.003	0.003	0.004	0.003	0.004	0.004	***0.973***	21
57		0.038	***0.962***	0.013	0.028	0.071	0.048	0.033	***0.712***	0.022	0.058	0.015	18
61	Clade β, Lemon Gap form	0.014	***0.986***	0.009	0.007	0.019	0.009	0.011	***0.927***	0.006	0.006	0.005	53
64		0.078	***0.922***	0.019	0.021	0.024	0.028	0.026	***0.825***	0.016	0.028	0.012	17
66		0.008	***0.992***	0.003	0.002	0.002	0.003	0.003	***0.981***	0.002	0.002	0.002	14
36	γ clade	0.003	***0.997***	0.003	0.070	0.012	0.018	0.010	***0.631***	***0.109***	0.023	***0.123***	7
73		0.007	***0.993***	0.004	0.004	0.003	***0.974***	0.003	0.003	0.005	0.002	0.002	10
89	*Desmognathus conanti*	0.004	***0.996***	0.002	***0.949***	0.014	0.020	0.003	0.003	0.003	0.003	0.003	16
Type loc.		0.003	***0.997***	0.001	***0.980***	0.002	0.003	0.002	0.002	0.003	0.002	0.004	30
85	*Desmognathus santeetlah*	0.009	***0.991***	0.005	0.040	***0.799***	0.049	0.015	0.031	0.015	0.012	0.034	12
91		0.003	***0.997***	0.001	0.002	***0.983***	0.003	0.002	0.002	0.002	0.003	0.002	34

Proportions exceeding 0.100 are indicated by boldface italicized type.

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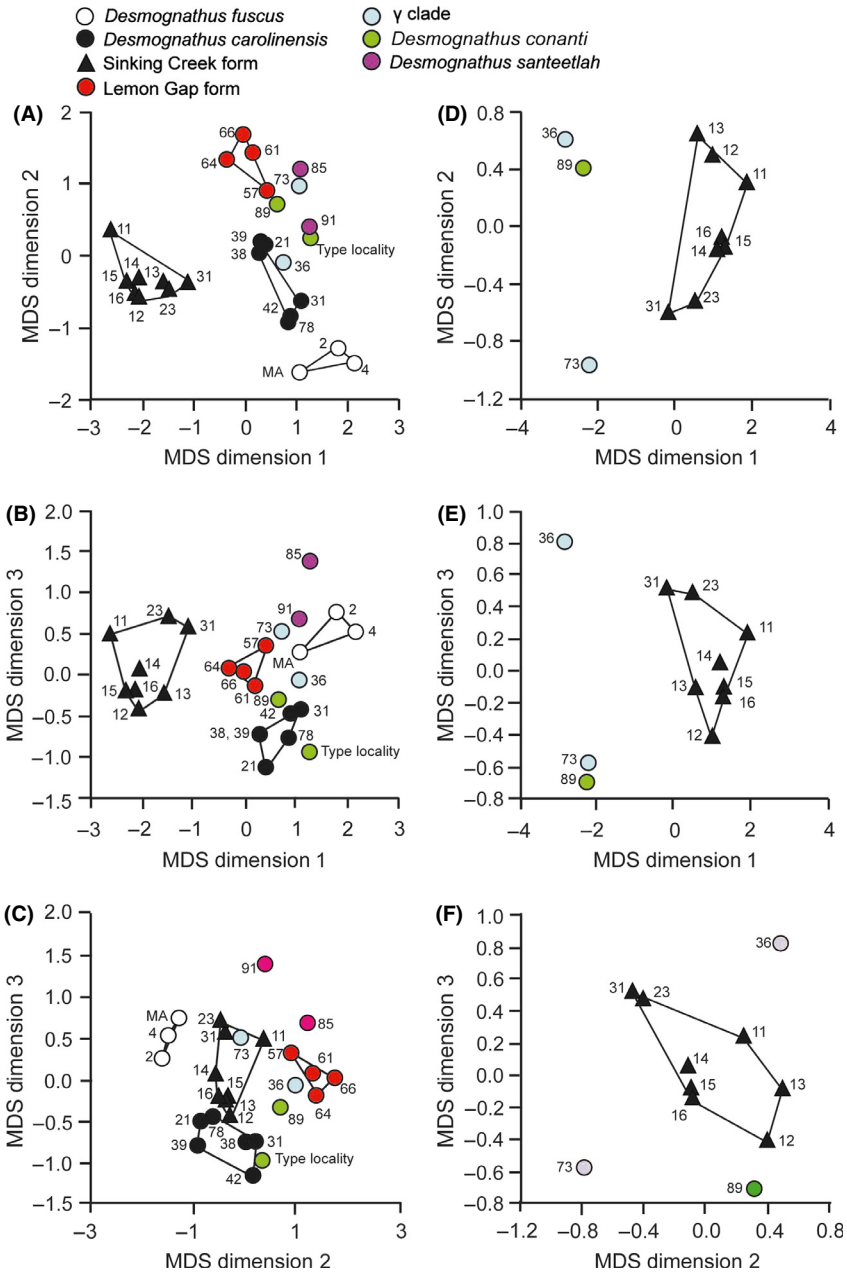

Figure 5. Results of multidimensional scaling analyses. (A–C) The analysis including the major population groups, Massachusetts *Desmognathus fuscus*, and topotypic *Desmognathus conanti*. (D–F) The analysis including populations of the Sinking Creek form, the γ clade at Localities 36 and 73, and topotypic *D. conanti*.

Nevertheless, the Nei distance of 0.18 (Table S4) between topotypic *D. conanti* and the population at Locality 89 is relatively modest for two populations separated by about 422 km.

The Sinking Creek Form (SCF)

Patterns of allozymic resemblance indicate that SCF is distinct from both *D. carolinensis* and *D. fusc*us. The three form distinct clusters in MDS space (Fig. 5A–C). Nei distances average 0.56 (range = 0.39–0.80) between SCF (Locs. 11–16, 23, and 31) and *D. carolinensis* (Locs. 21, 31, 38, 39, 42, 78, and 0.85 (range = 0.625–1.17) and between SCF and *D. fuscus* (Locs. 2 and 4; Table S4). In the vicinity of their contact zone, SCF and *D. fuscus* differ completely or nearly completely with respect to allozyme frequencies at 13 of the 22 presumptive loci that we have surveyed (Fig. 6). *D. fuscus* and SCF at Localities 9 and 12, respectively, which are 4.2 km apart, differ completely with respect to allozyme variants at GAPDH (n = 8, 24), GPI (n = 19, 20), HBDH (n = 8, 24), IDH-2 (n = 18, 27), LDH-1 (n = 19, 29), MPI (n = 19, 27), PEP (n = 19,

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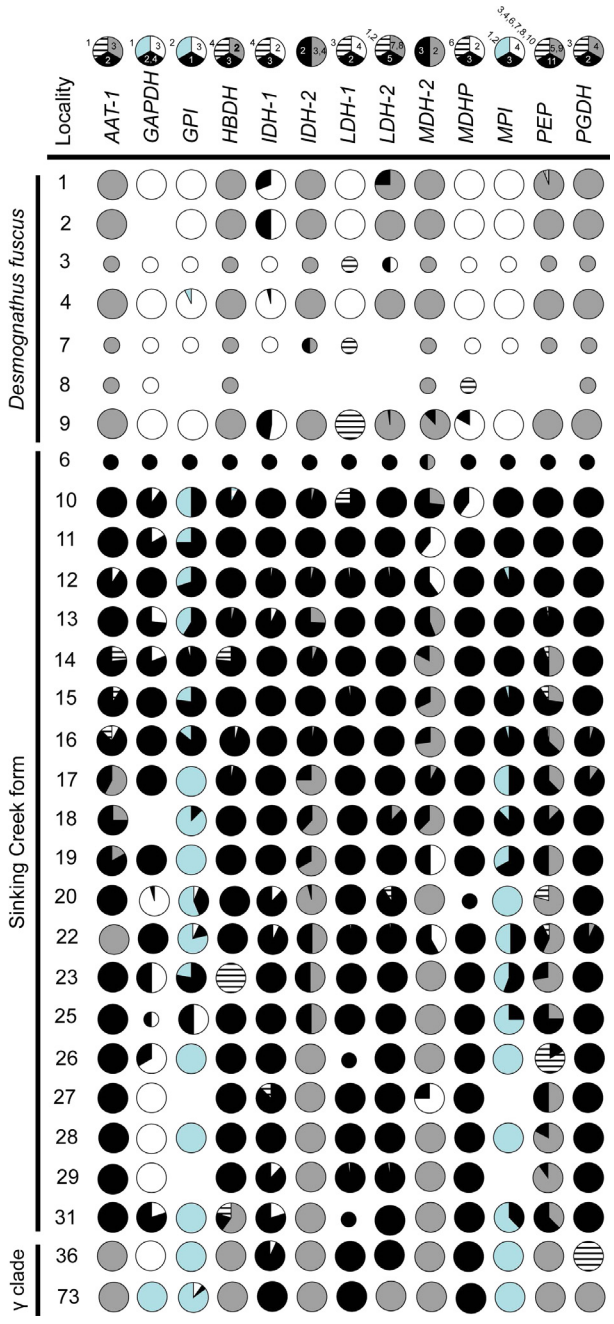

Figure 6. Variation in allozyme frequencies at diagnostic loci among populations of *Desmognathus fuscus*, the Sinking Creek Form, and the γ-clade populations at Localities 36 and 73. Shadings indicate variants characteristic of *D. fuscus* (white), the Sinking Creek form (black), γ-clade populations (blue), *D. fuscus* and γ-clade populations (gray), and other variants (cross-hatching). Small disks indicate single specimens.

7), and PGDH (n = 18, 27). Locality 4 *D. fuscus* and Locality 13 SCF, separated by 22 km, exhibit an unbiased Nei genetic distance of 0.80 (Fig. 7, Table S4).

Genetic distance increases with geographic distance for comparisons among SCF populations (Fig. 8; $r = 0.89$, $P < 0.00{,}001$, permutation test). The same is true when SCF populations are compared with the γ-clade populations at Localities 36 and 73 and with *D. conanti* at Locality 89 ($r = 0.85$, 0.89, and 0.76; $P = 0.0024$, 0.00,003, and 0.0146, respectively; permutation tests). The points for comparisons among SCF populations and between them and Localities 73 and 89 appear to lie on the same regression line. The points for comparisons between SCF and the γ-clade population at Locality 36 (which is actually closer to the range of SCF) appear higher, but the bootstrapped 95% confidence intervals around the vertical intercepts include zero for both for comparisons among the SCF populations and between them and Localities 36, 73, and 89. Overall, the genetic distance data indicate that SCF, the γ-clade populations at Localities 36 and 73, and *D. conanti* at Locality 89 form a complex within which genetic distances are largely explained by an isolation-by-distance model (Wright 1943). This pattern is also evident when these populations are plotted in MDS space (Fig. 5D–F). The SCF populations are well separated from the Localities 36, 73, and 89 populations along MDS Dimension 1, but the two southernmost SCF populations (Localities 31 and 23) are divergent from the others, toward Locality 73 along the first two dimensions (Fig. 5D) and toward Locality 36 along the third (Fig. 5E–F). The gap separating SCF from the Localities 36, 73, and 89 populations along MDS Dimension 1 corresponds to substantial geographic gaps between the southwestern-most SCF locality (31) and Localities 36 and 73 (37 and 93 km, respectively).

The relationships between genetic and geographic distance also reveal a peculiar pattern: For comparisons between *D. fuscus* and SCF, genetic distance actually declines with geographic distance (Fig. 8, $r = -0.833$, $P \sim 0$, permutation test). The average Nei unbiased distance between *D. fuscus* at Localities 2 and 4 and SCF north of the Nolichucky River (Localities 11-16) is 0.90 (range = 0.74–1.17, Table S4), while that between those *D. fuscus* populations and SCF south of the Nolichucky River (Localities 23 and 31) is 0.68 (range = 0.62–0.74, Table S4). This unexpected pattern owes to variation at five of the eight loci (AAT-1, GPI, HBDH, IDH-2, and PEP) that distinguish SCF and *D. fuscus* (Fig. 6). At these loci the *D. fuscus* variants also occur at substantial frequencies in populations of SCF south of the Nolichucky River, as well as the γ-clade populations at Localities 36 and 73 (Fig. 6). This causes genetic distances to decline for comparisons involving *D. fuscus* and progressively more southerly SCF populations.

Sinking Creek form populations north of the Nolichucky River (Locs. 11–16) are well differentiated from other forms with α-clade haplotypes (Nei D's = 0.388–0.800, mean = 0.579 for comparisons with *D. carolinensis*;

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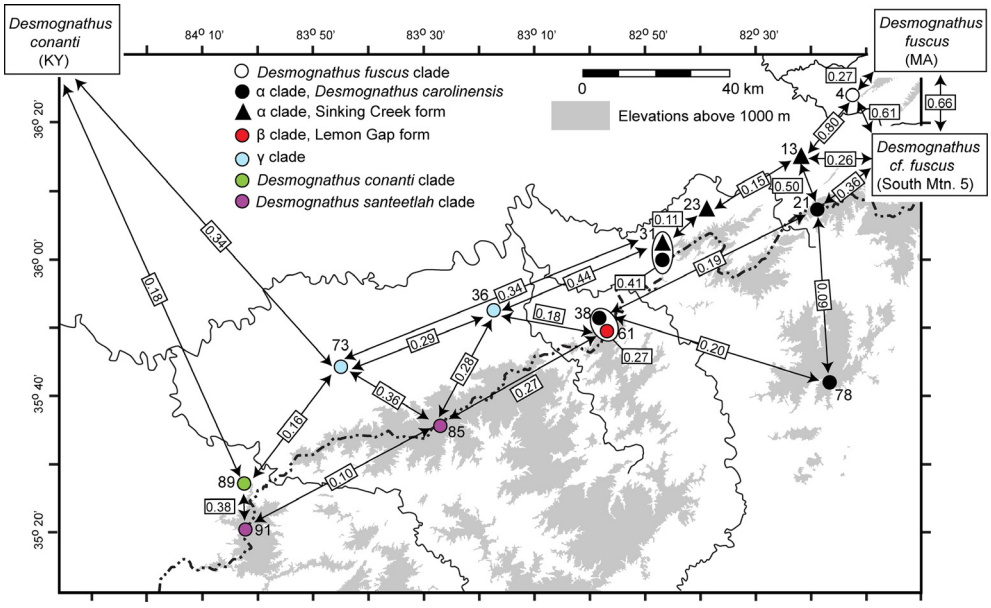

Figure 7. Unbiased Nei genetic distances for selected population comparisons. Clade memberships for Localities 31, 38, 57, 78, 85, 89, and 91 are inferred from allozymes.

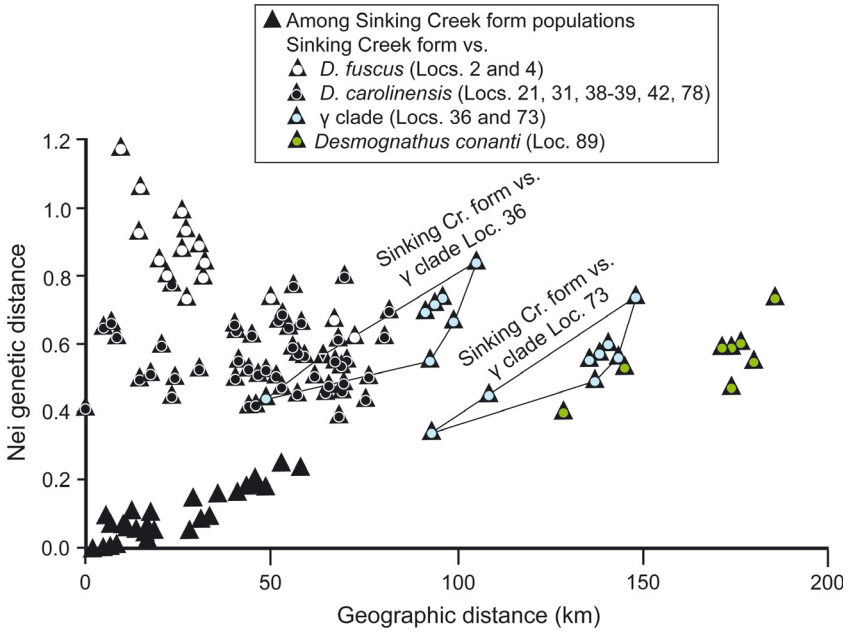

Figure 8. Nei unbiased genetic distance plotted against great circle distance between populations for comparisons involving the Sinking Creek form.

0.256–0.435, mean = 0.323 for comparisons with South Mtn. 5; Table S4). Genetic and geographic distances are unrelated for comparisons between SCF and *D. carolinensis* populations ($r = -0.027$; $P = 0.426$, permutation test).

Localities 36 and 73

The γ-clade populations at Localities 36 and 73 are anomalous in several respects. They are quite divergent from each other in MDS space (Fig. 5) and the Nei distance

between them (0.29) appears higher than would be expected from their geographic distance (44 km; Figs. 7, 8). The Locality 73 population is allozymically similar to *D. conanti* at Locality 89 (Nei D = 0.16), and it and the Locality 36 population are actually more similar to that population (Nei D's = 0.20 and 0.16, respectively) than to each other. Taken as a whole, the genetic distance data indicate that the population at Locality 73 might represent *D. conanti* despite its distinctive γ-clade haplotypes and that SCF and the populations at Localities 36, 73, and 89 form a complex within which genetic distance accumulates with increasing geographic separation between populations.

The Lemon Gap Form (LGF)

The four LGF populations for which we have complete allozyme data are genetically very similar (mean Nei D = 0.09, range = 0.03–0.12, Table S4). They form a cluster in MDS space that excludes all other populations along the second dimension and *D. carolinensis* populations along both the second and third (Fig. 5A–C). LGF populations are well differentiated from *D. carolinensis* allozymically, despite their phenotypic similarity (mean Nei distance = 0.39, range = 0.21–0.60), and from γ-clade and *D. conanti* populations (mean Nei D's = 0.27 and 0.26, ranges = 0.17–0.34 and 0.22–0.29; respectively; Table S4, Fig. 7).

Contact zones

SCF and *D. carolinensis*

The Sinking Creek form and *D. carolinensis* are essentially parapatric. *D. carolinensis* is generally a montane species, but we have collected it as low as 430 and 511 m in the Ridge and Valley Province (Locs. 30 and 32), and it and SCF are syntopic at Locality 31 (Locality 30 in Tilley and Mahoney 1996), where they differ by a Nei distance of 0.4 (Fig. 7; Table S4) and do not share variants at five presumptive loci.

SCF and *D. fuscus*

Desmognathus fuscus, which exhibits little genetic diversity or phylogenetic structure over its extensive range (Bernardo et al. 2007), is abruptly replaced by SCF in the vicinity of the Watauga River in Sullivan, Washington, and Carter Cos., TN. We have never taken the two forms in the same stream. *D. fuscus* Locality 3 ($n = 4$) and our northernmost locality for SCF (Loc. 6, $n = 1$) are only 1.55 km apart, along small, adjacent streams draining into the Watauga River along its northern shore. South of the Watauga River, the two specimens from Locality 8 and the 27 from Locality 9 have been identified as *D. fuscus* on the basis of haplotypes and/or allozymes, as have the 15 from Locality 10, about 3 km to the southeast.

SCF and the γ haplotype clade

The Sinking Creek form appears to be replaced by populations with γ-clade haplotypes in the vicinity of the French Broad River near its confluence with the Nolichucky River in Cocke Co., TN (Fig. 3). The southwestern- and northeastern-most localities for the α- (SCF) and γ-clade haplotypes (Localities 29 and 33, respectively) are 10 km apart on opposite sides of the French Broad River. SCF populations and those with γ-clade haplotypes are well-differentiated allozymically. Nei distances for all comparisons between the two forms average 0.59 (range = 0.34–0.84, Table S4). The two nearest SCF and γ-clade populations with complete allozyme data (Localities 31 and 36, respectively) exhibit a Nei distance of 0.44 (Fig. 7), and fixed or complete differences at 6 presumptive loci (AAT-1, AAT-2, AK, GDH, PEP, and PGDH). The Nei distance between SCF at Locality 31 and the γ-clade population at Locality 73, further to the south, is actually somewhat lower (0.34, Fig. 7). This may reflect gene exchange between γ clade Locality 36 and LGF (see below). Northern SCF populations (Localities 10–16) and the γ-clade population at Localities 36 and 73 are completely differentiated at 8 presumptive loci (AAT-1, AGPDH, HBDH, IDH-2, LDH-2, MPI, PEP, and PGDH) and exhibit substantial frequency differences at GPI and MDH-2 (Fig. 6). To the south, γ-clade variants appear in SCF populations at MPI and PEP and increase in frequency at GPI and MDH-2 (Fig. 6). Interestingly, some of these variants also occur in *D. fuscus*, as explained above.

In order to visualize genetic variation across the contact zone between SCF and the γ clade we performed a STRUCTURE analysis on the SCF populations at Localities 10–20, 22–23, 25–29, and 31 and the γ-clade populations at Localities 36 and 73 (Fig. 9). This analysis employed only the loci that exhibited strong differentiation between the northeastern-most SCF and γ-clade populations. The HARVESTER analysis indicated a single, steep ΔK mode at $K = 2$, consistent with our a priori selection of loci that distinguished two forms. More than 95% of the individuals in each of the two γ-clade populations were assigned to a single cluster (blue shading in Fig. 9) and more than 95% of the SCF populations at Localities 10–15 to the alternative cluster (black shading in Fig. 9). Among the remaining populations, membership in those clusters shifts along a southwest–northeast axis (Fig. 9A and B). We interpret this as a signature of admixture between SCF and γ-clade populations, which is

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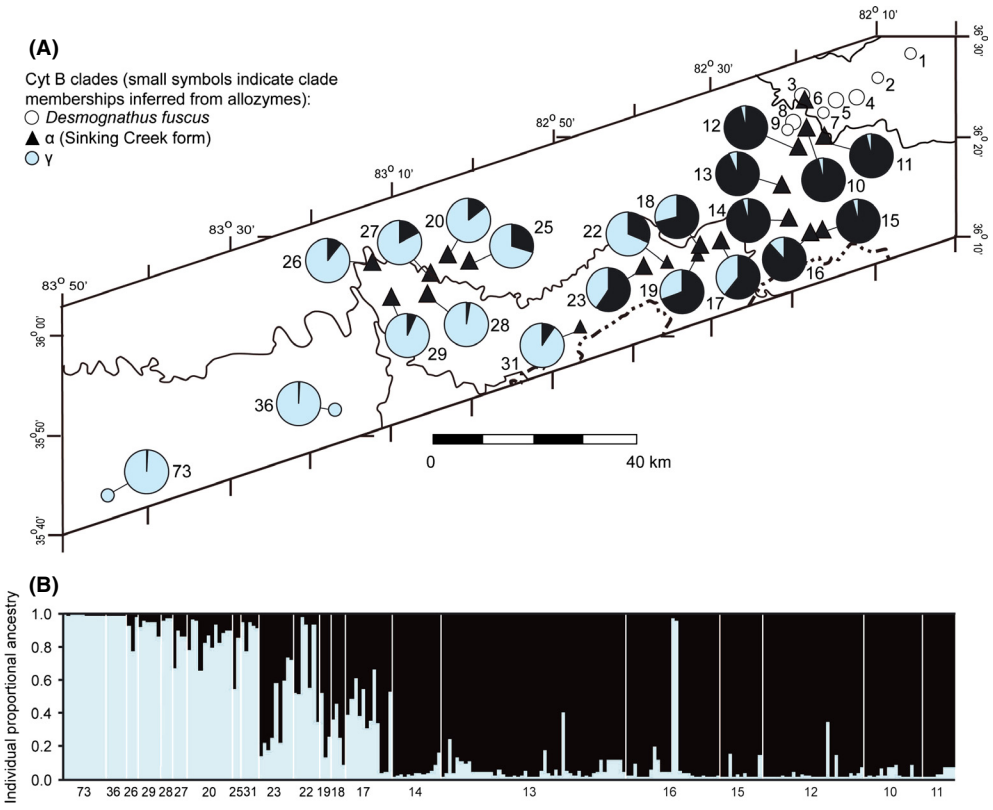

Figure 9. STRUCTURE analysis of the presumptive zone of admixture between γ clade at Localities 36 and 73 and the Sinking Creek form based on the diagnostic allozymes shown in Figure 6. Blue and black shadings denote the clusters corresponding to the γ-clade localities and SCF, respectively. Small symbols represent clade assignments inferred from allozymes. (A) Proportional cluster assignments at each locality averaged over individuals. (B) Inferred proportional ancestries with respect to STRUCTURE clusters. Each vertical bar represents an individual. Locality numbers are shown below the horizontal axis, arranged from southwest to northeast.

also evident in the shifts in frequencies of variants across the individual loci (Fig. 6).

LGF and *D. carolinensis*

The Nei unbiased genetic distance between LGF (Loc. 61) and *D. carolinensis* (Loc. 38) is 0.27 (Fig. 7). A contact zone between the two forms occurs in the headwater tributaries of Big Creek northwest of Lemon Gap (Fig. 10) in the southern Bald Mountains. In this region, haplotype sequences and allozymic variation at four marker loci (IDH-1, LDH-1, PGDH, and SOD) indicate that the populations at Localities 38, 40, 42, and 44 are assignable to *D. carolinensis* and those at Localities 58–61 to LGF, although one of the two haplotypes from Locality 60 is an α-clade (*D. carolinensis*) sequence (Fig. 10A). The other populations appear to exhibit admixture between these two forms. A HARVESTER analysis of STRUCTURE results based on the four maker loci indicated a single, steep ΔK mode at $K = 2$, as expected. The STRUCTURE analysis for $K = 2$ assigns nearly all the individuals at Localities 38, 40, 42, and 44 to one cluster. Nearly all the individuals at Localities 58–61 are assigned to the other cluster, although a few appear to be admixed (Fig. 10B and C). The remaining populations in the vicinity of Lemon Gap exhibit haplotypes and allozyme variants of both *D. carolinensis* and LGF (Fig. 10A) and a narrow zone of admixture appears to occur in the headwaters of Big Creek between *D. carolinensis* Locality 44 and LGF Localities 60 and 61 (Fig. 10B and C).

LGF and *D. santeetlah*

Haplotypes of the β clade occur south of the Pigeon River at Locality 67 in the foothills of the Great Smoky Mountains, near Cosby, Cocke Co., TN (Fig. 3). This indicates that the hybrid zone between *D. santeetlah* and "*D. fuscus*" along Cosby Creek described by Tilley (1988) actually involves hybridization between *D. santeetlah* and LGF. The mean Nei genetic distance between LGF populations (Locs. 57, 61, 64, and 66) and *D. santeetlah* at

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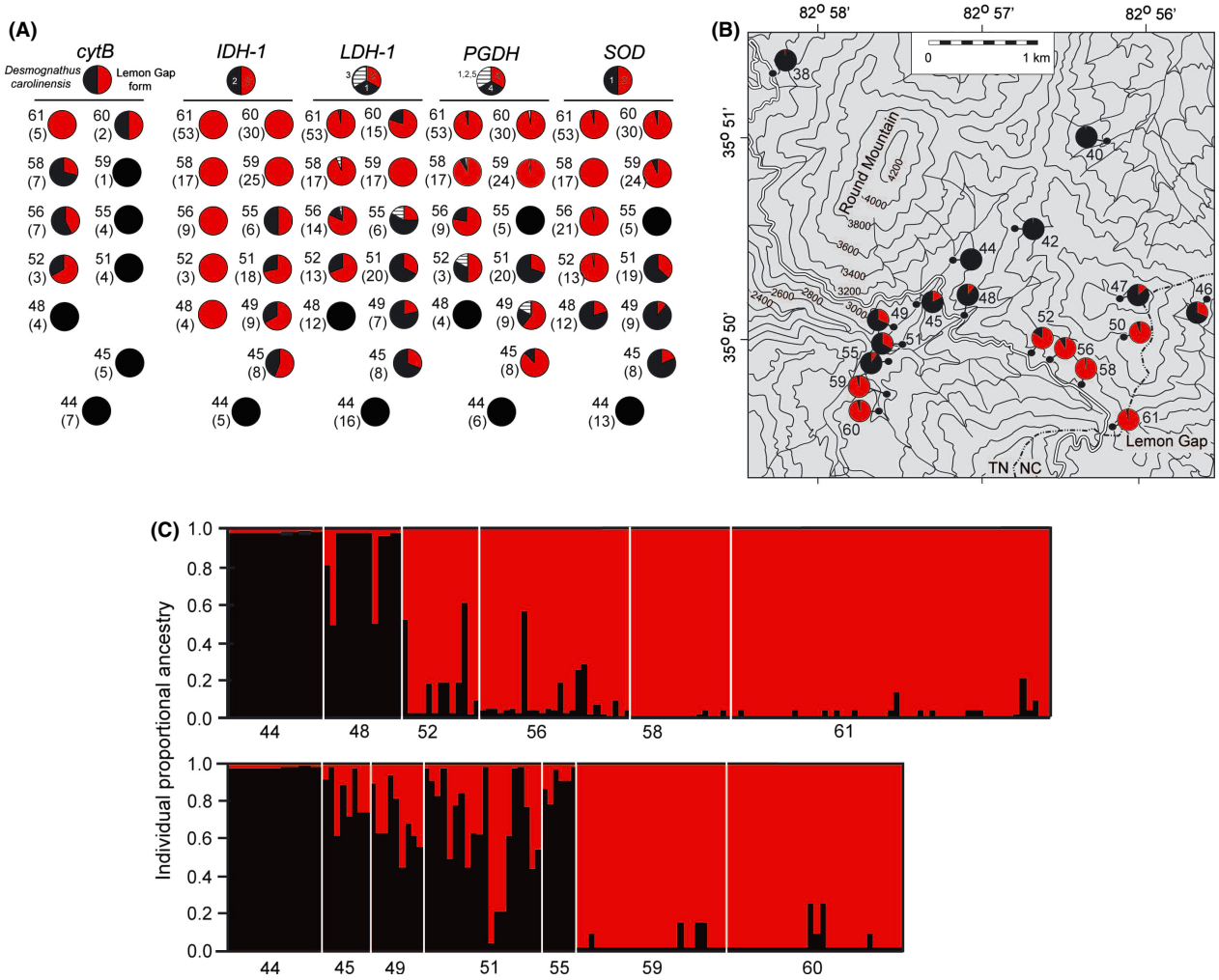

Figure 10. Haplotype and allozymic variation in the southern Bald Mountains near Lemon Gap, across a presumptive zone of admixture between *Desmognathus carolinensis* and the Lemon Gap form. Black and red shadings denote genetic markers and STRUCTURE clusters corresponding to *D. carolinensis* and LGF, respectively. (A) Diagnostic Haplotype and allozyme frequencies across transects between Localities 61 or 60 and 44. Sample sizes are shown in parentheses. Cross-hatching represents nondiagnostic allozyme variants. (B) Proportional STRUCTURE cluster assignments averaged over individuals. (C) Inferred proportional ancestries with respect to STRUCTURE clusters along transects between Localities 44 and 61 (upper) and Localities 44 and 60 (lower). Each vertical bar represents an individual. Locality numbers are shown below the horizontal axes, arranged from northwest to southeast (upper) and northeast to southwest (lower).

Locality 85 in the Great Smokies is 0.26 (range = 0.18–0.31, Table S4).

LGF and the γ haplotype clade

The contact zone between LGF and populations with γ-clade haplotypes is evidently located near the Pigeon River, although LGF haplotypes occur south of the river at Locality 67 (Figs. 3 and 11A). The genetic distance between LGF at Locality 61 and the γ-clade population at Locality 36, 32 km away across the Pigeon River, is 0.18 (Fig. 7).

Allozyme variants at GDH, PEP, and SOD exhibit pronounced frequency shifts in the vicinity of the Pigeon River northeast of the Great Smokies that are geographically concordant with each other and the shift from LGF to Locality 36 haplotypes (Fig. 11A–D). A HARVESTER analysis based on STRUCTURE results for those loci indicated a single, steep ΔK mode at $K = 2$, as expected. The STRUCTURE analysis for $K = 2$ indicates a zone of admixture between the γ-clade populations at Localities 36 and 37 just southeast of English Mountain in the Ridge and Valley Physiographic Province and LGF at Locality 66 on Snowbird Mountain in the southern Bald Mountains.

Gene exchange between LGF and Locality 36 may explain some anomalous patterns reported above. The

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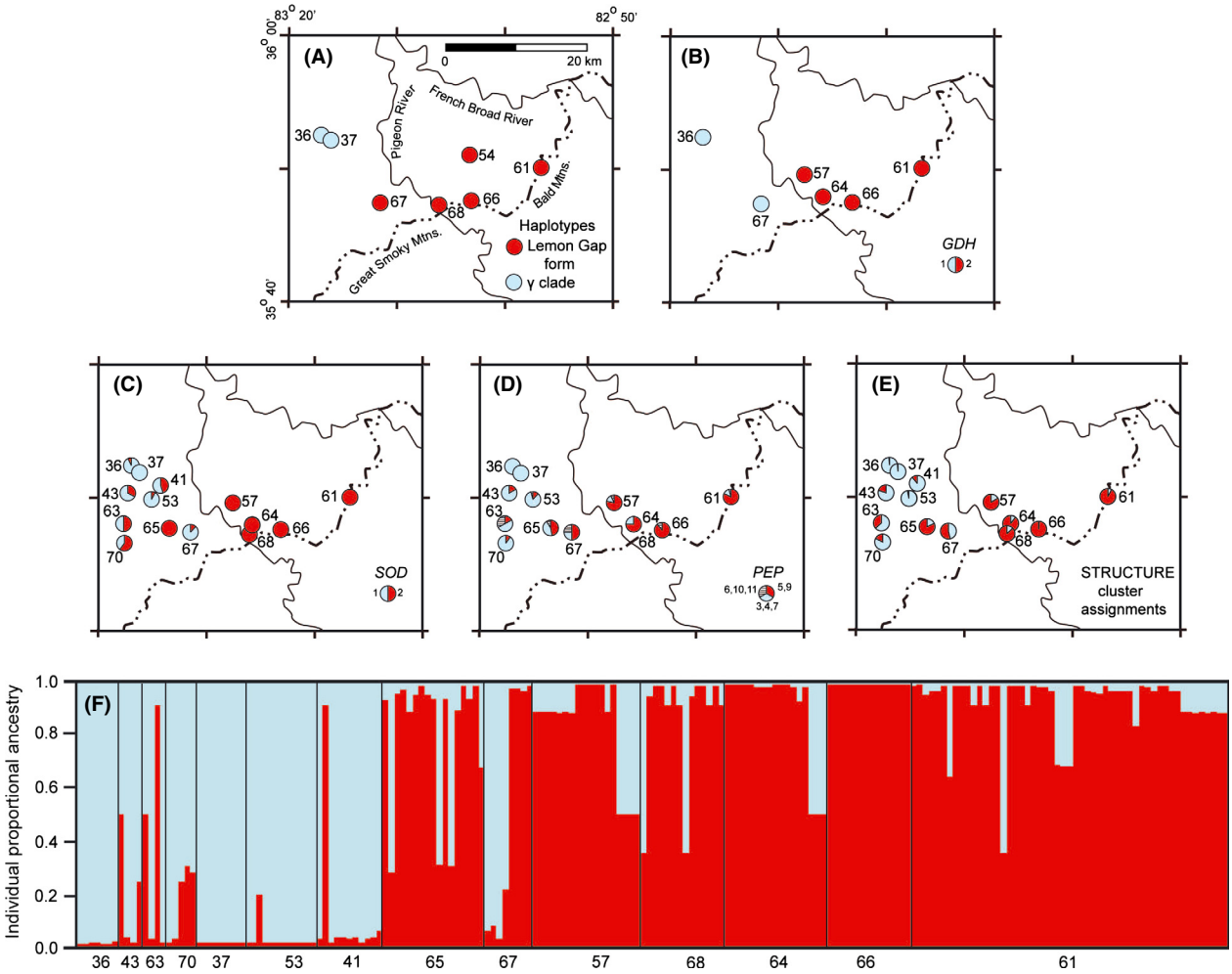

Figure 11. Haplotype and allozymic variation in the foothills of the southern Bald and Great Smoky Mountains, across a presumptive zone of admixture between the γ clade at Locality 36 and the Lemon Gap form. Blue and red shadings denote STRUCTURE clusters and genetic markers corresponding to the γ clade at Locality 36 and LGF at Locality 61, respectively. (A–D) Diagnostic haplotype and allozyme frequencies. (E) Proportional cluster assignments, averaged over individuals. (F) Inferred proportional ancestries with respect to clusters. Each vertical bar represents an individual. Locality numbers are shown below the horizontal axis, arranged from west to east.

population at Locality 36 is unexpectedly divergent from the γ-clade population at Locality 73 (Nei D = 0.29) and is actually genetically more similar to LGF at Locality 61 (Nei D = 0.18). The populations at Localities 36 and 73 are fixed or nearly fixed for the same variants at 16 presumptive loci, but exhibit complete differentiation at the remaining six (AK, GAPDH, GDH, LDH-2, PEP, and PGDH). At each of these diagnostic loci, the population at Locality 36 shares a variant with LGF at Locality 61 (Table 2). A second anomaly mentioned earlier is that SCF at Locality 31 is more divergent from the Locality 36 population (Nei D = 0.44) than from the more distant Locality 73 population (Nei D = 0.34). Three loci contribute to this pattern, and at two of them (AK and PEP) the Locality 36 population shares a variant with LGF at Locality 61 that is absent in SCF at Locality 31 (Table 2).

Discussion

We can confidently assign some populations to named species (*D. fuscus* and *D. conanti*) and firmly establish the southern range limit for *D. fuscus* in northeastern Tennessee. Our results confirm current taxonomic treatment of *D. fuscus* and *D. conanti* as full species (Titus and Larson 1996; Tilley 2000a; Frost 2011; Tilley et al. 2011). While Conant and Collins (1991) portrayed a broad zone of hybridization between *D. conanti* and *D. fuscus*, our data provide no evidence of gene exchange between *D. fuscus* and any form that might be referred to *D. conanti* in eastern Tennessee. The "poorly delineated" contact zone between *D. fuscus* and *D. conanti* indicated by Petranka (1998) roughly corresponds, in eastern Tennessee, to the contact between *D. conanti* and our γ-clade populations.

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Table 2. Allozyme variation at diagnostic loci in the locality 36 and 73 populations, the Lemon Gap form, and the Sinking Creek form.

	Clade γ Locality 73	Clade γ Locality 36	Lemon Gap form Locality 61	Sinking Creek form Locality 31
AK	$N = 10$	$N = 7$	$N = 39$	$N = 5$
2	0	1	1	0
3	1	0	0	1
GAPDH	$N = 10$	$N = 7$	$N = 45$	$N = 5$
1	1	0	0.000	0
2	0	0	0.022	0
3	0	1	0.978	1
GDH	$N = 10$	$N = 6$	$N = 29$	$N = 5$
1	0	1	0.759	0
2	1	0	0.241	1
LDH2	$N = 9$	$N = 7$	$N = 45$	$N = 5$
2	0	0.429	0.022	0
5	0	0.571	0.200	1
6	0	0	0.000	0
7	0.833	0	0.567	0
8	0.167	0	0.211	0
PEP	$N = 10$	$N = 6$	$N = 45$	$N = 4$
4	0	0.417	0.000	0
5	0.250	0	0.311	0
6	0	0	0.011	0
7	0	0.583	0.133	0
9	0.750	0	0.511	0.375
10	0	0	0.033	0
11	0.000	0	0.000	0.625
PGDH	$N = 10$	$N = 7$	$N = 53$	$N = 5$
1	0	0	0.009	0
2	0	0	0.009	0
3	0	1	0.840	1
4	1	0	0.142	0

Our results raise three major challenges to species delineation: (1) unexpectedly high levels of lineage divergence in the absence of morphological differentiation and physiographic barriers (*D. fuscus* vs. SCF, *D. carolinensis* vs. LGF, γ-clade populations and *D. conanti*); (2) discordance between *cytB* clades and allozyme profiles (SCF and *D. carolinensis*); and 3) evidence of gene exchange between differentiated lineages (SCF and the γ clade, LGF and *D. carolinensis*, LGF and γ-clade populations).

SCF

SCF populations are an allozymically distinctive group whose *cytB* sequences further distinguish them from *D. fuscus*, the γ clade, and *D. conanti* (but not from *D. carolinensis* or Piedmont *D. cf. fuscus*). Nevertheless, geographic variation (Fig. 9) across the contact zone between SCF and γ-clade populations indicates admixture between two allozymically distinctive forms, across a zone whose width (ca. 73 km) is about four times the distance (ca. 18 km) between our most distant "pure" SCF populations (Locs. 11 and 16). Isolation-by-distance patterns (Fig. 8) across SCF, the γ clade, and *D. conanti* further indicate that these populations form an assemblage in which gene flow significantly affects the genetic structures of populations. Admixture between SCF and γ-clade populations to the south, together with allozyme variants shared between γ-clade and *D. fuscus* populations, have generated a peculiar pattern in which genetic distance between SCF and *D. fuscus* populations actually decreases with geographic distance over the set of allozyme loci employed in this study.

Kozak et al. (2005) and Tilley et al. (2008) offered two obvious explanations of these discordances between *cytB* sequences and morphology. On the one hand, the phylogenetic histories of mitochondria and populations might coincide, implying that the distinctive morphologies and allozyme profiles of *D. carolinensis* and SCF have evolved within the same clade. In that case, the lack of differentiation between the haplotypes of the two forms would suggest an extremely close relationship and rapid morphological and allozymic divergence. Alternatively, "cytoplasmic capture" (Avise 2004) via hybridization might have led to discordance between mitochondrial and population phylogenies. Kozak et al. (2005) favored the former hypothesis in light of the apparent allopatry between *D. carolinensis* and Piedmont populations resembling *D. fuscus* with similar haplotypes. The cytoplasmic capture hypothesis seems most consistent with (a) the extreme similarity between *D. carolinensis*, SCF, and Piedmont (South Mountain) α-clade haplotypes; (b) the failure of *D. carolinensis*, Piedmont *cf. D. fuscus*, and SCF haplotypes to form reciprocally monophyletic *cytB* clades; (c) the relationship between southwestern SCF and *D. carolinensis* haplotypes from geographically adjacent localities in the Ridge and Valley; and (d) sympatry between *D. carolinensis* and SCF in the Ridge and Valley adjacent the Blue Ridge in eastern Tennessee. Invoking this hypothesis does not, however, suggest the actual mechanism by which cytoplasmic capture is achieved.

Discordances between allozyme and *cytB* variants across zones of admixture have been reported in *Desmognathus* (two forms of *D. orestes*, Mead et al. 2001) and *Plethodon* (*P. metcalfi* and *P. jordan;* Chatfield et al. 2010). In both cases mitochondrial haplotypes were more broadly distributed than allozyme markers into the range of one of the two forms, and asymmetric mating preferences were consistent with the discordances. Chatfield et al. (2010) proposed a model in which such asymmetries could cause directional shift in a zone of admixture, leading to the replacement of one form's nuclear genome while its mitochondrial genome remained as a "footprint" of that form's former range. Female philopatry with respect to egg brooding sites, demonstrated in *D. ocoee* by Forester

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(1977), could inhibit the spread of that footprint. Similar asymmetries in zones of contact could have enhanced the spread of SCF nuclear genes into the former range of *D. carolinensis*, which might have extended further into the Ridge and Valley in an earlier time.

Other studies have attributed cytonuclear discordance to higher rates of introgression among cytoplasmic variants. Chan and Levin (2005) found that mitochondria introgressed faster than nuclear variants when hybridizing forms differed in relative abundance in their zones of contact and exhibit frequency-dependent prezygotic reproductive isolation. Petit and Excoffier (2009) proposed that mitochondrial introgression should be enhanced (paradoxically) in forms subject to low rates of intraspecific gene flow, which results in smaller effective population sizes and higher probabilities that introgressed variants will increase in local populations.

All these hypotheses suffer from two serious difficulties: (1) lack of evidence for current hybridization between SCF and *D. carolinensis,* or an isolation-by-distance pattern for allozymic comparisons between the two forms (Fig. 8), and (2) the apparent absence of populations that combine a different mitochondrial genome with the allozymic and morphological attributes of SCF or *D. carolinensis*. These problems could be addressed by experimental studies of mate choice and more intensive sampling at localities where SCF and *D. carolinensis* are sympatric, and further west in the Ridge and Valley Province, where populations with SCF nuclear genomes might be associated with different haplotypes.

A second problem involves the evolutionary relationships among desmognathans resembling *D. fuscus* but with *D. carolinensis* haplotypes. Genetic distances between SCF and South Mountain *cf. D. fuscus* populations are rather high, but suggest, along with morphological similarities, that those forms are more closely related to each other than either is to *D. carolinensis*. Under this hypothesis, SCF and *D. cf. fuscus* populations in the South Mountains might be members of a clade distributed on both sides of the Blue Ridge that has engaged in cytoplasmic exchange with *D. carolinensis,* perhaps as it displaced lowland populations of that form. The level of allozyme differentiation between SCF and South Mountain *D. cf. fuscus* seems more consistent with separate exchanges involving divergent forms resembling *D. fuscus* on opposite sides of the Blue Ridge. A relatively distant phylogenetic relationship between those two forms inferred from nuclear sequences would provide further confirmation.

LGF

Tilley (1981, 1988) found that *D. santeetlah* hybridized with a lowland form that he called "*D. fuscus*" along the northwestern escarpment of the Great Smokies. Tilley and Huheey (2001) and Dodd (2004) treated those low-elevation populations as *D. conanti*. Our LGF Locality 53 is located in the lower end of that hybrid zone, just 0.9 km SE of Tilley's (1988) Area B, Population 3 in the Cosby Creek drainage. LGF thus appears to be the low-elevation form that hybridizes with *D. santeetlah* and to thus exchange genes with three forms: (1) *D. santeetlah* along the northwestern escarpment of the Great Smokies, (2) *D. carolinensis* over a much narrower zone in the vicinity of Lemon Gap in the southern Bald Mountains, and (3) lowland populations with γ-clade haplotypes in the vicinity of the Pigeon River northeast of the Great Smokies.

Tilley (1988) found that salamanders in the Cosby Creek hybrid zone were smaller than both *D. santeetlah* and "*D. fuscus*" to the northwest in the Ridge and Valley, near our γ clade at Locality 36. He proposed that this might reflect the interaction of genetic and environmental effects at intermediate elevations. It now appears that these shifts in body sizes correspond to two different contact zones: between *D. santeetlah* and LGF and between LGF and the larger lowland form with γ-clade haplotypes at Locality 36.

Tilley (1988) reported that gene exchange between *D. santeetlah* and "*D. fuscus*" appeared to diminish from northeast to southwest along the northwestern escarpment of the Great Smokies, and appeared to be absent between *D. santeetlah* and "*D. fuscus*" populations further to the southwest in the Unicoi Mountains. It now appears that this pattern owes to genetic interactions between *D. santeetlah* and up to three different lowland forms: (1) LGF, with which it extensively hybridizes in the Cosby Creek drainage; (2) populations with γ-clade haplotypes with which it appears to hybridize less extensively; and (3) *D. conanti* south of the Little Tennessee River where there is no evidence of gene exchange.

The γ and *D. conanti* clades

Salamanders whose *cytB* sequences fall into the *D. conanti* clade are relatively robust desmognathans with tails that are weakly keeled or triangular in cross section. Their dorsal patterns are often bold and colorful, consisting of pronounced and typically wavy dorsolateral stripes and retained larval spots that often enclose bright yellow-to-orange pigment. Yellow-to-orange postocular stripes are often present. These are all characteristics of *D. conanti* (Rossman 1958*;* Karlin and Guttman 1986*;* Bonett 2002) and, together with their haplotypes and allozyme frequencies, identify the populations at our Localities 86–92 as that species.

The γ-clade populations are more problematic. Assigning them to *D. conanti* would be consistent with their relatively low sequence divergence from the *D. conanti*

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clade (Table S2) and the relatively low Nei distance (D = 0.16, Fig. 7) between the Locality 73 γ-clade population and *D. conanti* at Locality 89. However, the Nei distance between the Locality 73 population and topotypic *D. conanti* (0.34, Fig. 7) is relatively high. Furthermore, assigning the γ-clade populations to *D. conanti* would render haplotypes of *D. conanti* paraphyletic with respect to those of *D. santeetlah* (Fig. 2). *D. conanti* and *D. santeetlah* are sister clades on our *cytB* genealogy, yet apparently occur parapatrically without hybridization in the Unicoi Mountains (Tilley 1981).

The location of the type locality of *D. conanti*, on the periphery of the species' range (Rossman 1958), complicates the assignment of populations to that taxon on the basis of molecular data (Bonett 2002; Kozak et al. 2005; Beamer and Lamb 2008). Allozymic and haplotype similarity between populations at the type locality and our Locality 89 suggest that *D. conanti* does exhibit genetic cohesion (Templeton 1989) over substantial distances.

Beamer and Lamb (2008) showed the range of *D. conanti* extending northward in the Ridge and Valley Province to the Tennessee Valley drainage divide in southwestern Virginia. However, they did not actually obtain *D. conanti* haplotypes from Virginia and we seriously doubt that it occurs in that state. A comprehensive phylogeographic analysis of "*D. conanti*" populations throughout that species' putative range is sorely needed and might clarify the taxonomy of some of the populations in this study.

Species delimitation

Employing evidence of nuclear gene exchange to delimit species in this complex would combine distinct haplotype clades (SCF with populations of the γ clade; LGF with *D. carolinensis*, *D. santeetlah*, or the γ clade; assigning the γ-clade population at Locality 73 to *D. conanti*). Assigning populations of SCF to *D. carolinensis* on the basis of their similar *cytB* haplotypes would combine forms that are otherwise genetically, ecologically, and morphologically distinct. In these cases, nomenclature simply cannot convey the complex patterns of discordance among *cytB* sequences, allozyme patterns, morphology, and reproductive isolation and we caution against basing species delimitations on any single type of data (Bauer et al. 2011; Bernardo 2011). We propose that SCF, LGF, and perhaps the γ-clade populations be regarded as "failed species:" genetically distinctive lineages that now exchange genes with other forms at levels that compromise their evolutionary independence. For convenience they might be referred to as *Desmognathus cf. conanti*, but we prefer to currently treat them as innominate forms. Returning to Darwin's (1859) words that began this study, this may not offer "a cheering prospect" for delimiting species and we may have engaged in a "vain search" for species boundaries, at least among these Appalachian salamanders.

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Conflict of Interest

None declared.

References

Agapow, P.-M., O. R. P. Bininda-Emonds, K. A. Crandall, J. L. Gittleman, G. M. Mace, and J. C. Marshall. 2004. The impact of species concept on biodiversity studies. Q. Rev. Biol. 79:161–179.

Anderson, J. A., and S. G. Tilley. 2003. Systematics of the *Desmognathus ochrophaeus* complex in the Cumberland Plateau of Tennessee. Herpetol. Monogr. 17:75–110.

Avise, J. C. 1989. A role for molecular genetics in the recognition and conservation of endangered species. Trends Ecol. Evol. 4:279–281.

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Avise, J. C. 2004. Molecular markers, natural history, and evolution, 2nd ed.. Sinauer Associates, Sunderland, MA.

Ballard, J. W. O., and D. M. Rand. 2005. The population biology of mitochondrial DNA and its phylogenetic implications. Annu. Rev. Ecol. Evol. Syst. 36:621–642.

Barber, B. R., J. Xu, M. Pérez-Losada, C. G. Jara, and K. A. Crandall. 2012. Conflicting evolutionary patterns due to mitochondrial introgression and multilocus phylogeography of the Patagonian freshwater crab *Aegla neuquensis*. PLoS ONE 7(6):e37105. doi: 10.1371/journal.pone.0037105.

Bauer, A. M., J. F. Parham, R. M. Brown, B. L. Stuart, L. Grismer, and T. J. Papenfuss. 2011. Availability of new Bayesian-delimited gecko names and the importance of character-based species descriptions. Proc. Biol. Sci. 278:490–492.

Beamer, D. A., and T. Lamb. 2008. Dusky salamanders (*Desmognathus*, Plethodontidae) from the Coastal Plain: multiple independent lineages and their bearing on the molecular phylogeny of the genus. Mol. Phylogenet. Evol. 47:143–153.

Bernardo, J. 2011. A critical appraisal of the meaning and diagnosability of cryptic evolutionary diversity, and its implications for conservation in the face of climate change. Pp. 380–438 *in* T. R. Hodkinson, M. , B. Jones, S. Waldren, J. A. N. Parnell, eds. Climate change, ecology, and systematics. Cambridge Univ. Press, Cambridge, U. K. and New York, NY.

Bernardo, J., R. Ossola, J. Spotila, and K. A. Crandall. 2007. Validation of interspecific physiological variation as a tool for assessing global warming-induced endangerment. Biol. Lett. 3:695–698.

Bonett, R. M. 2002. Analysis of the contact zone between the dusky salamanders *Desmognathus fuscus fuscus* and *Desmognathus fuscus conanti* (Caudata: Plethodontidae). Copeia 2002:344–355.

Camp, C. D., S. G. Tilley, R. M. Jr Austin, and J. L. Marshall. 2002. A new species of black-bellied salamander (Genus *Desmognathus*) from the Appalachian mountains of northern Georgia. Herpetologica 58:471–484.

Camp, C. D., W. E. Peterman, J. R. Milanovich, T. Lamb, J. C. Maerz, and D. B. Wake. 2009. A new genus and species of lungless salamander (family Plethodontidae) from the Appalachian highlands of the south-eastern United States. J. Zool. (Lond.) 279:86–94.

Chaitra, M. S., K. Vasudevan, and K. Shanker. 2004. The biodiversity bandwagon: the splitters have it. Curr. Sci. India 86:897–899.

Chan, K. M. A., and S. A. Levin. 2005. Leaky prezygotic isolation and porous genomes: rapid introgression of maternally inherited DNA. Evolution 59:720–729.

Chatfield, M. W. H., K. H. Kozak, B. M. Fitzpatrick, and P. K. Tucker. 2010. Patterns of differential introgression in a salamander hybrid zone: inferences from genetic data and ecological niche modeling. Mol. Ecol. 19:4265–4282.

Chippindale, P. T., R. M. Bonett, A. S. Baldwin, and J. J. Wiens. 2004. Phylogenetic evidence for a major reversal of life-history evolution in plethodontid salamanders. Evolution 58:2809–2822.

Conant, R., and J. T. Collins. 1991. A field guide to reptiles and amphibians. Eastern and central North America. Houghton Mifflin, Boston, MA.

Crespi, E. J., L. J. Rissler, and R. A. Browne. 2003. Testing Pleistocene refugia theory: phylogeographical analysis of *Desmognathus wrighti*, a high-elevation salamander in the southern Appalachians. Mol. Ecol. 12:969–984.

Crespi, E. J., R. A. Browne, and L. J. Rissler. 2010. Taxonomic revision of *Desmognathus wrighti* (Caudata: Plethodontidae). Herpetologica 66:283–295.

Darwin, C. R. 1859. On the origin of species by means of natural selection, or the preservation of favoured races in the struggle for life. 1st ed., 1st issue, John Murray, London.

Dodd, C. K. Jr. 2004. The amphibians of Great Smoky Mountains national park. University of Tennessee Press, Knoxville, TN.

Dunn, E. R. 1926. The salamanders of the family plethodontidae. Smith College, Northampton, MA.

Earl, D. A., and B. M. vonHoldt. 2012. STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. Conserv. Genet. Res. 4:359–361.

Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32:1792–1797.

Evanno, G., S. Regnaut, and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol. Ecol. 14:2611–2620.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Fisher-Reid, M. C., and J. J. Wiens. 2011. What are the consequences of combining nuclear and mitochondrial data for phylogenetic analysis? Lessons from *Plethodon* salamanders and 13 other vertebrate clades. BMC Evol. Biol. 11:300.

Forester, D. C. 1977. Comments on the female reproductive cycle and philopatry by *Desmognathus ochrophaeus* (Amphibia, Urodela, Plethodontidae). J. Herpetol. 11:311–316.

Frost, D. R. 2011. Amphibian Species of the World: an Online Reference. Version 5.5 (31 January 2011). Available at http://research.amnh.org/vz/herpetology/amphibia/American Museum of Natural History, NY (accessed 31 May 2013).

Galtier, N., M. Gouy, and C. Gautier. 1996. SEAVIEW and PHYLO_WIN: two graphic tools for sequence alignment and molecular phylogeny. Comput. Appl. Biosci. 12:543–548.

Gouy, M., S. Guindon, and O. Gascuel. 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Biol. Evol. 27:221–224.

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Hall, M. S., and L. A. Katz. 2011. On the nature of species: insights from *Paramecium* and other ciliates. Genetica 139:677–684.

Highton, R. 1989. Biochemical evolution in the slimy salamanders of the *Plethodon glutinosus* complex in the eastern United States. I Geographic protein variation. Ill. Biol. Monogr. 57:1–78.

Highton, R. 1990. Taxonomic treatment of genetically differentiated populations. Herpetologica 46:114–121.

Highton, R. 1998. Is *Ensatina* a ring-species? Herpetologica 54:254–278.

Highton, R. 2000. Detecting cryptic species using allozyme data. Pp. 215–241 *in* R. C. Bruce, R. G. Jaeger and L. D. Houck, eds. The biology of plethodontid salamanders. Kluwer Academic/Pleneum Publishers, New York, NY.

Highton, R., and R. B. Peabody. 2000. Geographic protein variation and speciation in salamanders of the *Plethodon jordani* and *Plethodon glutinosus* complexes in the southern Appalachian Mountains with the description of four new species. Pp. 31–94 *in* R. C. Bruce, R. G. Jaeger and L. D. Houck, eds. The biology of plethodontid salamanders. Kluwer Academic/Pleneum Publishers, New York, NY.

Irwin, D. E. 2002. Phylogeographic breaks without geographic barriers to gene flow. Evolution 56:2382–2394.

Isaac, N. J. B., J. Mallet, and G. M. Mace. 2004. Taxonomic inflation: its influence on macroecology and conservation. Trends Ecol. Evol. 19:464–469.

Karlin, A. A., and S. I. Guttman. 1986. Systematics and geographic isozyme variation in the plethodontid salamander *Desmognathus fuscus* (Rafinesque). Herpetologica 42:283–301.

Kozak, K. H., A. Larson, R. M. Bonett, and L. J. Harmon. 2005. Phylogenetic analysis of ecomorphological divergence, community structure, and diversification rates in dusky salamanders (Plethodontidae: *Desmognathus*). Evolution 59:2000–2016.

Kozak, K. H., R. W. Mendyk, and J. J. Wiens. 2009. Can parallel diversification occur in sympatry? Repeated patterns of body-size evolution in coexisting clades of North American salamanders. Evolution 63:1769–1784.

Kruskal, J. P., and M. Wish. 1978. Multidimensional Scaling. Sage Publications, Newbury Park, CA.

Lessa, E. 1990. Multidimensional analysis of geographic genetic structure. Syst. Zool. 39:242–252.

Linnen, C. R., and B. D. Farrell. 2007. Mitonuclear discordance is caused by rampant mitochondrial introgression in *Neodiprion* (Hymenoptera: Diprionidae) sawflies. Evolution 61:1417–1438.

Mead, L. S., and S. G. Tilley. 2000. Ethological isolation and variation in allozymes and dorsolateral pattern between parapatric forms in the *Desmognathus ochrophaeus* complex. Pp. 181–198 *in* R. C. Bruce, R. G. Jaeger and L. D. Houck, eds. The Biology of Plethodontid Salamanders. Kluwer Academic/Pleneum Publishers, New York, NY.

Mead, L. S., S. G. Tilley, and L. A. Katz. 2001. Genetic structure of the Blue Ridge Dusky Salamander (*Desmognathus orestes*): inferences from allozymes, mitochondrial DNA, and behavior. Evolution 55:2287–2302.

Moritz, C., C. J. Schneider, and D. B. Wake. 1992. Evolutionary relationships within the *Ensatina eschscholtzii* complex confirm the ring species interpretation. Syst. Biol. 41:273–291.

Murphy, R. W., J. W. Jr Sites, D. G. Buth, and C. H. Haufler. 1996. Proteins: isozyme electrophoresisis. Pp. 51–120 *in* D. M. Hillis, C. Moritz and B. K. Mable, eds. Molecular systematics. Sinauer Associates, Sunderland, MA.

Nei, J. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583–590.

Peakall, R., and P. E. Smouse. 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes 6:288–295.

Petit, R. J., and L. Excoffier. 2009. Gene flow and species delimitation. Trends Ecol. Evol. 24:386–393.

Petranka, J. W. 1998. Salamanders of the United States and Canada. Smithsonian Institution Press, Washington, DC.

Pfenniger, M., and K. Schwenk. 2007. Cryptic animal species are homogeneously distributed among taxa and biogeographical regions. BMC Evol. Biol. 7(121):6.

Pritchard, J. K., M. Stephens, and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. Genetics 155:945–959.

Pritchard, J. K., X. Wen, and D. Falushb. 2010. Documentation for structure software: Version 2.3. Available at http://pritch.bsd.uchicago.edu/software/structure2_1.html (accessed 5 March 2013).

Rambaut, A., and A. J. Drummond. 2009. Tracer: MCMC trace analysis tool. 1.4.1 ed. Institute of Evolutionary Biology, Edinburgh. Available at http://tree.bio.ed.ac.uk/software/tracer/ (accessed 30 June 2011).

Richmond, J. Q., and E. L. Jockusch. 2007. Body size evolution simultaneously creates and collapses species boundaries in a clade of scincid lizards. Proc. Biol. Sci. 274:1701–1708.

Ronquist, F., and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574.

Rossman, D. A. 1958. A new race of *Desmognathus fuscus* from the south-central United States. Herpetologica 14:158–160.

Rubinoff, D., and B. S. Holland. 2005. Between two extremes: mitochondrial DNA is neither the panacea nor the nemesis of phylogenetic and taxonomic inference. Syst. Biol. 54:952–961.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory, New York.

 © 2013 The Authors. *Ecology and Evolution* published by John Wiley & Sons Ltd.

Schmidt, K. P. 1953. A check list of North American amphibians and reptiles, 6th ed.. University of Chicago Press, Chicago, IL.

Shaw, K. L. 2002. Conflict between nuclear and mitochondrial DNA phylogenies of a recent species radiation: what mtDNA reveals and conceals about modes of speciation in Hawaiian crickets. Proc. Natl Acad. Sci. USA 99:16122–16127.

Sites, J. W. Jr, and J. C. Marshall. 2003. Species delimitation: a Renaissance issue in systematic biology. Trends Ecol. Evol. 18:462–470.

Sites, J. W. Jr, and J. C. Marshall. 2004. Operational criteria for delimiting species. Annu. Rev. Ecol. Evol. Syst. 35:199–229.

Stamatakis, A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690.

Stamatakis, A., P. Hoover, and J. Rougemont. 2008. A rapid bootstrap algorithm for the RAxML web-servers. Syst. Biol. 57:758–771.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28:2731–2739.

Templeton, A. R. 1989. The meaning of species and speciation: a genetic perspective. Pp. 3–27 *in* D. Otte and J. A. Endler, eds. Speciation and its consequences. Sinauer Associates, Inc., Sunderland, MA.

Tilley, S. G. 1981. A new species of *Desmognathus* (Amphibia: Caudata: Plethodontidae) from the southern Appalachian Mountains. Occas. Pap. Mus. Zool. Univ. Mich. 695:1–23.

Tilley, S. G. 1988. Hybridization between two species of *Desmognathus* Amphibia: Caudata: Plethodontidae) in the Great Smoky Mountains. Herpetol. Monogr. 2:27–39.

Tilley, S. G. 1997. Patterns of genetic differentiation in Appalachian desmognathine salamanders. J. Hered. 88:305–315.

Tilley, S. G. 2000a. *Desmognathus santeetlah* Tilley. Santeetlah Dusky Salamander. Cat. Am. Amphib. Reptil. 703-1–703-3.

Tilley, S. G. 2000b. Systematics of *Desmognathus imitator*. Pp. 121–147 *in* R. C. Bruce, R. G. Jaeger and L. D. Houck, eds. The biology of plethodontid salamanders. Kluwer Academic/ Pleneum Publishers, New York, NY.

Tilley, S. G., and J. E. Huheey. 2001. Reptiles and amphibians of the smokies. Great Smoky Mountains Natural History Association, Gatlinburg, TN.

Tilley, S. G., and M. J. Mahoney. 1996. Patterns of genetic differentiation in salamanders of the *Desmognathus ochrophaeus* complex (Amphibia: Plethodontidae). Herpetol. Monogr. 10:1–42.

Tilley, S. G., R. B. Merritt, B. Wu, and R. Highton. 1978. Genetic differentiation in salamanders of the *Desmognathus ochrophaeus* complex (Plethodontidae). Evolution 32:93–115.

Tilley, S. G., R. L. Eriksen, and L. A. Katz. 2008. Systematics of dusky salamanders, *Desmognathus* (Caudata: Plethodontidae), in the mountain and Piedmont regions of Virginia and North Carolina, USA. Zool. J. Linn. Soc. 152:115–130.

Tilley, S. G., R. Highton, and D. B. Wake. 2011. Caudata – Salamanders *in* B. I. Crother, ed. Scientific and Standard English Names of Amphibians and Reptiles of North America North of Mexico, With Comments Regarding Confidence In Our Understanding. Edition 6.1. Available at http://www.ssarherps.org/pages/comm_names/Caudata.php (accessed 31 May 2013).

Titus, T. A., and A. Larson. 1996. Molecular phylogenetics of desmognathine salamanders (Caudata: Plethodontidae): a reevaluation of evolution in ecology, life history, and morphology. Syst. Biol. 45:451–472.

Trontelj, P., and C. Fišer. 2009. Cryptic species diversity should not be trivialised. Syst. Biodivers. 7:1–3.

Trontelj, P., C. J. Douady, C. Fišer, J. Gibert, Š. Gorički, T. LeFébure, et al. 2007. A molecular test for cryptic diversity in ground water: how large are the ranges of macro-stygobionts? Freshw. Biol. 54:727–744.

Wake, D. B. 2009. What salamanders have taught us about evolution. Annu. Rev. Ecol. Evol. Syst. 40:33–352.

Wake, D. B., and C. J. Schneider. 1998. Taxonomy of the plethodontid salamander genus *Ensatina*. Herpetologica 54:279–298.

Weisrock, D. W., and A. Larson. 2005. Testing hypotheses of speciation in the *Plethodon jordani* species complex with allozymes and mitochondrial DNA sequences. Biol. J. Linn. Soc. 89:25–51.

Wiens, J. J., C. A. Kuczynski, and P. R. Stephens. 2010. Discordant mitochondrial and nuclear gene phylogenies in emydid turtles: implications for speciation and conservation. Biol. J. Linn. Soc. 99:445–461.

Wilder, I. W., and E. R. Dunn. 1920. The correlation of lunglessness in salamanders with a mountain brook habitat. Copeia 84:63–68.

Wright, S. 1943. Isolation by distance. Genetics 28:114–138.

Zink, R. M., and G. F. Barrowclough. 2008. Mitochondrial DNA under siege in avian phylogeography. Mol. Ecol. 17:2107–2121.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Sample locailties.

Table S2. GenBank accession numbers and numbers of base differences per site between sequences over 55 nucleotide sequences. All ambiguous positions were removed for each sequence pair.

Table S3. Allozyme genotypes in GenAlEx format. Zeros indicate missing data. EC numbers follow Murpy et al., 1996.

Table S4. Nei unbiased genetic distances based on 22 presumptive allozyme loci.

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