Zoological Journal of the Linnean Society, 2008, **152**, 115–130. With 8 figures

Systematics of dusky salamanders, *Desmognathus* (Caudata: Plethodontidae), in the mountain and Piedmont regions of Virginia and North Carolina, USA

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Received 22 December 2006; accepted for publication 14 May 2007

We analysed mitochondrial (cytochrome *b*) nucleotide sequences, nuclear allozyme markers, and morphometric characters to investigate species boundaries and phylogenetic relationships among dusky salamanders (*Desmognathus*) in the southern Blue Ridge and adjacent Piedmont Physiographic Provinces of Virginia and North Carolina. Our results revealed four distinct mitochondrial DNA clades that are also characterized by distinct allozyme markers. One clade consists of sequences derived from populations distributed from New England to south-western Virginia that are referable to *Desmognathus fuscus* Rafinesque, 1820, although there is considerable sequence and allozyme divergence within this clade. A second clade consists of sequences derived from populations referable to *Desmognathus planiceps* Newman, 1955, a form that we resurrect from its long synonymy under *D. fuscus*. *Desmognathus planiceps* and *D. fuscus* also differ in mandibular tooth shape. Two other cytochrome *b* sequences recovered from populations along the Blue Ridge escarpment in southern Virginia are quite divergent from those of the previous two clades, and these populations may represent yet another distinct species. Sequences from a population in the Brushy Mountains in the Piedmont of northern North Carolina are similar to those of *Desmognathus carolinensis*. Population groupings indicated by allozyme data generally correspond to the cytochrome *b* clades. Cryptic diversity in Appalachian desmognathan salamanders clearly requires further study.

ADDITIONAL KEYWORDS: allozymes – cytochrome *b* – *Desmognathus planiceps* – mitochondrial DNA – morphometrics – phylogeography – taxonomy.

INTRODUCTION

Species boundaries and evolutionary relationships in plethodontid salamanders have long been obscured by morphological conservatism and homoplasy (i.e. Wake, 1966; Chippindale *et al*., 2004; Mueller *et al*., 2004). Recent application of molecular systematic and phylogeographical techniques have revealed unexpected relationships and evolutionary patterns (Chippindale *et al*., 2004; Mueller *et al*., 2004), and forms that have traditionally been treated as widely distributed species have been subdivided into assemblages of parapatric units that qualify as species under evolutionary or phylogenetic species concepts (Highton, 1989, 2000; Frost & Hillis, 1990; Tilley & Mahoney, 1996; Highton & Peabody, 2000; Anderson & Tilley, 2003).

One taxon of putatively wide distribution is *Desmognathus fuscus*, which Petranka (1998) recognized as a single species with three subspecies, *D. f. fuscus*, *D. f. conanti*, and *D. f. santeetlah*. Tilley (2000) argued for recognizing both *Desmognathus santeetlah* and *Desmognathus conanti* as distinct species, and there is now considerable molecular evidence that these two forms are sister taxa distinct from *D. fuscus* (Titus & Larson, 1996; Kozak *et al*., 2005). Studies by Bonett (2002) and Kozak *et al*. (2005) have revealed additional differentiation among populations of *D. fuscus*, exclusive of *D. conanti* and *D. santeetlah*. In the most recent and extensive analysis (Kozak *et al*., 2005), mitochondrial DNA (mtDNA) sequences from populations putatively assigned to *D. fuscus* fell into three

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distinct clades: one in the Cumberland Plateau of Tennessee ('*fuscus* A'), one that is distributed from North Carolina to Pennsylvania and Massachusetts ('*fuscus* B'), and one that includes both *Desmognathus carolinensis* and putative *D. fuscus* from the Piedmont of the Carolinas and Virginia.

The discovery of substantial evolutionary divergence within *D. fuscus* has led us to investigate the taxonomic status of *Desmognathus planiceps* Newman (1955), which was reduced to synonymy under *D. fuscus* by Martof & Rose (1962). Newman's (1955) description of *D. planiceps* was based on collections from two localities on the south-eastern escarpment of the Blue Ridge in Virginia. He described *D. planiceps* as 'A large, heavy-bodied *Desmognathus* with [a] broad, flattened head; spatulate and strongly depressed snout; enlarged and recurved premaxillary teeth in adult males; conspicuous, and normally straight-edged, dorsal band sharply margined with blackish; chest and anterior two-thirds of belly immaculate; chin, throat, and posterior third of belly lightly spotted with brownish-tan.' The holotype and five topotypes were collected in '. . . a portion of the stream (approximate elevation 2800 feet) dropping down into the gorge below the Dan River Dam near Meadows of Dan, Patrick County, Va.' Thirteen paratypes '. . . were collected in a mountain stream [the headwaters of Rock Castle Cr.] along Route 8, 5.5 miles north-west of Woolwine, Patrick County, Va.' In Bonett's (2002) study, these localities lie within a putative hybrid zone between his Groups A and C of *D. fuscus*. In the Kozak *et al*. (2005) study, they lie within the general ranges of two forms that they identify as '*fuscus*': their '*fuscus* A' and a form in which mtDNA sequences cluster with *D. carolinensis*.

Martof & Rose (1962) examined the paratypes of *D. planiceps* (USNM 143560-75) and a series of specimens from about a mile south-west of the Rock Castle Creek paratype locality. They concluded that the morphological and colour pattern characteristics cited by Newman (1955) did not distinguish *D. planiceps* from *D. fuscus*, and the former name has subsequently been treated as a junior synonym of the latter (Frost, 2006). Martof & Rose did not examine the holotype (USNM 143559), which may have still been in Newman's personal collection, and although they reported 'inspecting' the type locality, they did not collect specimens there. They identified one specimen in the collection of paratypes from the Rock Castle Creek locality (USNM 143576) as a *Desmognathus ochrophaeus* (examined by SGT and here referred to *Desmognathus orestes* on the basis of its geographical origin), and the remaining specimens and the type specimen (based upon Newman's illustration) as *D. fuscus*. They reported collecting only *D. fuscus*, *Desmognathus monticola*, and *Desmognathus quadramaculatus* at the Rock Castle Creek paratype locality.

In June 1990, Richard L. Hoffman, who helped Walter and Jaine P. Newman collect the type and paratypes of *D. planiceps*, guided SGT to a locality along Shooting Creek, Floyd County, VA (Population 12, this study) where he believed salamanders of the form described by Newman occurred. Allozyme analyses (unpublished) of these specimens revealed that they were indeed distinct from *D. fuscus* in the vicinity of Mt Rogers in south-western Virginia, and elsewhere in the range of that species. We now report the results of allozyme, mtDNA sequence, and morphological comparisons that document the distinctness of *D. planiceps*, and clarify its geographical distribution and phylogenetic relationships. Our analyses also reveal additional, unsuspected diversity among desmognathan salamanders in this region.

MATERIAL AND METHODS

Sampling localities

We collected a series of specimens of *D. fuscus* (according to current taxonomy) from 20 localities in Massachusetts, south-western Virginia, and north-eastern North Carolina (Fig. 1; Appendix S1). These included the type locality of *D. planiceps* (Population 15), Rock Castle Creek, near the paratype locality (Population 13), other streams draining the Blue Ridge escarpment (Populations 8, 11, 12, 14, 16, 18, and 19), the interior Blue Ridge Physiographic Province (Populations 5–7, 10, and 17), the Virginia Piedmont (Population 9), the North Carolina Piedmont (Population 20), and the Ridge and Valley Physiographic Province (Populations 2 and 4). The exact location of the type locality (Population 15) is not clearly explained in the type description, but we were guided to it by Richard Hoffman, 54 years after he assisted with the collection of the type series in 1951. The locality is located at 821 m above sea level in a tributary of Haunted Branch below County Road 602, 1.07-km west of Townes Dam on the Dan River, Patrick County, VA, 36°41.127′N, 80°26.523′W.

Specimens were anaesthetized with a 0.1% solution of tricaine methansulphonate buffered to pH 7.0 with sodium bicarbonate, digitally photographed, measured (see below), and killed with 2-phenoxyethanol (8–10 drops per 500 mL). Allozyme analyses were performed on samples of stomach, liver, and ventral musculature frozen in distilled water at –70 °C. Tail tips were also frozen at –70 °C for DNA extraction. The holotype and paratypes of *D. planiceps* in the National Museum of Natural History were examined and digitally photographed. Specimens collected during this study will be deposited in the collection

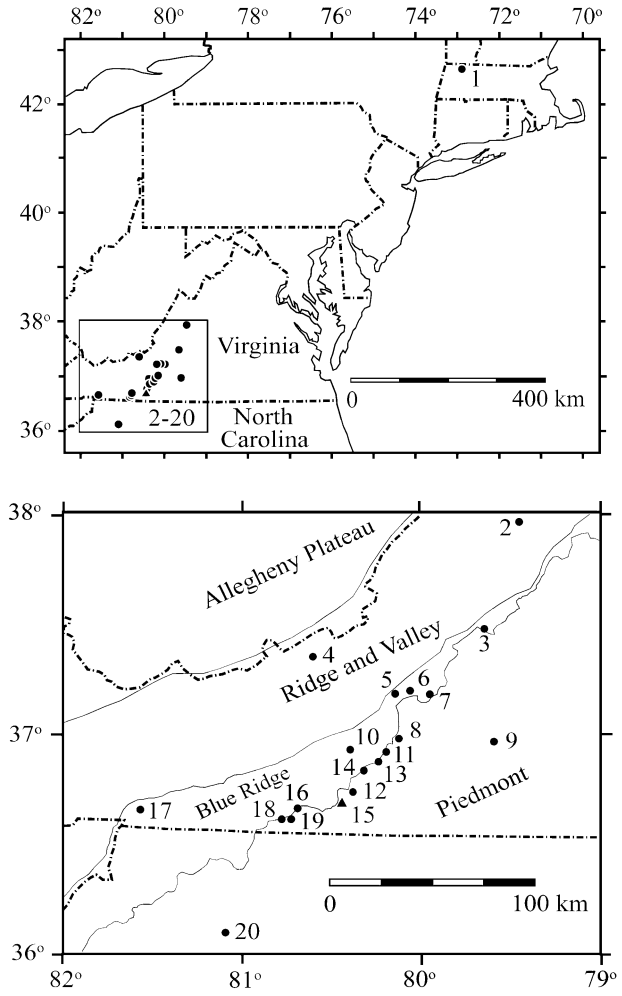

Figure 1. Sample localities. The triangle (Population 15) indicates the type locality of *Desmognathus planiceps*. Solid lines in the lower map indicate approximate boundaries of physiographic provinces.

at the Museum of Comparative Zoology, Harvard University.

Mitochondrial DNA sequencing

Sequencing was performed on samples from all the study populations except Populations 14 and 17. Genomic DNA was extracted from tail tips, which had been stored at –70 °C, using liquid nitrogen to grind the tissue and a standard phenol extraction protocol (Sambrook, Fritsch & Maniatis, 1989). A 387-bp fragment of the cytochrome *b* (cyt B) region was amplified using primers and protocols described by Mead, Tilley & Katz (2001). Both strands of each PCR product were sequenced using BigDye terminator follwing the manufacturer's protocol (PE Applied BioSystems, Foster City, CA, USA). Sequencing products were purified using Performa gel filtration cartridges, and were run on an ABI 3100 automated sequencer at Smith College.

To assess variation accurately, we assembled pairs of sequencing reads, resolved ambiguities, generated a multisequence alignment, and rechecked resulting polymorphisms by eye. Complementary sequencing reads for each individual sampled were assembled and edited in SeqMan (DNA Star Inc., Madison, WI, USA) to generate a contiguous 387-bp fragment of the cyt B gene. The resulting 96 edited sequences were aligned using CLUSTALW (Thompson, Higgins & Gibson, 1994) implemented in MegAlign (DNA Star), and chromatograms for all variable sites were confirmed by eye. These 96 sequences, representing 23 haplotypes, were then aligned with sequences from GenBank from *D. fuscus*, *D. ochrophaeus*, *D. orestes*, *Desmognathus ocoee*, *D. carolinensis*, *Desmognathus imitator*, and *Desmognathus wrighti* (Appendix S2) for phylogenetic analysis.

Phylogenetic analyses were performed on a single representative sequence of each haplotype using both maximum-parsimony (MP) and maximum-likelihood (ML) analyses, as implemented in PAUP* version 4.0b10 (Swofford, 2002). ML analyses relied on a model and parameters estimated from the data using a hierarchical likelihood ratio test and models from MODELTEST v. 3.7 (Posada & Crandall, 1998). Heuristic searches were performed using ten random addition sequence replicates, and bootstrap values were estimated from 1000 pseudoreplicates for both MP and ML analyses. Average pairwise uncorrected distances among haplotypes were calculated in PAUP* version 4.0b10 (Swofford, 2002).

Allozyme analyses

Allozyme analyses were conducted using the loci and procedures of Tilley & Mahoney (1996). A survey of the 22 allozyme systems employed by Tilley & Mahoney (1996) revealed six that exhibited significant variation among populations: aspartate aminotransferase-1 and -2 (*AAT-1*, *-2*, EC 2.6.1.1), glyceraldehyde-3-phosphate dehydrogenase (*G3PDH*, EC 1.2.1.12), isocitrate dehydrogenase-2 (*IDH-2*, EC 1.1.1.42), lactate dehydrogenase-2 (*LDH-2*, EC 1.1.1.27), and phosphogluconate dehydrogenase (*PGDH*, EC 1.1.1.44). Variant designations tentatively correspond to those of Tilley & Mahoney (1996). These correspondences are based on relative mobilities, using the single variant at each locus in Massachusetts *D. fuscus* as standards; because alleles from Tilley & Mahoney (1996) were not rerun, these inferred similarities should be treated cautiously. Allele frequencies were calculated with the web-based version of GENEPOP 3.4 (Raymond & Rousset, 1995).

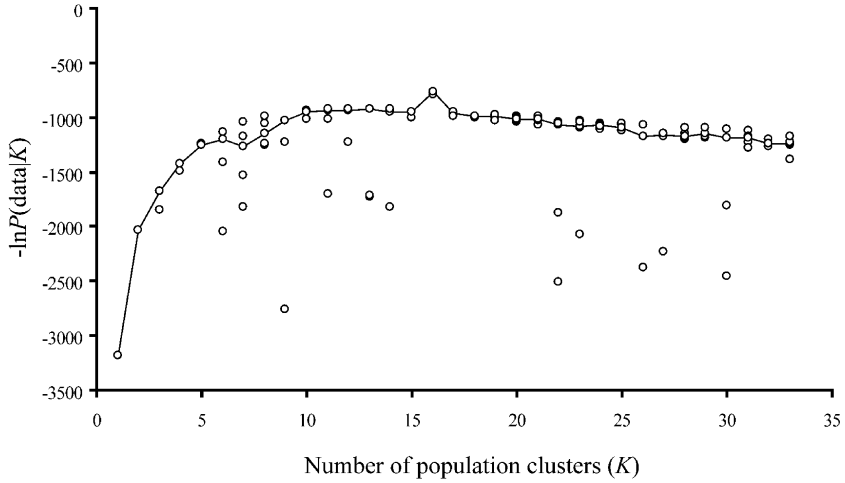

Figure 2. Estimated natural logarithm of the probability of obtaining the genotypic data across 19 variable loci plotted against the number of designated population clusters. Lines connect median values of $-\ln P(\text{data}\,|\,K)$.

Nei unbiased genetic distances were calculated with GENALEX 6 (Peakall & Smouse, 2006) for populations with data for all 22 loci.

STRUCTURE software (version 2; Pritchard, Stephens, & Donelly, 2000) was employed to discern population clustering patterns revealed by the allozyme data, and to compare them with patterns suggested by the sequence data. This program uses a Markov chain Monte Carlo procedure to determine the proportions of individuals in each of the actual populations that can be assigned to each of K population clusters. K can be varied over multiple runs of the program to find the value that maximizes the quantity $P(\text{data}\,|\,K)$, the probability of obtaining the actual genotypic data set given K population clusters. Using genotypic data for the 19 loci at which at least two variants were detected, we conducted five runs each using K-values of 1–30 with Markov chain burn-in lengths of 10^5 steps, and 10^5 subsequent steps to determine the K-value that maximized $P(\text{data}\,|\,K)$ (Fig. 2). $P(\text{data}\,|\,K)$ appears to attain a maximum at $K = 10$ clusters and then slowly declines (we interpret the spike at $K = 16$ to be an artifact). In such situations, Pritchard & Wen (2004) suggest that when $P(\text{data}\,|\,K)$ remains relatively constant across a range of K-values, choosing '. . . the smallest of these is often "correct".' We thus determined the proportions of individuals in each population that could be assigned to each of ten clusters, across ten replicates using burn-ins of 10^5 steps, and 10^6 subsequent steps.

Morphometric analyses

Shortly after being anaesthetized and prior to preservation, each specimen was measured for the 26 linear dimensions employed by Tilley (1981) and Camp *et al*. (2002), plus the height of the snout just anterior to the orbits. A digital ocular micrometer was employed to record dimensions smaller than *c*. 5 mm; digital calipers were used to measure larger dimensions. Only specimens with complete sets of measurements were employed in subsequent analyses. Sample sizes are shown in Appendix S3.

Each measurement was regressed against the standard length (tip of snout to posterior margin of the vent) for adult specimens. Standardized ('studentized') residuals from least-squares quadratic regressions were then calculated, and entered into factor analysis (with varimax rotation) and principal components analysis (MINITAB, release 14). Both types of analysis employed correlation matrices. Mann–Whitney tests were employed to compare the scores on the first principal component of Clades A and B.

Tooth morphology

Tooth morphology was examined in one mature female *D. fuscus* (Population 2), seven mature male *D. fuscus* (one from Population 1 and two each from Populations 2, 3, and 5), four female *D. planiceps* (one from Population 9 and three from Population 11), four mature male *D. planiceps* (one each from Populations 7, 9, 12, and 15, the type locality), and one mature male desmognathan from Population 18 (Clade C below). Lower jaws were dissected out with scissors, and the tongue tissue and hyoid apparatus were removed from the dentary bones with scissors and forceps. Jaws were rinsed five times with water to remove the ethanol in which the specimens had been stored, and were then immersed in 3% hydrogen peroxide for 30 min. The hydrogen peroxide was then removed and the jaws were rinsed five times with water, and immersed in

10% household bleach solution for 30 min or until the tissue became a dark yellow colour but had not disappeared. The jaws were occasionally removed and inspected by eye during this process to ensure that the teeth were still in place. If it appeared that the teeth were no longer in place, the jaw was removed from the bleach solution. When the tissue surrounding the teeth was dark yellow, the bleach solution was removed and the jaws were rinsed five times with water. They were next immersed in acetone for drying. When the acetone in the Petri dish was evaporated the drying cycle was repeated. The jaws were then mounted on a scanning electron microscope stub with mounting tape, and sputter coated. Gaps between the bone and the stub were carefully filled with a generous quantity of carbon paint. This paint was left to dry for a minimum of 2 h. It was then sputter coated and viewed on a JEOL 6400 Scanning Electron Microscope.

Tooth heights and widths were measured for the five posteriormost teeth that were still present on the posterior half of each dentary in adults representing Clades A, B, and C. Measurements were taken from digitized scanning electron microscope images using IMAGE J software (Abramoff, Magelhaes & Ram, 2004). Height was measured from the tip to the horizontal groove that marks the level of soft tissue. Width was measured at the widest point.

RESULTS

Mitochondrial DNA sequence variation

Phylogenetic analysis of the 23 haplotypes (found among 96 individuals) and other sequences available on GenBank generated similar tree topologies under both MP and ML analyses (Fig. 3). The analyses reveal a distinct, highly supported clade (clade A, with MP and ML bootstrap values $\geq 95\%$) that includes a haplotype (A7, Population 15) from the type locality of *D. planiceps*, and closely related sequences (A1–A6, A8–A11) from Populations 6–13. We will argue (in the Discussion) that populations of Clade A are taxonomically referable to *D. planiceps* Newman, 1955. A second clade (Clade B), which includes haplotypes from Massachusetts *D. fuscus* (Population 1) and Populations 2–5 (Fig. 1; Appendix S1), is moderately supported in the ML but not in the MP analysis. This clade includes a haplotype (B1) that is identical to a published sequence from Massachusetts *D. fuscus* (GenBank sequence AY728227, Mueller *et al*., 2004). Average genetic divergences among haplotypes within Clades A and B are 1.01% and 0.86%, respectively, whereas the average divergence between these clades is 9.19% (Table 1). We will argue (in the Discussion) that populations of Clade B are referable to *D. fuscus* Rafinesque, 1820.

Haplotype C1 from Populations 18 and 19 clusters with Clade B, but only with moderate bootstrap support (64% ML, 88% MP). This sequence is 7.68% divergent from sequences within clade B. Two additional haplotypes (D1 and D2, Table 1) from Population 20 in the Brushy Mountains, an isolated range in the North Carolina Piedmont, nest within a moderately supported clade (84% ML, 71% MP) that also includes *D. carolinensis* sequences. These sequences differ by an average of only 4.2% from the *D. carolinensis* sequences, but by 12.0% and 9.8% from Clade A and B sequences, respectively (Table 1).

We also analysed cyt B sequences from *D. monticola* and *D. quadramaculatus* (Table 1). We found no sequence variation among *D. monticola* individuals sampled over a broad geographical area (localities of Populations 4, 5, 7, 12 and 13, Fig. 1). Levels of bootstrap support for the deep nodes in the phylogram are too weak to clarify relationships among *D. quadramaculatus*, *D. monticola*, and other *Desmognathus*.

Allozyme variation

Table 2 shows the results for one of the STRUCTURE runs specifying that allozyme genotypes be assigned to ten population clusters [the *K*-value associated with high values of $-\ln P(\text{data}\,|\,K)$]. This run produced the lowest $-\ln P(\text{data}\,|\,K)$ value (–943.1) of ten runs using $K = 10$. Table 2 shows the proportions of each population that were allocated to each of the ten clusters, with proportions exceeding 10% emphasized in bold italics. These results illustrate three patterns that were evident in all the runs, and generally support the haplotype clades suggested by the sequence data (Fig. 4; Appendix S4).

1. Most specimens (88–99%) from each *D. fuscus* population were apportioned among two or three clusters, whereas most specimens (86–99%) from each *D. planiceps* population were apportioned among a different array of five or six clusters.
2. In nine of the ten runs, at least 91% of the individuals in each of the Clade C populations (Populations 18 and 19) were assigned to the same cluster. In six of those nine runs essentially all the individuals in that cluster were from Clade C. In three runs it also contained a substantial contribution from Population 17. One run assigned Clade C populations to two clusters, one of which also contained substantial contributions from Populations 5 (Clade B) and 17.
3. In all ten STRUCTURE runs virtually all (> 98%) of the Clade D (Population 20) individuals were assigned to a cluster, to which the other populations contributed very small (< 6%) percentages.

Allocation of specimens from Population 17 (near Mt Rogers, VA), for which we lack sequence data,

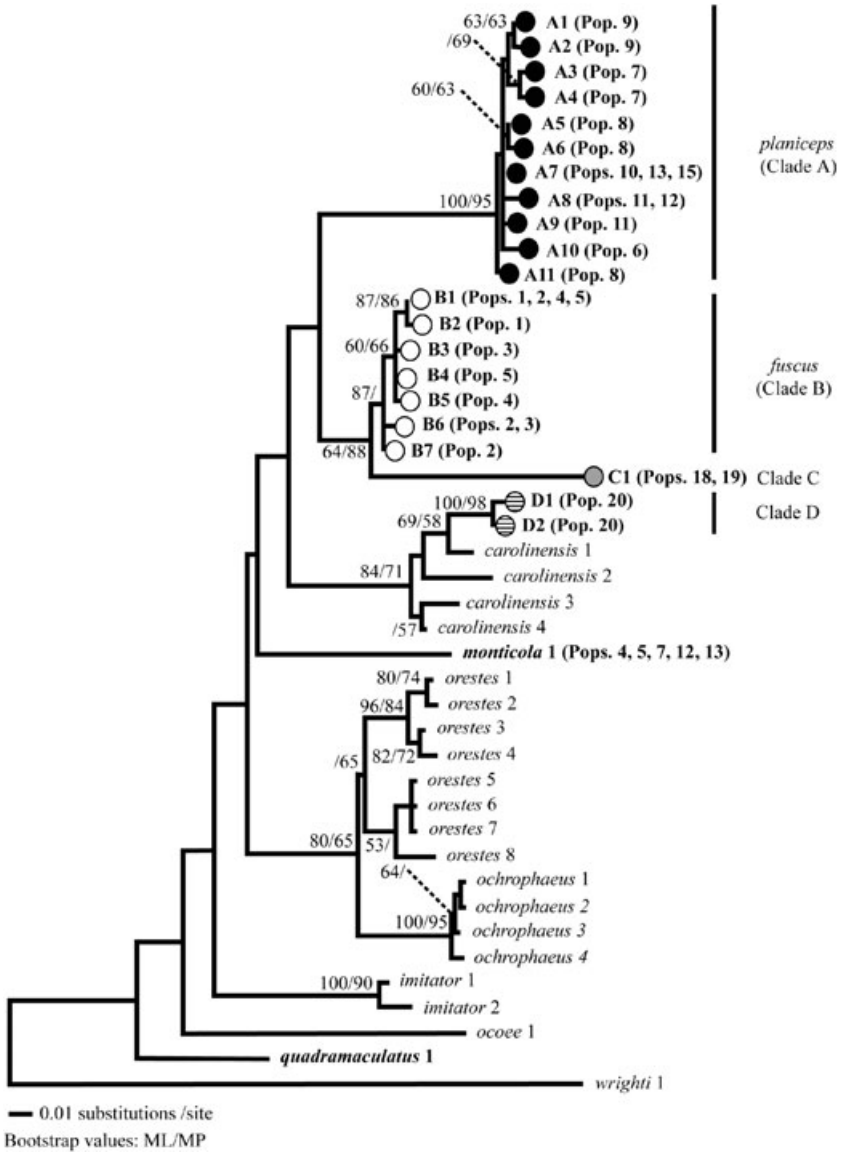

Figure 3. Phylogeny generated by maximum-likelihood analysis of cytochrome *b* sequences. Bootstrap percentages for ML/MP analyses are shown for nodes where either or both the values exceeded 50%. Boldface type indicates sequences generated in this study.

varied among the runs. They fell predominantly into a unique cluster (one run) or into clusters dominated by *D. fuscus* (five runs), Clade C (three runs) or both (one run).

Examination of geographical variation in allozyme frequencies further supports the haplotype clades. At five of the marker loci, populations of *D. planiceps* (Clade A) exhibit variants (*AAT-2*^{b}, *G3PDH*^{d}, *IDH-2*^{b}, *LDH-2*^{d}, and *PGDH*^{c}) that were not detected in *D. fuscus* (Clade B). At *AAT-1*, Population 2 of *D. fuscus* exhibits a variant that otherwise characterizes *D. planiceps*. Populations 18 and 19, which form the sister clade to Clade B (*D. fuscus*) on the cyt B phylogeny, exhibit the variant typical of *D. fuscus* at *IDH*-2, *G3PDH*, and *PGDH*, variants occurring in both *D. planiceps* and *D. fuscus* at *AAT-1* and *AAT*-2, and unique variants at *AAT-1* and *LDH-2*. The population (20) in the Brushy Mountains of North Carolina exhibits the variant typifying *D. planiceps* at *IDH-2*, the variant typifying *D. fuscus* at *AAT-2* and *G3PDH*, both variants at *AAT-1*, and unique variants at *LDH-2* and *PGDH*.

Table 3 shows unbiased Nei genetic distances among populations for which there were allozyme data for all 22 loci. Nei distances for comparisons between *D. fuscus* and *D. planiceps* populations

Table 1. Average uncorrected percentage divergences among unique haplotypes within and between clades. Divergence between *Desmognathus planiceps* and *Desmognathus fuscus* is indicated in bold italic type. Divergences between sympatric, reproductively isolated taxa are indicated by non-italicized bold type

	D. planiceps (Clade A)	*D. fuscus* (Clade B)	Clade C	*D. ochrophaeus*	*D. orestes*	*D. monticola*	Clade D	*D. carolinensis*	*D. ocoee*	*D. quadramaculatus*	*D. imitator*	*D. wrighti*
D. planiceps (Clade A)	1.01	***9.19***	11.25	12.59	**10.19**	**11.58**	11.96	10.94	13.32	**11.84**	12.92	16.38
D. fuscus (Clade B)		0.86	7.68	**10.74**	**8.13**	**10.19**	9.78	9.16	11.85	**10.93**	10.16	16.83
Clade C			–	12.92	**12.47**	**13.70**	14.21	12.73	13.70	**13.95**	14.04	18.35
D. ochrophaeus				0.95	6.22	**11.11**	13.57	12.73	12.14	**11.24**	12.10	16.28
D. orestes					3.41	**11.08**	11.31	10.87	12.34	**9.21**	11.46	16.51
D. monticola						–	**9.56**	**9.04**	**13.44**	**10.59**	**11.54**	**17.31**
Clade D							0.52	4.20	13.95	11.37	11.76	14.99
D. carolinensis								3.49	13.31	**10.53**	10.57	**14.28**
D. ocoee									–	**11.89**	**12.66**	**17.05**
D. quadramaculatus										–	**10.42**	**15.25**
D. imitator											**1.21**	**14.73**
D. wrighti												–

(mean = 0.535) are substantially greater than those for comparisons within *D. fuscus* (mean = 0.181) or *D. planiceps* (mean = 0.031). For comparisons within *D. fuscus*, the highest values (> 0.2) are for comparisons involving the Massachusetts population. Population 17 is most similar to *D. fuscus* populations, but is quite divergent from Populations 2 and 4.

The comparisons among Populations 5, 6, and 7 are of particular interest because Population 5 (*D. fuscus*) is separated from Populations 6 and 7 (*D. planiceps*) by only 7.4 and 16.4 km, respectively. The single specimen from Population 6 is homozygous for variants typifying *D. planiceps* at all but one of the six loci that distinguish the two species. Populations 5 and 7 do not share variants at three allozyme loci (*AAT-2*, *G3PDH*, and *LDH-2*, Fig. 4). We lack allozyme data for *GDH* and *MPI* for Population 7, but if these loci are assumed to be monomorphic for their respective *b* alleles, as they are in other populations of *D. planiceps*, the genetic distance between Populations 5 and 7 is 0.456, well within the range for other comparisons between the two species.

Clade C populations (18 and 19) differ strongly from both *D. fuscus* and *D. planiceps*, particularly from the latter (Table 3, mean Nei distances = 0.269 and 0.631, respectively). We were unable to obtain allozyme data for the MPI locus in the sample from Clade D (Population 20). As several alleles occur at this locus among the populations, it is not possible to assign tentative variants to Population 20. Nei distances calculated across the remaining 21 loci are all very large for comparisons involving Population 20 and the populations in Table 3 (Population 20 vs *D. fuscus*, Nei distance = 0.685–1.079, mean = 0.822; vs *D. planiceps*, Nei distance = 0.848–1.013, mean = 0.941; vs. Clade C, Nei distance = 0.652–0.654, mean = 0.653).

Morphometric analyses

In the multivariate analyses specifying three factors or principal components, those quantities accounted for about half (55%) of the total variance. The eigenvalues declined steeply over these first three factors or principal components, and much more slowly thereafter. Factor analysis character loadings after varimax rotation on the first factor declined gradually across the entire set of morphometric measurements. Five of the six characters with the heaviest (and positive) loadings on the first factor were toe-length residuals, and the three heaviest loadings were contributed by anterior toe residuals (Table 4). These characters also contribute relatively strong (and negative) first principal component coefficients. With respect to variation along the first three principal components (Fig. 5), there is almost complete overlap among the scatterplots for representatives of the

Table 2. Results of the STRUCTURE analysis that yielded the highest probability of obtaining the allozyme data with ten population clusters. Proportions exceeding 0.1 are shown in bold italic type

		Proportions of individuals assigned to cluster									
		1	2	3	4	5	6	7	8	9	10
D. planiceps (Clade A)	6	0.003	0.003	0.002	0.078	0.005	***0.879***	0.019	0.005	0.003	0.004
	7	0.048	0.004	0.005	0.025	0.026	***0.774***	0.007	0.032	0.019	0.059
	8	0.003	0.002	0.002	0.013	0.073	0.004	0.004	***0.895***	0.003	0.001
	9	0.002	0.002	0.002	0.007	0.005	***0.958***	0.008	0.007	0.002	0.007
	10	0.009	0.006	0.007	***0.206***	***0.220***	***0.204***	0.008	***0.322***	0.007	0.011
	11	0.002	0.009	0.002	0.006	0.042	0.004	0.003	***0.925***	0.002	0.004
	12	0.005	0.003	0.007	***0.517***	***0.164***	0.015	0.007	***0.277***	0.003	0.002
	13	0.005	0.011	0.012	0.011	***0.604***	0.005	***0.229***	***0.118***	0.004	0.002
	14	0.002	0.002	0.004	0.026	***0.716***	0.010	0.004	***0.233***	0.002	0.002
	15	0.005	0.004	0.069	***0.180***	***0.246***	***0.171***	0.007	***0.305***	0.006	0.005
D. fuscus (Clade B)	1	***0.003***	***0.981***	0.004	0.001	0.001	0.001	0.002	0.001	0.002	0.004
	2	***0.968***	0.004	0.006	0.003	0.004	0.002	0.002	0.006	0.004	0.001
	3	***0.978***	0.004	0.003	0.002	0.002	0.002	0.002	0.002	0.003	0.001
	4	***0.721***	***0.188***	0.074	0.002	0.003	0.002	0.004	0.002	0.003	0.001
	5	***0.493***	0.011	***0.455***	0.005	0.005	0.006	0.009	0.005	0.005	0.006
Mt Rogers	17	0.051	0.009	***0.444***	0.087	0.061	0.095	0.094	0.034	0.051	0.075
Clade C	18	0.036	0.013	0.012	0.003	0.004	0.004	0.004	0.004	***0.910***	0.010
	19	0.014	0.007	0.010	0.003	0.003	0.003	0.005	0.003	***0.946***	0.005
Clade D	20	0.002	0.003	0.002	0.002	0.001	0.002	0.002	0.001	0.002	***0.981***

different clades. The first principal component score distributions do differ significantly (Mann–Whitney test, $P = 0.05$) for males of the two species, but not for females ($P = 0.14$).

Adult standard lengths are significantly larger in *D. planiceps* than in Clade B *D. fuscus* (Mann–Whitney tests, $N = 10$ and 5, $P = 0.024$ for females; $N = 25$ and 8, $P = 0.014$ for males). These analyses included additional data for specimens that lacked measurements for all of the morphometric variables.

Tooth morphology

Figure 6 illustrates right lateral and anterior views of the lower jaws of two *D. planiceps* (Fig. 6A–F) and two *D. fuscus* (Fig. 6G–L) individuals. The teeth of *D. planiceps* are noticeably broader than those of *D. fuscus*, especially near the posterior margins of the dentary bones, and the zone of distal crown expansion begins closer to the base of the tooth in *D. planiceps.* Figure 7A indicates virtually no overlap between the scatterplots of tooth width versus height for adult male *D. fusc*us and *D. planiceps*. Population 18 (Clade C) males appear intermediate between those of *D. fuscus* and *D. planiceps*. The teeth of adult females are relatively narrower than those of males, especially in *D. planiceps*, and the height–width relationships are similar for the two species (Fig. 7B). Tooth shape is thus sexually dimorphic in *D. planiceps*, but not in *D. fuscus*.

Colour patterns

Figure 8 shows the dorsal aspects of a series of *D. planiceps* from the type locality (Population 15) and of *D. fuscus* from Population 5 near Roanoke, VA. The holotype of *D. planiceps* is an especially large and robust adult male, with a broad dorsal stripe enclosing irregular melanophore patches, and bordered laterally by a series of parallel melanophore stripes. These stripes grade ventrolaterally into small, widely spaced melanophore patches, and the venter is relatively immaculate. Our series of *D. planiceps* from the type locality indicates that the colour patterns are highly variable, particularly with respect to the distinctness of the dorsal stripe, the tendencies of melanophores to aggregate dorsally and laterally into stripes and blotches, and ventral pigmentation. The dorsal stripe frequently contains reddish-brown pigment, and small patches of melanophores are scattered over the venters of most specimens. White ventral iridophore patches are also often present and produce a 'salt-and-pepper' effect (not evident in the type specimen). Our samples of *D. fuscus* exhibit these same characteristics to varying degrees, and we are unable to discern or quantify any colour pattern

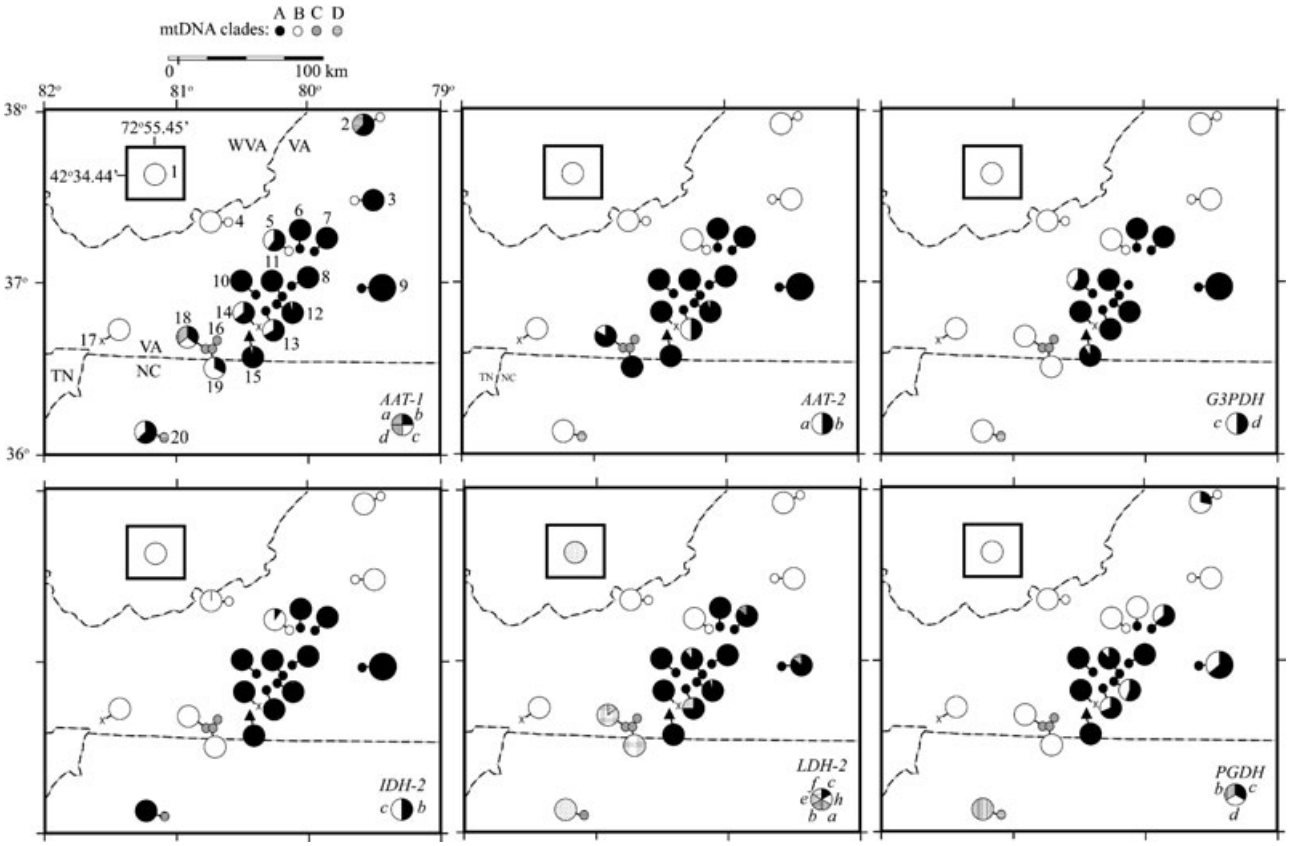

Figure 4. Variation in allozyme frequencies at six marker loci that differ between *Desmognathus planiceps* and *Desmognathus fuscus* (Clades A and B, Fig. 3). The shading of the small circles (collecting localities) indicates mitochondrial DNA (mtDNA) sequence clades. 'Xs' indicate localities where sequence data are lacking. Insets show allozyme frequencies in Population 1 (Massachusetts).

Table 3. Unbiased Nei distances for comparisons between *Desmognathus planiceps* (Clade A) and *Desmognathus fuscus* (Clade B) populations with allozyme data for all 22 loci

		planiceps (Clade A)				*fuscus* (Clade B)					Clade C	
	Pop.	7	8	11	12	1	2	4	5	17	18	19
D. planiceps	7	0.000	0.111	0.101	0.094	0.598	0.541	0.596	0.456	0.559	0.617	0.580
(Clade A)	8		0.000	0.006	0.074	0.619	0.478	0.552	0.484	0.504	0.651	0.694
	11			0.000	0.075	0.590	0.472	0.544	0.444	0.470	0.647	0.678
	12				0.000	0.666	0.585	0.660	0.519	0.441	0.549	0.629
D. fuscus (Clade B)	1					0.000	0.272	0.202	0.204	0.218	0.336	0.294
	2						0.000	0.093	0.106	0.268	0.226	0.215
	4							0.000	0.113	0.223	0.321	0.294
	5								0.000	0.112	0.277	0.245
	17									0.000	0.238	0.242
Clade C	18										0.000	0.049
	19											0.000

Table 4. Character loadings on Factor 1 after varimax rotation and first principal component coefficients. Characters are residuals from regressions on standard length

Character	Loading on Factor 1	First PC coefficient
Posterior toe 1 length	0.892	–0.294
Posterior toe 4 length	0.838	–0.303
Posterior toe 2 length	0.837	–0.302
Anterior toe 4 length	0.780	–0.277
Tail height at base	0.718	–0.248
Anterior toe 1 length	0.660	–0.218
Head length	0.629	–0.259
Orbitonarial distance	0.626	–0.225
Interorbital width	0.619	–0.213
Head width at jaw angle	0.548	–0.238
Tail height at fifth caudal fold	0.501	–0.182
Internarial width	0.459	–0.214
Hind limb length	0.378	–0.205
Anterior toe 3 length	0.367	–0.200
Anterior toe 2 length	0.268	–0.189
Head width at jaw musculature	0.258	–0.177
Forelimb length	0.229	–0.163
Tail width at fifth caudal fold	0.189	–0.103
Tail width at base	0.150	–0.121
Posterior toe 5 length	0.104	–0.108
Posterior toe 3 length	0.103	–0.107
Snout height	0.027	–0.122
Pelvic width	0.024	–0.006
Head height at jaw angle	–0.026	–0.084
Head height at jaw musculature	–0.186	–0.008
Axillary width	–0.254	–0.007

characters that consistently distinguish members of the two clades.

DISCUSSION

In interpreting our analyses we regard species as metapopulational lineages on independent evolutionary trajectories. This corresponds to the general lineage or metapopulation lineage concept of de Queiroz (1998, 2005), and elements of the evolutionary species concept of Wiley (1978) and Frost & Hillis (1990). This general concept has been applied to desmognathan salamanders by Tilley & Mahoney (1996) and Anderson & Tilley (2003). We interpret the following as evidence of evolutionary independence: reciprocally monophyletic and substantially divergent mtDNA haplotype clusters; substantial differentiation with respect to allozyme frequencies at multiple loci; concordant variation among mtDNA sequences, allozyme frequencies at different loci, and morphology.

Employing these criteria for recognizing separate metapopulation lineages on independent evolutionary trajectories, we assign the populations of haplotype Clade A (Fig. 3) to *D. planiceps* Newman, 1955, which we resurrect from synonymy under *D. fuscus*. Haplotypes of this clade include one from Population 15 at the type locality of *D. planiceps*. *Desmognathus planiceps* haplotypes are distinct and reciprocally monophyletic with respect to all other congeners, including those of Clade B (Fig. 3), which includes Population 1 in Massachusetts and clearly represents *D. fuscus* Rafinesque, 1820, type locality 'the northern parts of the state of New York' (Rafinesque, 1820).

Desmognathus planiceps is distinguishable from *D. fuscus* on the basis of allozyme as well as mitochondrial markers. However, none of the allozyme loci we examined are fixed for alternative variants in our *D. planiceps* and *D. fuscus* samples, which could reflect either gene exchange, ancestral polymorphism, or convergence of mobility classes. The two forms are differentiated at a level (9.2% cyt B sequence divergence, Nei genetic distance based on allozymes ~0.35) that characterizes other reproductively isolated forms of *Desmognathus* (Table 1; Tilley & Mahoney, 1996). Hence, *D. planiceps* and *D. fuscus* appear to represent independent evolutionary trajectories, and distinct species under evolutionary or metapopulation lineage concepts. We are unable to confirm Newman's (1955) assertion that the heads of *D. planiceps* are relatively flattened, or to identify any other morphological characters other than tooth shape that reliably distinguish the species from *D. fuscus*.

Desmognathus planiceps and *D. fuscus* appear to be parapatrically distributed, with the range of the former extending from the interior of the Blue Ridge Physiographic Province in Floyd County, VA, eastward at least as far as Population 9 in the Virginia Piedmont. The two taxa have not been found in sympatry. If they are parapatric, the range of *D. planiceps* may be contained within that of *D. fuscus*, which apparently extends south-east into the North Carolina Piedmont (Kozak *et al*., 2005). Levels of differentiation and apparent lack of gene flow between Population 5 (*D. fuscus*) and Populations 6 and 7 (*D. planiceps*) indicate that the contact zone between the two species may be quite abrupt. These two species may represent yet another example of forms that have achieved a level of divergence sufficient to restrict gene flow, yet insufficient to permit sympatric coexistence (Arnold, 2000).

Our analyses suggest that genetically distinct taxa remain to be described. The Clade C cyt B haplotype is 7.68% divergent from those of *D. fuscus*, its closest relative surveyed in this study. This level of divergence exceeds that between *D. orestes* and *D. ochrophaeus* (6.22%, Table 1), and approaches that

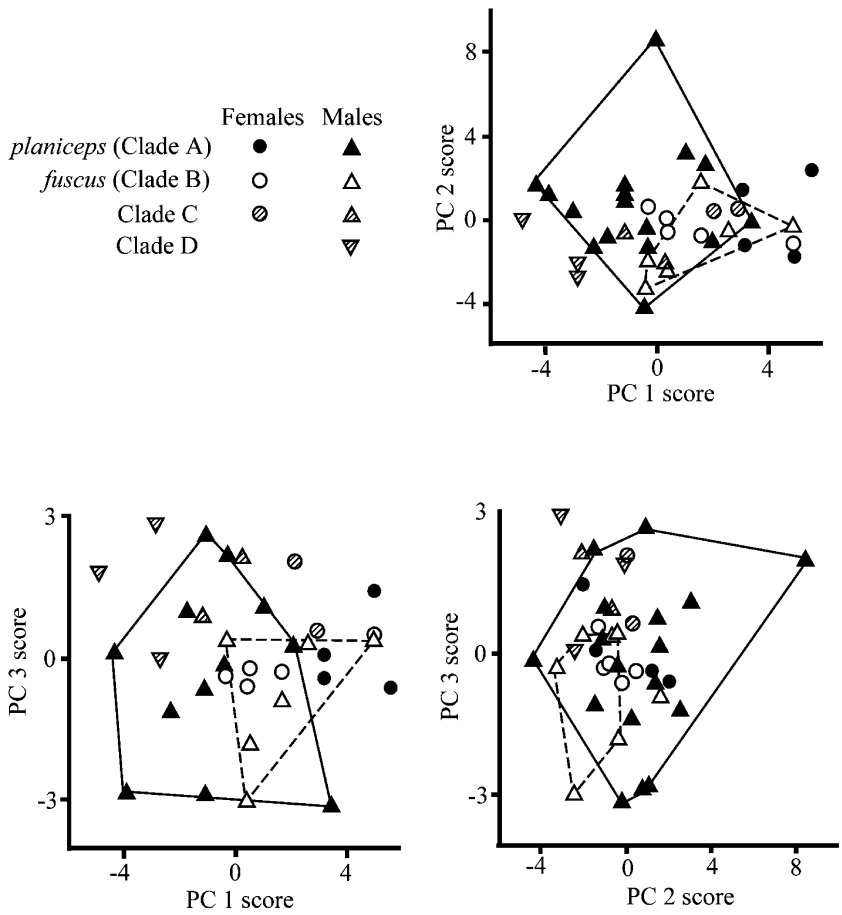

Figure 5. Results of principal components analysis on adult specimens. Polygons enclose points for adult male *Desmognathus planiceps* (solid lines) and *Desmognathus fuscus* (dashed lines).

between *D. orestes* and *D. fuscus* (8.13%, Table 1), which occur sympatrically. Nei genetic distances based on allozymes between Clade C and populations of the other clades are also substantial. All of them exceed the minimum Nei distances between sympatric desmognathans (Tilley & Mahoney, 1996: table 6). Population 20, Clade D, also exhibits strong haplotype and allozyme divergence from the other populations sampled here. Although these levels of divergence are suggestive, determining whether Populations 18, 19, and 20 represent undescribed species will require more geographically extensive sampling and analysis of contact zones.

Allozymic divergence among some populations of Clade B (*D. fuscus*) is also rather high. Nei distances for Population 1 versus 2, 5, and 17 (mean = 0.224) and for Population 17 versus 2 and 4 (mean = 0.246), exceed levels of differentiation between northern and southern populations of *D. ochrophaeus*, and approximate some distances between sympatric species of *Desmognathus* (Tilley & Mahoney, 1996).

Two previous studies (Bonett, 2002; Kozak *et al*., 2005) deal with some of the populations sampled here (Table 5). Bonett (2002) analysed allozyme data to explore relationships among populations putatively referred to *D. fuscus*, and to document the distinctness of *D. fuscus* and *D. conanti*. His 'Group A' is clearly the *D. fuscus* (Clade B) of our study, whereas his 'Group C' appears to be a heterogeneous collection of populations inhabiting the North Carolina and Virginia Piedmont. Bonett proposed that his 'Group C' Population 58 might reflect hybridization with his Group A (*D. fuscus*), but our analyses of specimens from that locality (Population 9, Clade A) clearly assign their cyt B sequences to *D. planiceps*. The cyt B sequences from Bonett's Population 24 (our Population 20, Clade D) form a clade within those of *D. carolinensis* (Fig. 3), whereas the sequence from Bonett's Population 25 (our Population 19, Clade C) do not suggest a clear relationship to either *D. fuscus* or *D. planiceps*.

Kozak *et al*. (2005 and pers. comm.) included two populations that we also sampled: their Populations 54 and 60 (which correspond to our Population 1 and a locality near our Population 17, respectively, Table 5). They assigned both these populations to

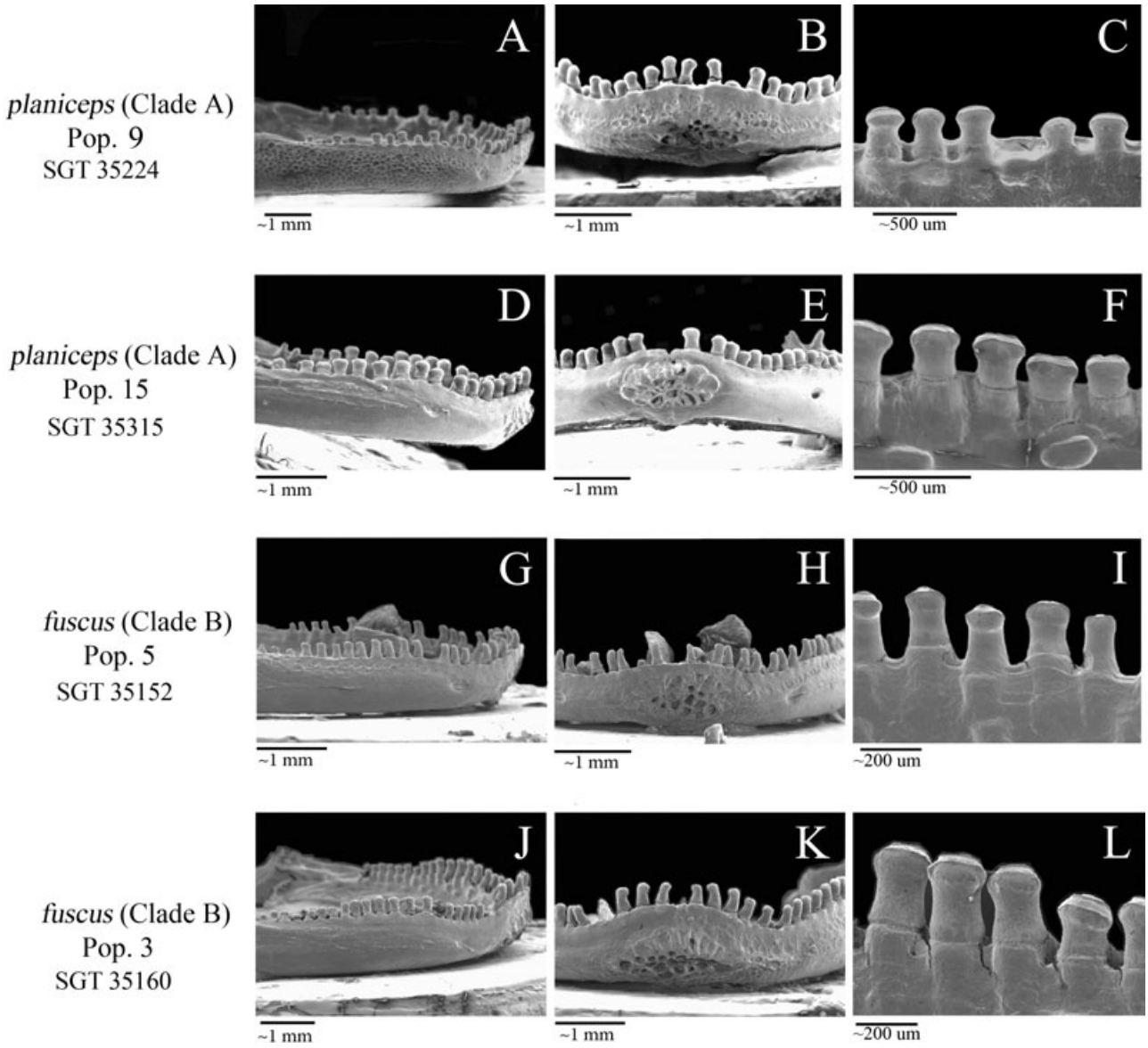

Figure 6. Tooth morphology in adult male *Desmognathus*. A–F, *Desmognathus planiceps* (Clade A). G–L, *Desmognathus fuscus* (Clade B). Left to right: lateral views of left dentaries, anterior views of dentaries, and lingual views of teeth near posterior margin of right dentaries.

Table 5. Correspondences between populations and population groupings in this paper and in published studies

This paper	Bonett (2002)	Kozak *et al*. (2005)
Clade B (*D. fuscus*)	*D. fuscus* Group A	*fuscus* B
Pop. 9, Clade A (*D. planiceps*)	Pop. 58 Group C	–
Pop. 20, Clade D	Pop. 24 Group C	–
Pop. 19, Clade C	Pop. 25 Group C	–
Pop. 1 (Clade B, *D. fuscus*)	–	Pop. 54, *fuscus* B
Pop. 17 (Clade B, *D. fuscus*)	–	Pop. 60, *fuscus* B

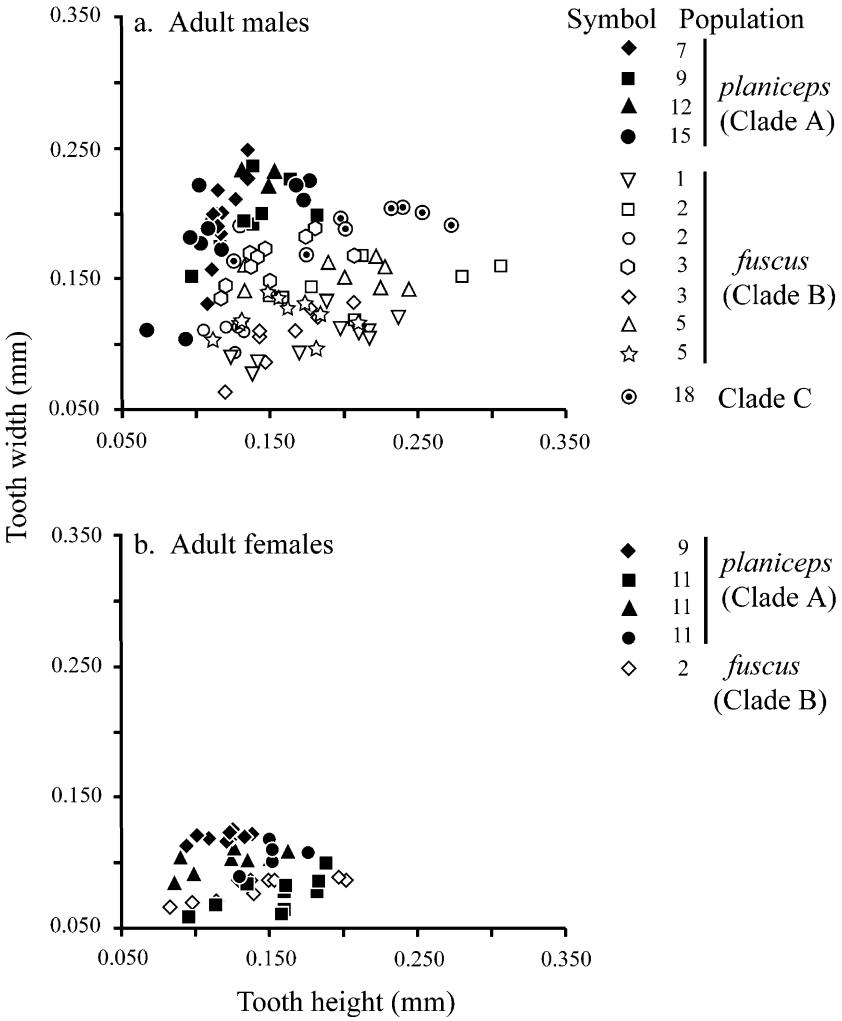

Figure 7. Scatterplots of width (ordinate) vs height (abscissa) of teeth in the posterior dentaries of individuals representing mature male (A) and female (B) *Desmognathus planiceps* (solid symbols), *Desmognathus fuscus* (open symbols) and Clade C (circled dots). Symbol shapes distinguish different individuals.

their '*fuscus* B' clade, which corresponds to our Clade B, and our data support this treatment.

Haplotypes in Clade D are distinct from those of *D. fuscus* or *D. planiceps*, but fall within the clade of *D. carolinensis* sequences. Kozak *et al*. (2005) also found haplotype similarities between *D. carolinensis* and some populations of '*D. fuscus*' from Virginia, North Carolina, and South Carolina, and speculated that these might reflect gene exchange between these forms. This explanation deserves exploration, as does the alternative possibility that *D. carolinensis* is more closely related to some lowland Piedmont *Desmognathus* than to other mountain forms. The diversity of dusky salamanders in the southern Piedmont and adjacent Blue Ridge is clearly more extensive and complex than previously appreciated.

Relationships among the desmognathan taxa on our phylogeny largely support previously published analyses (Mead *et al*., 2001; Rissler & Taylor, 2003; Kozak *et al*., 2005). These relationships include the occurrence of two distinct clades of *D. orestes*, and a sister relationship between *D. ochrophaeus* and *D. orestes*. The single sequence from *D. ocoee* (Population 37 in Tilley & Mahoney, 1996) falls outside the clade comprising *D. imitator* and all the previously mentioned taxa, but this relationship has low bootstrap support. Kozak *et al*. (2005) speculated that low levels of bootstrap support for basal relationships among desmognathans might reflect rapid, early radiation involving species with aquatic larval periods.

ACKNOWLEDGEMENTS

We are heavily indebted to Richard L. Hoffman, who for more than half a century maintained and shared his suspicion that *D. planiceps* was a distinct species.

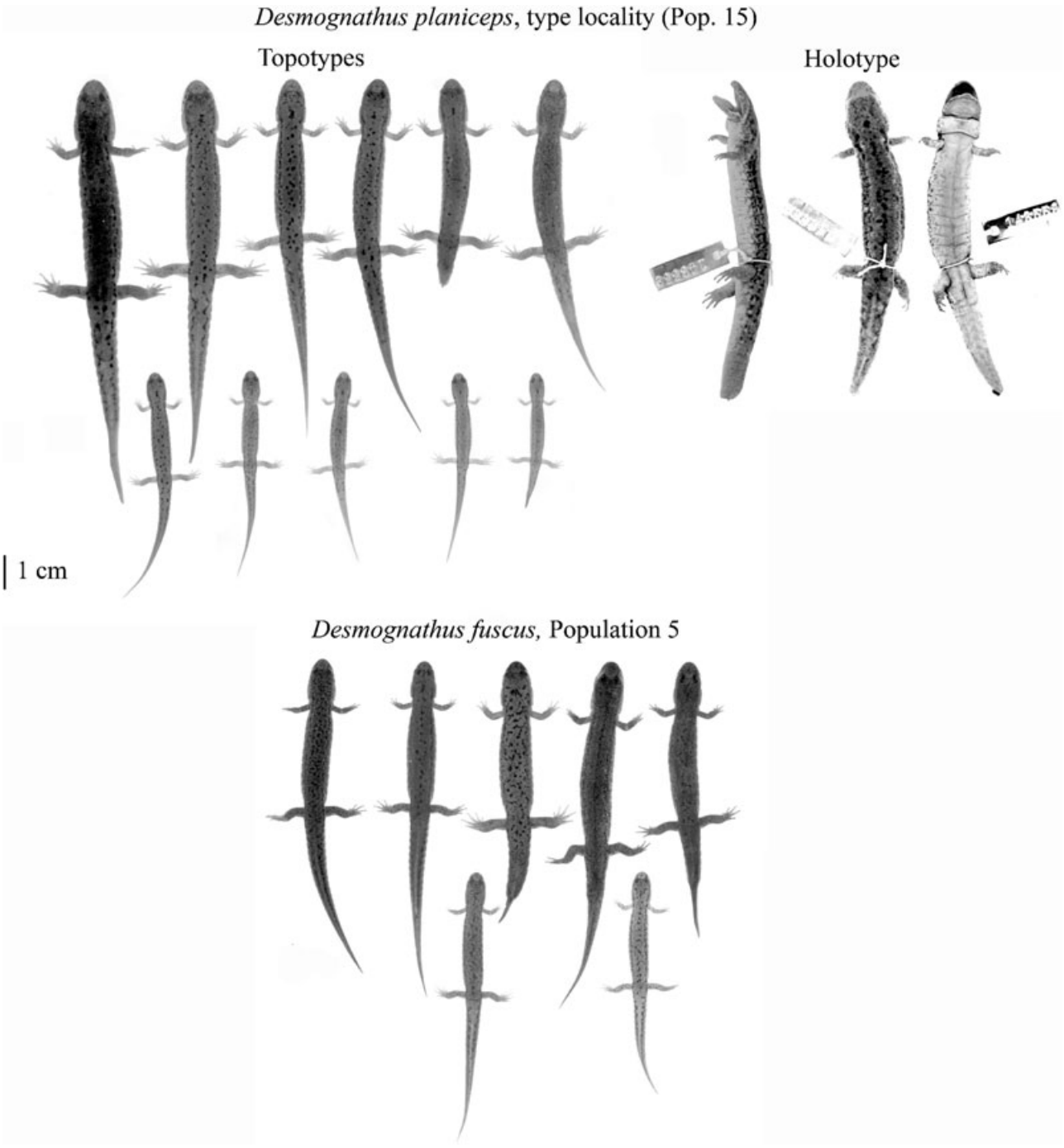

Figure 8. Specimens of *Desmognathus planiceps* (Clade A) from the type locality, including the holotype (USNM 143559), and a series of *Desmognathus fuscus* (Clade B) from Population 5. The contrast has been adjusted to bring out details of the dorsal patterns.

He and Joseph Bernardo assisted in the collection of specimens. Addison Wynn kindly provided access to the specimens of *D. planiceps* in the National Museum of Natural History. Nicholas Horton advised us on the use of multivariate morphometric analyses. Richard Briggs, Judith Wopereis, and Lizmarie Lopez provided invaluable help with the SEM preparations and tooth measurements. Ronald M. Bonett provided exact localities for some of his collections so we could resample them in our study. Joseph Bernardo, Ronald

Bonett, Richard Highton, and Kenneth Kozak offered helpful suggestions on the manuscript. SGT and RLE were supported by the Blakeslee Genetics Research Fund at Smith College. LAK was supported by NSF grants DEB-0092908, DEB-0079325, and DEB-043115. Collecting permits were provided by the states of Massachusetts, North Carolina, and Virginia.

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SUPPLEMENTARY MATERIAL

The following material is available for this article online:

Appendix S1. Sampling localities. GenBank accession numbers are shown in parentheses.
Appendix S2. Individuals (identified by SGT field tag numbers) sampled for mitochondrial DNA (mtDNA) sequences, sorted by haplotype (Fig. 3) and population. Sequences with GenBank accession numbers were used in genealogical analysis.
Appendix S3. Sizes of samples employed in morphometric analyses.
Appendix S4. Allozyme frequencies at loci that distinguish Clades A and B.

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