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MOLECULAR PHYLOGENETICS OF DESMOGNATHINE SALAMANDERS (CAUDATA: PLETHODONTIDAE): A REEVALUATION OF EVOLUTION IN ECOLOGY, LIFE HISTORY, AND MORPHOLOGY

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Abstract.—Phylogenetic relationships were reconstructed for salamanders of the plethodontid subfamily Desmognathinae to examine evolution of morphology, ecology, and life history. Mitochondrial DNA sequences encoding 12S and 16S ribosomal RNA and the valine transfer RNA provided 259 phylogenetically informative sites from approximately 1,200 nucleotide positions for 21 specimens representing 15 species and subspecies. These data were analyzed in conjunction with 13 morphological and reproductive characters to generate phylogenetic hypotheses. The directly developing terrestrial desmognathines *Phaeognathus hubrichti* and *Desmognathus wrighti* represent, respectively, the first and second phylogenetic branching events within the subfamily, and the remaining terrestrial lineage, *D. aeneus*, also branches near the base of the phylogenetic tree. These results challenge earlier hypotheses that within *Desmognathus* the small nonmetamorphosing species, *D. aeneus* and *D. wrighti*, represent the end of a graded phylogenetic decrease in size and decrease in use of aquatic habitats. In contrast to previous hypotheses, our results suggest that desmognathine evolution includes transformations in the direction of larger body sizes, lengthened larval periods, and greater use of aquatic habitats. [Desmognathinae; ecology; life history; mitochondrial DNA; molecular phylogenetics; ribosomal DNA; salamanders.]

Evaluating the causal basis of character evolution requires knowledge of ecology, functional morphology, and phylogeny (Brooks, 1985; Mayden, 1987; Brooks and McLennan, 1991; Gorman, 1992; Losos, 1992). Studies of ecology and functional morphology reveal the contributions of characters to organismal survival and reproduction, and phylogenies provide the historical framework needed to examine a character's evolutionary origin and transformation. This information permits testing of hypotheses of adaptation (Baum and Larson, 1991; Larson and Losos, 1996).

Desmognathine salamanders of the family Plethodontidae constitute a small evolutionary radiation (15 recognized species and subspecies) in the aquatic and terrestrial communities of eastern North American forests (Tilley and Bernardo, 1993). Desmognathine communities in the southern Appalachian Mountains commonly contain three to five and as many as seven species (Bruce, 1991). These communities exhibit considerable ecological structure, primarily along an aquatic-to-terrestrial gradient. The position of each species along this gradient has important correlates in morphology and life history (reviewed by Tilley and Bernardo, 1993). The most aquatic species have the largest body size, a pronounced tail fin, a larval period of ≥2 years, and large clutches of eggs. Terrestrial species have the smallest body size, no tail fin, no aquatic larval stage, and small clutches. Species inhabiting edges of streams and seepages are intermediate for all of these characteristics.

A semiaquatic habitat generally has been considered ancestral and a terrestrial habitat has been considered derived for desmognathines. This hypothesis can be traced to the proposal of Wilder and Dunn (1920) that lunglessness in plethodontids arose as an adaptation for minimizing stream drift in mountain brook habitats. Dunn (1917, 1926) proposed that the ancestral desmognathine occupied a semi-

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aquatic habitat similar to that of the largest species, *Desmognathus quadramaculatus*. He suggested that from this common ancestor evolution proceeded in two different directions. One lineage included the remaining species of *Desmognathus* known at that time (*D. "phoca"* = *D. monticola, D. fuscus, D. auriculatus, D. brimleyorum,* and *D. ochrophaeus*) and followed an evolutionary progression toward increased terrestriality. The other lineage included only the entirely aquatic *Leurognathus marmoratus* and was characterized by evolution toward increased use of aquatic habitats. The tiny terrestrial species, *D. wrighti* and *D. aeneus,* were subsequently described (King, 1936; Brown and Bishop, 1947) and later hypothesized to represent the culmination of evolution toward terrestriality in *Desmognathus,* with the other extant species illustrating intermediate conditions (Organ, 1961b).

The structuring of desmognathine communities and its causal basis have been studied intensively (reviewed by Hairston, 1987). Hairston (1949) first postulated that the observed community structure is maintained by competition. This hypothesis appeared to gain support from some subsequent studies (e.g., Organ, 1961a; Means, 1975; Krzysik, 1979). However, Tilley (1968) suggested that size differences among species arose initially in allopatry, with predation driving smaller species into increasingly terrestrial habitats following secondary geographic contact among species. Hairston (1986) then showed experimentally that predation is the dominant factor structuring desmognathine communities, with competition between larger species being a secondary factor, a conclusion that has been supported by additional experiments (e.g., Southerland, 1986a, 1986b; Roudebush and Taylor, 1987).

Study of desmognathine communities can contribute to our understanding of the evolution of life history traits. In a community of five desmognathine taxa, Organ (1961b) found that with increased terrestriality there was a concomitant decrease in body size, fecundity, and length of the larval period and an increase in survivorship, culminating in direct development and miniaturization. He interpreted these trends as adaptive responses for reducing mortality associated with predation in aquatic environments. Tilley (1968) also documented a strong positive correlation between body size and fecundity. Smaller size may result from earlier maturation or lower rates of growth in larvae and juveniles (Tilley, 1974, 1977; Bruce, 1988, 1989, 1990).

Community structure is hypothesized to generate selective regimes that drive the evolution of body size and length of the larval period. Predation may select for earlier maturation, with small body size being an indirect outcome (Bruce, 1990). However, there are problems in applying the predation/competition model to evolution of desmognathine body size; predation on larvae and juveniles would select for earlier maturation, but the concomitant reduction in body size would make the adults more vulnerable to predation (Bruce, 1990). If predation by aquatic congeners has selected for decreased larval period and body size, the predicted phylogenetic pattern is a transformation in the direction of decreasing size and length of larval period that has occurred within an aquatic adaptive zone.

Evolutionary interpretations of data on community structure and life history evolution invoked the hypothesis that the evolution of terrestriality within the genus *Desmognathus* proceeded in an ordered transformation series, the primitive condition being the semiaquatic state exhibited by the large *D. quadramaculatus,* and the most derived condition being the most terrestrial state as seen in the smallest species, *D. aeneus* and *D. wrighti.* This hypothesis predicts a phylogenetic tree compatible with ordered character changes leading toward increased terrestriality (Fig. 1). Likewise, the hypothesis of an aquatic-to-terrestrial assembly of desmognathine communities predicts that phylogenetic mapping will reveal an ordered transformation representing evolutionary occupation of increasingly terrestrial habitats.

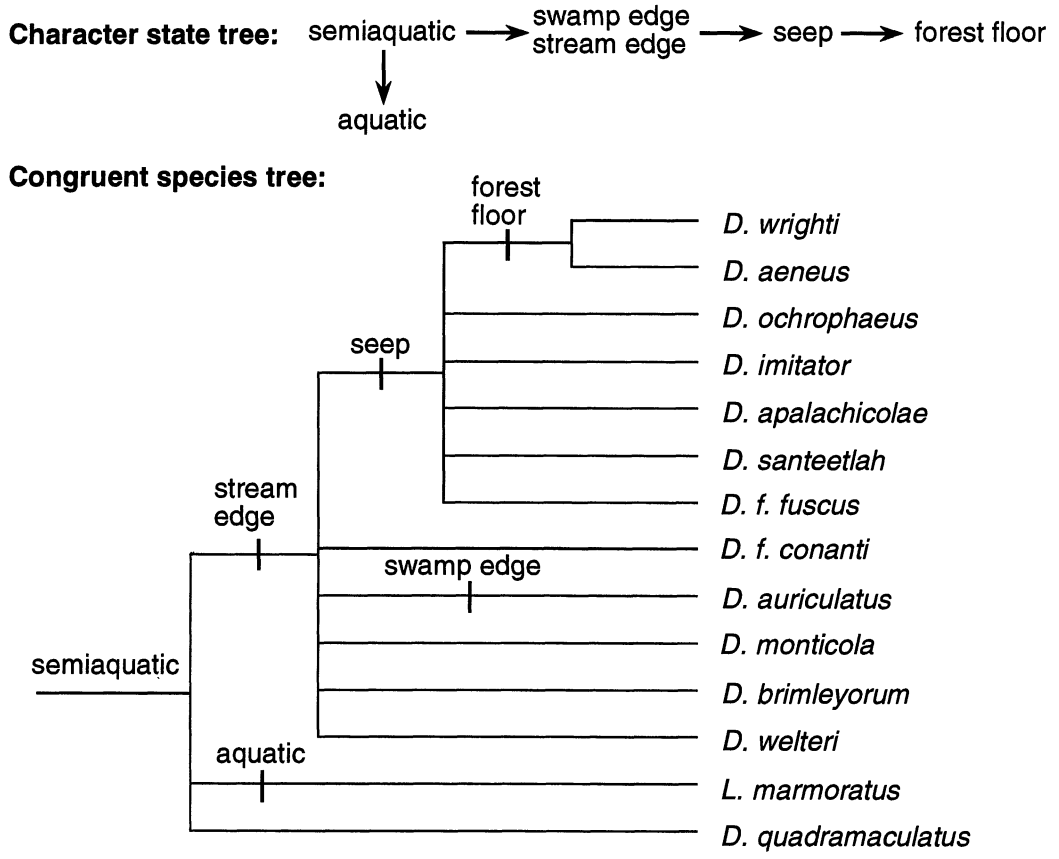

FIGURE 1. Character state tree representing an ordered transformation series from primitively semiaquatic to increasingly derived terrestrial and aquatic species and a partially resolved cladogram of the Desmognathinae (excluding *Phaeognathus*) congruent with the character state tree. *D.* = *Desmognathus*; *L.* = *Leurognathus*.

Despite the phylogenetic predictions made by these adaptive hypotheses of desmognathine evolution, no explicit phylogenetic hypothesis has been proposed for the Desmognathinae. DNA sequences offer a potentially large set of characters for phylogenetic reconstruction in morphologically problematic groups such as plethodontid salamanders (Larson and Chippindale, 1993). We used mitochondrial DNA (mtDNA) sequences encoding the 12S and 16S ribosomal RNA (rDNA) and the valine transfer RNA ($tDNA_{VAL}$) to generate a phylogenetic hypothesis for the Desmognathinae. This phylogeny was then used to test various hypotheses of desmognathine evolution and the monophyly of desmognathine taxa.

MATERIALS AND METHODS

Specimens examined for the analysis of mtDNA sequences, locality data, and voucher numbers are listed in Table 1. All desmognathine species were represented by at least one individual in the molecular analyses. *Eurycea wilderae* was chosen as a representative of the first outgroup because it is within the plethodontid subfamily Plethodontinae, the sister taxon to the Desmognathinae (Lombard and Wake, 1986). Plethodontids have no obvious sister group among the remaining salamander families, but the other families having internal fertilization (including the Salamandridae) appear more closely related to the Plethodontidae than those having ex-

TABLE 1. Identity, locality data, and voucher numbers for salamander specimens examined in the analysis of mtDNA sequences. KU = Museum of Natural History, University of Kansas; MVZ-FC = Museum of Vertebrate Zoology Frozen Tissue Collection, University of California–Berkeley.

Species	Locality	Voucher no.
Desmognathus aeneus	Macon Co., North Carolina	KU 218959
D. apalachicolae	Barbour Co., Alabama	KU 218963
D. auriculatus	North Carolina	MVZ-FA 13580
D. brimleyorum	Montgomery Co., Arkansas	KU 218982
D. fuscus conanti	Walker Co., Georgia (=Georgia *D. f. conanti*)	KU 219023
D. f. conanti	Lyon Co., Kentucky (=Kentucky *D. f. conanti*)	KU 218690
D. f. fuscus	McDowell Co., North Carolina (=North Carolina *D. f. fuscus*)	KU 219012
D. f. fuscus	Orleans Co., Vermont (=Vermont *D. f. fuscus*)	KU 211348
D. imitator	Swain Co., North Carolina	KU 219042
D. monticola	Macon Co., North Carolina	KU 219087
D. ochrophaeus	Washington Co., Virginia (=Virginia *D. ochrophaeus*)	KU 219159
D. ochrophaeus	Macon Co., North Carolina (=North Carolina *D. ochrophaeus*)	KU 219140
D. ochrophaeus	Nottingham City, Pennsylvania (=Pennsylvania *D. ochrophaeus*)	KU 219161
D. quadramaculatus	Avery Co., North Carolina (=Northern *D. quadramaculatus*)	KU 219187
D. quadramaculatus	Macon Co., North Carolina (=Southern *D. quadramaculatus*)	KU 219186
D. santeetlah	Swain Co., North Carolina	KU 219206
D. welteri	Harlan Co., Kentucky	KU 219238
D. wrighti	Haywood Co., North Carolina	KU 219241
Leurognathus marmoratus	Watauga Co., North Carolina (=Northern *L. marmoratus*)	KU 219255
Leurognathus marmoratus	Macon Co., North Carolina (=Southern *L. marmoratus*)	KU 219251
Phaeognathus hubrichti	Butler Co., Alabama	MVZ-FC 13612
Eurycea wilderae	Macon Co., North Carolina	no voucher
Notophthalmus viridescens	Macon Co., North Carolina	KU 219309

ternal fertilization (Larson and Dimmick, 1993). *Notophthalmus viridescens* (Salamandridae) was chosen as a relatively distant outgroup to the desmognathines.

Genomic DNA was extracted following standard methods (Hillis et al., 1996). A 1.8-kb segment including portions of the 12S and 16S rDNA and the $tDNA_{VAL}$ was amplified from genomic DNA using the polymerase chain reaction (PCR). Base sequences and relative locations of primers used for amplification and sequencing of DNA are presented in Table 2 and Figure 2. The amplification profile entailed denaturation at 94°C for 30 sec, annealing at 55°C for 35 sec, and extension at 70°C for 150 sec, with 4 sec added to the extension time per cycle, for 30 cycles. The amplified products were electrophoresed in 2.5% Nusieve GTG agarose and reamplified under identical conditions. This second double-stranded product was electrophoresed on a 2.5% acrylamide gel (Maniatis et al., 1982), and the DNA was recovered by electroelution and ethanol precipitation. The purified product was suspended in 7 μl of distilled H_2O, 1 μl of a 2 pmol/μl concentration of sequencing primer, and 2 μl of Sequenase reaction buffer, heated to 95°C for 5 min, and immediately cooled in ice for at least 10 min. Sequencing followed the protocols of Hillis et al. (1996).

An approximate sequence alignment was made using the program CLUSTAL

TABLE 2. Composition of primers used for amplification and sequencing of salamander mtDNA. Primer designations correspond to those used in Figure 2. Position is the most 3′ base in the numbered *Xenopus* sequence (Roe et al., 1985).

Primer	Sequence (5′ → 3′)	Strand	Position	Reference
A	GGGTTGGTAAATCTCGTGC	light	2307	Titus, 1992
B	AAACTGGGATTAGATACCCCACTA	light	2508	Kocher et al., 1989
C	TAGAGCACCGCCAAGTCCTTTG	heavy	2576	Titus, 1992
D	GTCAGGTCAAGGTGTAGCAAT	light	2758	Titus, 1992
E	AGGAGGGTGACGGGCGGTGTGT	heavy	2897	Kocher et al., 1989
F	TAAAGCATTTTGCTTACACC	light	3059	Titus, 1992
G	AGGTTTTCTGTCGCCCTTAC	heavy	3174	Titus, 1992
H	GCATAATAATCTAGCCAG	light	3307	Titus, 1992
I	GGTGGCTCGTTGAAGGGC	heavy	3626	Titus, 1992
J	AGATAGAAACCGACCTGGAT	heavy	4577	Titus, 1992

(Higgins and Sharp, 1988, 1989). Use of secondary structural models in the alignment of rRNA and tRNA genes can increase alignment and phylogenetic accuracy (Kjer, 1995; Titus and Frost, 1996). Therefore, alignments were adjusted manually to maintain length-invariant stem regions according the structural models for 12S rRNA (Van de Peer et al., 1994), $tRNA_{VAL}$ (Kumazawa and Nishida, 1993), and 16S rRNA (Gutell and Fox, 1988). Regions of uncertain alignment were excluded from phylogenetic analyses (Swofford and Olsen, 1990). Gaps spanning more than one nucleotide position were scored as a single character in phylogenetic analyses. Divergences among paired sequences were calculated following Mindell and Honeycutt (1990). A complete sequence alignment is available from the Systematic Biology World Wide Web site (http://www.utexas.edu/depts/systbiol/). GenBank accession numbers are U71221–43.

Thirteen nonmolecular characters related to osteology and reproduction were included in some analyses (Appendix, Table 3). Among major lineages of plethodontids, the few morphological taxonomic characters appear highly variable and homoplastic (Wake, 1966, 1991; Larson, 1984; Wake and Larson, 1987). Consequently, phylogenetic relationships among major plethodontid lineages are not well resolved (Lombard and Wake, 1986; Presch, 1989; Wake, 1993). Nonmolecular characters therefore were studied only in the desmognathine taxa. Because all analyses of the molecular data strongly supported *Phaeognathus hubrichti* and *D. wrighti* as the

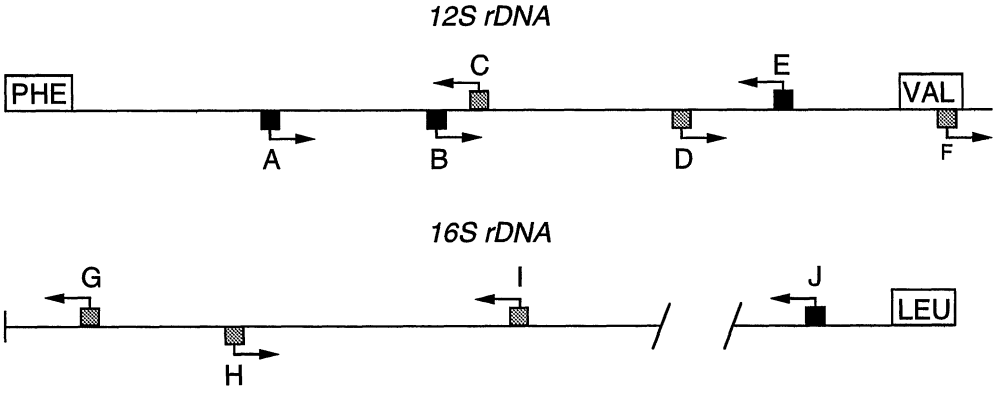

FIGURE 2. Primer locations for PCR and sequencing. ■ = primers used for both PCR and sequencing; □ = primers used for sequencing only. Letter designations for primers correspond to those in Table 2.

TABLE 3. Data matrix for 13 morphological characters of desmognathine salamander. Question marks denote unknown character states.

Taxon	Characters 1111 1234567890123
Phaeognathus hubrichti	0?00000000?00
Desmognathus wrighti	0001011010010
Leurognathus marmoratus S	0010010001101
L. marmoratus N	0110010001101
D. quadramaculatus S	001100?111000
D. quadramaculatus N	001100?111000
D. aeneus	0001011010110
D. ochrophaeus Virginia	1101011????10
D. ochrophaeus Pennsylvania	1101011????10
D. ochrophaeus North Carolina	1101011????10
D. imitator	11?101?????10
D. apalachicolae	11?1011110110
D. monticola	0?11000100110
D. auriculatus	01?2011110110
D. santeetlah	0101011110110
D. f. conanti Georgia	0101011110110
D. f. conanti Kentucky	0101011110110
D. f. fuscus North Carolina	0101111010110
D. f. fuscus Vermont	0101111010110
D. brimleyorum	01?1110100110
D. welteri	0101110100110

two most basal lineages within the Desmognathinae, they were used as functional outgroups (Watrous and Wheeler, 1981) for analyses incorporating nonmolecular characters.

Two parsimony analyses were performed using PAUP 3.0s (Swofford, 1991). The first used only the nucleotide sequence data with regions of ambiguous alignment removed and all substitutions and gaps equally weighted. The second used a combination of the unambiguously aligned nucleotide sites and the 13 morphological and reproductive characters, with all characters weighted equally and unordered. Heuristic searches were performed using 20 replications with random additions of taxa. In all analyses, *Notophthalmus viridescens* was designated the outgroup. The most-parsimonious cladograms obtained using the combined analysis of mtDNA sequences and nonmolecular characters were compared with previously proposed hypotheses and current taxonomies by incorporating a constraint tree congruent with the hypothesis in question and performing a PAUP analysis to find the most-parsimonious cladogram given this topological constraint. The resultant tree was then compared with the most-parsimonious tree(s) using the Wilcoxon rank sum test following Templeton (1983), except that probability values for the more conservative two-tailed test were used (Felsenstein, 1985). This test is appropriate for simultaneous analysis of diverse data sets (Larson, 1994). Significance of results was evaluated using the critical values of Rohlf and Sokal (1981:191) and modifications therein for $n > 50$.

Maximum likelihood estimates of sequence divergence were calculated under two models, one assuming that transitions are twice as likely as transversions and the other assuming that transitions are 10 times more likely than transversions, using PHYLIP version 3.4 (Felsenstein, 1989). Numbers of transitions and transversions for all pairwise comparisons were plotted against estimated sequence divergence under the two models. Widespread saturation (substitutions extensively superimposed at the same molecular site) should be evident when the number of substitutions no longer increases as a function of increasing sequence divergence.

Species were coded with respect to selective regime and length of the larval period using discrete categories. These categories were mapped a posteriori as characters on the trees resulting from the simultaneous analysis of molecular and nonmolecular characters. A summary of the data and sources used to construct these categories is provided in Table 4. Three categories of larval period were recognized: absence of a larval period (direct development), metamorphosis at <1 year, and metamorphosis after ≥2 years. Six adaptive zones and associated selective regimes were recognized based on modal habitats occupied by each species: aquatic, semiaquatic, stream edge, seepage, swamp edge, and forest floor. A large amount of variation exists within some species for some or all of these categories, and this coding is therefore an approximation. However, previous evolutionary hypothe-

TABLE 4. Characterization of length of the larval period, body size, and adaptive zone for 16 desmognathine species and populations (capital letters correspond to references[a]).

Species	Larval period (months)	Body size[b] (mm)	Adaptive zone
Phaeognathus hubrichti	direct development (T)	101[c] (C)	burrowing (C)
Leurognathus marmoratus	35 (T)	64[c] (I)	aquatic (D, E, J)
Desmognathus aeneus	direct development (T)	25.1 (B)	terrestrial (J)
D. apalachicolae	9 (T)	42.7 (M)	seep (M)
D. auriculatus	ca. 12 (L)	48.5[c] (M)	swamp edge (L)
D. brimleyorum	9–12 (T)	70.7 (W)	stream edge (D)
D. fuscus conanti	<12 (T)	49.4 (Q)	seep (R)
D. f. fuscus	7–12 (T)	46[c] (A)	stream edge (P, V)
D. imitator	unknown	37.4 (W)	seep (S)
D. monticola	9.5–12.5 (T)	57.7 (N)	stream edge (E, P)
D. ochrophaeus (PA, VA)	4–6 (T)	35.7 (W)	seep (A, O)
D. ochrophaeus (NC)	9–10 (T)	34.2 (K)	seep (E, K)
D. quadramaculatus	35–48 (T)	65.0 (W)	semiaquatic (E, O)
D. santeetlah	<12 (T)	38[c] (R)	seep (R)
D. welteri	24 (T)	62.0 (W)	stream edge (W)
D. wrighti	direct development (T)	19 (H)	terrestrial (E, O, P)

[a] A = Bishop, 1941; B = Bishop and Valentine, 1950; C = Brandon, 1965; D = Dunn, 1926; E = Hairston, 1949; F = Hairston, 1984; G = Juterbock, 1984; H = King, 1936; I = Martof, 1956; J = Martof, 1962; K = Martof and Rose, 1963; L = J. Harrison, pers. comm.; M = Means and Karlin, 1989; N = Means and Longden, 1970; O = Organ, 1961a; P = Organ, 1961b; Q = Rossman, 1958; R = Tilley, 1981; S = Tilley, 1985; T = Tilley and Bernardo, 1993; U = Valentine, 1963; V = Wilder, 1913; W = this study.

[b] Mean adult snout–vent length.

[c] Estimated value.

ses for desmognathines have implicitly used these discrete categories in their explanations, and our character analysis is therefore justified in a phylogenetic test of those hypotheses. Semiaquatic in our coding scheme refers to occupation of completely saturated substrates along stream edges by *D. quadramaculatus* (Hairston, 1949; Organ, 1961a). This definition differs from that used in previous studies, in which semiaquatic generally refers to all but the most completely aquatic desmognathines and the most terrestrial one, *P. hubrichti*.

Average snout–vent lengths (SVL) of sexually mature adults (Table 4) were optimized as a continuous variable on the most-parsimonious trees from the combined analysis using linear and squared-change parsimony options in MacClade, version 3.0 (Maddison and Maddison, 1992). When measurements were available in the literature for more than one population and/or males and females (e.g., Martof and Rose, 1963; Means and Karlin, 1989), measurements were pooled into a grand mean. In some cases, mean values and/or raw data were not provided, and average values were estimated from the available information. Mean SVL was calculated for the following species based upon measurements from preserved specimens in the University of Kansas Museum of Natural History (Titus, 1992): *D. brimleyorum*, Polk Co., Arkansas (15 males, 6 females); *D. imitator*, Swain Co., North Carolina (14 males, 6 females); Pennsylvania *D. ochrophaeus*, Armstrong Co., Pennsylvania (17 males, 8 females); *D. quadramaculatus*, Macon Co., North Carolina (13 males, 6 females); and *D. welteri*, Harlan Co., Kentucky (12 males, 6 females).

Choosing among equally parsimonious reconstructions of characters often requires assumptions about the evolutionary process (Swofford and Maddison, 1992). In the a posteriori mapping of changes in length of the larval period and selective regime, we did not order the transformation series but did assume that changes between character states adjacent to one another in these transformation series are more likely than changes between nonadjacent states. Therefore, although we did not impose ordering on the character mapping, we did

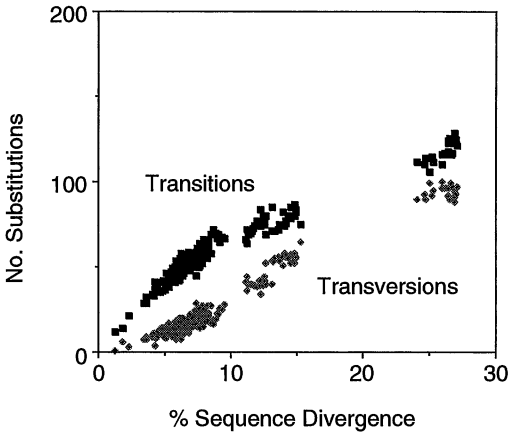

FIGURE 3. Transitions (■) and transversions (◆) in desmognathine sequences plotted as a function of corrected sequence divergence using a maximum likelihood model under which transitions are 10 times more frequent than transversions.

prefer optimizations that minimized unordered transformations.

RESULTS

Aligned Sequences

Alignment of sequences produced 1,204 nucleotide positions. Of the sites included in the phylogenetic analysis, 494 positions were variable and 259 positions were phylogenetically informative using the criterion of parsimony. The largest uncorrected divergence between paired sequences was 25.8% (*Notophthalmus viridescens–Eurycea wilderae*). Divergence among desmognathine species ranged from 13.4% (*P. hubrichti–D. wrighti*) to 1.9% (northern *D. quadramaculatus*–northern *L. marmoratus*). The smallest amount of divergence was 1.1%, which was between the two *D. fuscus conanti* sequences from animals separated by approximately 500 km.

Transitions and transversions increased in number with increasing sequence divergence under both maximum likelihood models (Fig. 3) and continued to increase at the highest levels of sequence divergence. This suggests that neither class of substitutions has been affected by widespread saturation.

Phylogenetic Analyses

Phylogenetic analysis using only the nucleotide sequences produced three equally most-parsimonious trees of 1,126 steps, with a consistency index for informative characters (CI) of 0.476 and a retention index (RI) of 0.444. The majority rule consensus of these three alternatives is illustrated in Figure 4. Two of the three trees supported North Carolina *D. f. fuscus* as the sister of *D. monticola* + *D. apalachicolae* and *D. brimleyorum* as the sister of all of these. Branch lengths for one of the mtDNA trees are illustrated in Figure 5. Bootstrap analysis indicates strong support (presence of a node in >70% of 1,000 pseudoreplicates) for eight branches on this tree, including those supporting the monophyly of the Desmognathinae, the genus *Phaeognathus* as the sister taxon to all other members of the subfamily, and the pygmy species *D. wrighti* as the sister lineage to the remaining taxa. Monophyly of *D. ochrophaeus* sequences from Virginia and Pennsylvania was strongly supported, but no tree placed all three *D. ochrophaeus* sequences together. Likewise, there was strong support for the monophyly of the Vermont *D. f. fuscus* and *D. auriculatus* sequences, but no tree supported monophyly of the two *D. f. fuscus* sequences. Monophyly of the *D. f. conanti* sequences and their sister-group relationship to *D. santeetlah* was well supported.

The combined analysis of mtDNA sequences and nonmolecular characters produced four equally most-parsimonious trees of 1,167 steps (CI = 0.470, RI = 0.443). The majority rule consensus tree is illustrated in Figure 6. Three of the four trees favored a sister-group relationship between *D. apalachicolae* and *D. monticola* and between Virginia + Pennsylvania *D. ochrophaeus* and *D. auriculatus* + Vermont *D. f. fuscus*. Branch lengths for one of the combined trees are illustrated in Figure 7. All four trees agreed in supporting *D. aeneus* as the sister taxon to all other species exclusive of *P. hubrichti* and *D. wrighti*. In all trees, the next lineage to diverge contained *D. quadramaculatus* and *L. marmora-*

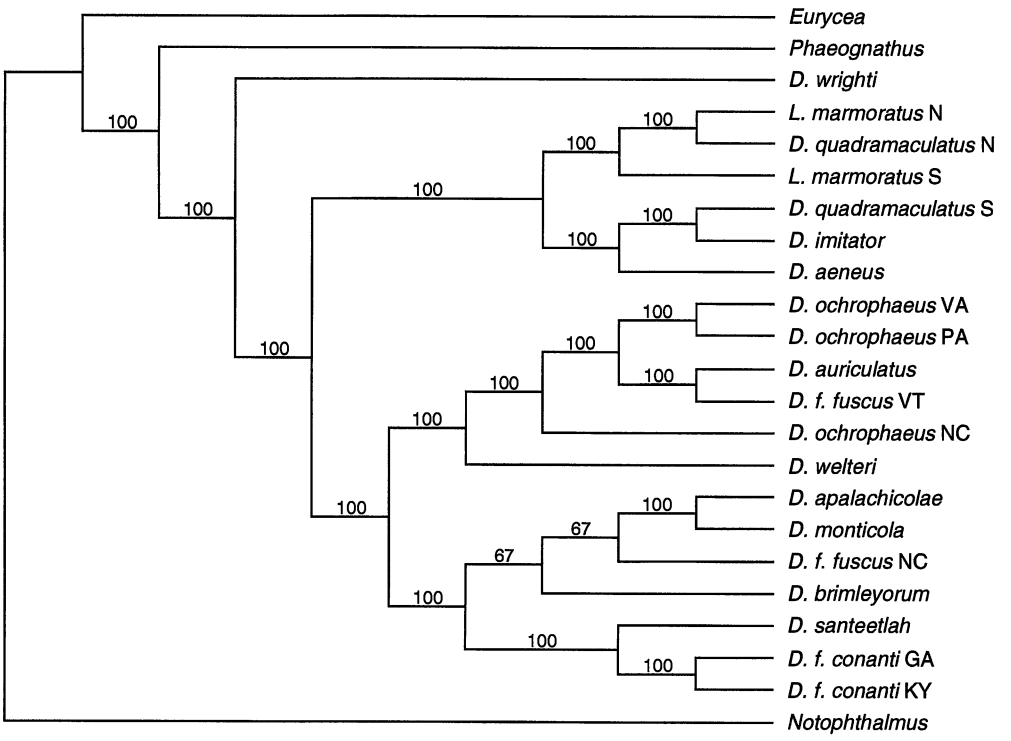

FIGURE 4. Majority rule consensus tree for desmognathines plus outgroups derived from three equally most-parsimonious trees based on mtDNA sequences only.

tus, within which the two *L. marmoratus* sequences formed a clade. None of the four trees supported monophyly of the *D. quadramaculatus*, *D. ochrophaeus*, *D. fuscus*, or *D. f. fuscus* sequences. Simultaneous analysis of molecules and morphology did not provide robust support for many branches of the desmognathine tree. Of the four trees derived from the simultaneous analysis of molecular and morphological data, only the one shown in Figure 7 provides the most-parsimonious optimization for larval period and selective regime; it is hereinafter referred to as the preferred tree.

Testing Evolutionary Hypotheses

Optimizations of larval period, selective regime, and body size on the preferred tree are illustrated in Figure 8. Optimization of selective regime required seven changes; homoplasy was the result of switching between the relatively similar seep and stream edge regimes. Of the equally parsimonious reconstructions, we preferred ACCTRAN (Farris, 1970) optimization because it required the fewest changes (one) between nonadjacent states of this transformation series. Three steps were required to accommodate evolutionary changes in larval period. All inferred changes represent acquisition or lengthening of the larval stage.

Changes in body size (SVL) were optimized on the preferred tree using linear and squared-change parsimony. For linear parsimony, both maximum and minimum values for ancestral nodes were obtained. Results of these optimizations were inspected to find branches that are inferred to show large changes (>7 mm) in body size using all three optimizations (squared change, linear minimum, linear maximum). Lineages for which large increases in body size are inferred on all optimizations include those immediately ancestral to *D. welteri* (9–16 mm), *D. monticola* (7–12

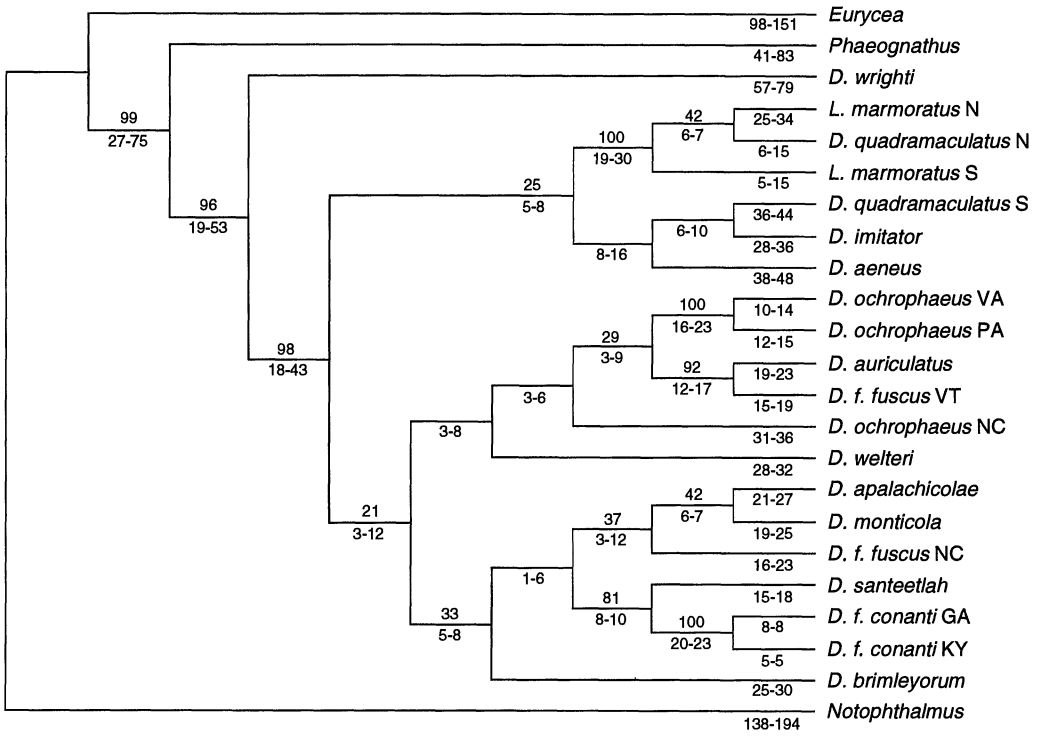

FIGURE 5. One of three equally most-parsimonious trees for desmognathines plus outgroups based on mtDNA sequences only. Numbers below each branch are minimum and maximum number of character changes. Number above each branch is the bootstrap value based on 1,000 pseudoreplications. Branches without bootstrap values did not appear in the resampling analysis.

mm), *D. brimleyorum* (13–24 mm), and *P. hubrichti* (47–75 mm) and the common ancestor of *D. quadramaculatus* and *L. marmoratus* (9–28 mm). Decreases in body size are inferred for lineages ancestral to *D. wrighti* (6–35 mm), *D. santeetlah* (7–11 mm), *D. apalachicolae* (3–8 mm), *D. ochrophaeus* from North Carolina (3–6 mm), and the remaining *D. ochrophaeus* (2–10 mm). Results of the different optimizations are ambiguous regarding evolutionary decrease in size on the lineage ancestral to *D. aeneus* (0–22 mm).

Alternative hypotheses of desmognathine phylogeny were tested against the preferred tree using the Wilcoxon rank sum test to ask whether these alternatives are significantly less parsimonious than the shortest trees (Templeton, 1983). The molecular and morphological characters were used together for these tests. A maximally parsimonious tree congruent with the ordered character state tree in Figure 1 representing aquatic-to-terrestrial evolution (Fig. 9) required a minimum of 28 additional steps and was significantly longer than the preferred tree ($n = 57$, $t_s = 3.1$, $0.01 < P < 0.001$). Monophyly of the directly developing pygmy species, *D. wrighti* and *D. aeneus*, required a minimum of 13 additional steps, and this tree was not significantly longer than the preferred tree ($n = 50$, $T = 475$, $P > 0.1$). Placing the genus *Leurognathus* as the sister taxon to *Desmognathus*, thus making *Desmognathus* monophyletic, added 25 steps and was significantly less parsimonious than the preferred tree ($n = 59$, $t_s = 3.47$, $P < 0.001$). Making *D. fuscus* monophyletic by placing the *D. f. fuscus* and *D. f. conanti* sequences together as monophyletic sister groups required 17 additional steps, and this tree

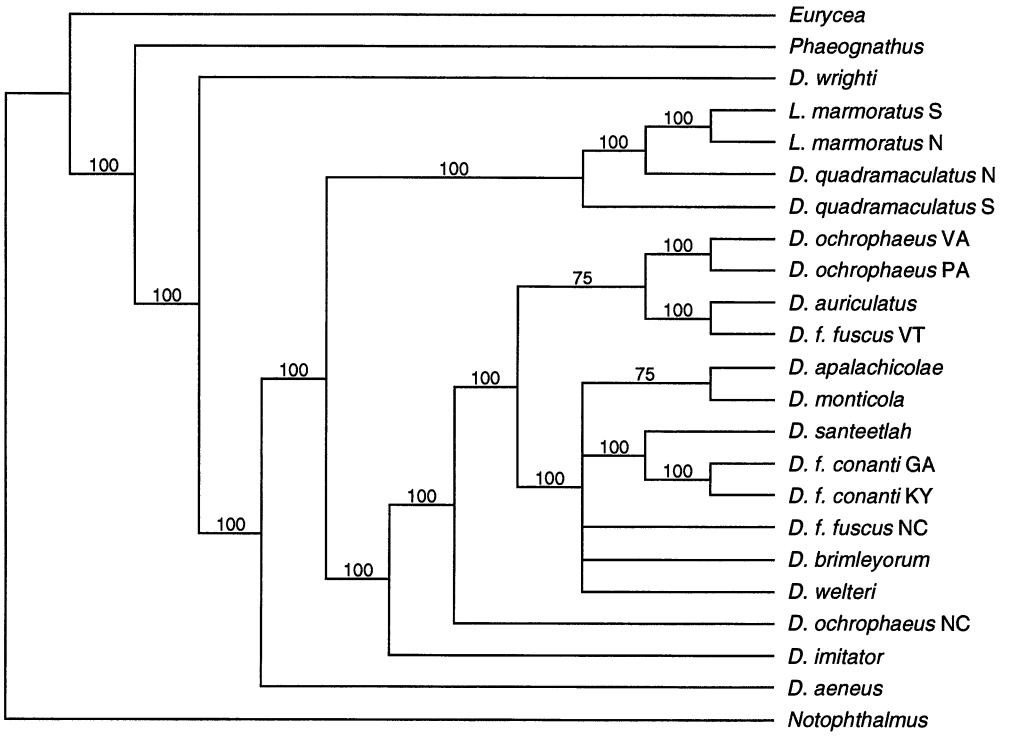

FIGURE 6. Majority rule consensus tree for desmognathines plus outgroups derived from four equally most-parsimonious trees based on simultaneous analysis of molecular and nonmolecular characters.

was not significantly less parsimonious than the preferred tree (n = 60, t_s = 1.6, $0.2 < P < 0.1$). Monophyly of the two *D. f. fuscus* sequences required 12 additional steps, and this tree was not significantly less parsimonious than the preferred tree (n = 37, T = 249.5, $P > 0.1$). Monophyly of the two *D. quadramaculatus* sequences required 15 additional steps, and this tree was not significantly less parsimonious than the preferred tree (n = 47, T = 400.5, $0.1 < P < 0.5$). Monophyly of the three *D. ochrophaeus* sequences required only two additional steps, and this tree was not significantly less parsimonious than the preferred tree (n = 23, T = 126.5, $P > 0.1$). Grouping *L. marmoratus* and species of *Desmognathus* that have large body size (*D. brimleyorum, D. auriculatus, D. monticola, D. quadramaculatus, D. welteri*) required 10 extra steps, and this tree was not significantly longer than the preferred tree (n = 46, T = 431.5, $P > 0.1$). The hypothesis that the small desmognathines, *D. aeneus, D. apalachicolae, D. ochrophaeus,* and *D. santeetlah,* form a monophyletic group was significantly rejected (19 extra steps, n = 51, t_s = 2.14, $0.05 > P > 0.02$).

The results indicate paraphyly for the genus *Desmognathus* because *Leurognathus* forms a clade with *D. quadramaculatus* and the cost to the most-parsimonious tree of placing *Leurognathus* outside a clade containing all species of *Desmognathus* is significant. A close relationship between *Leurognathus* and *D. quadramaculatus* has been suggested (Dunn, 1926; Noble, 1927; Martof, 1962; Wake, 1966; Hinderstein, 1971b), although this hypothesis was never framed in an explicitly phylogenetic context. Our analyses demonstrate that these taxa share derived attributes of morphology and life history, including extreme reduction of the cloacal tube in females and a larval period of 3–4 years. To eliminate confusion by the taxonomic implication that these features

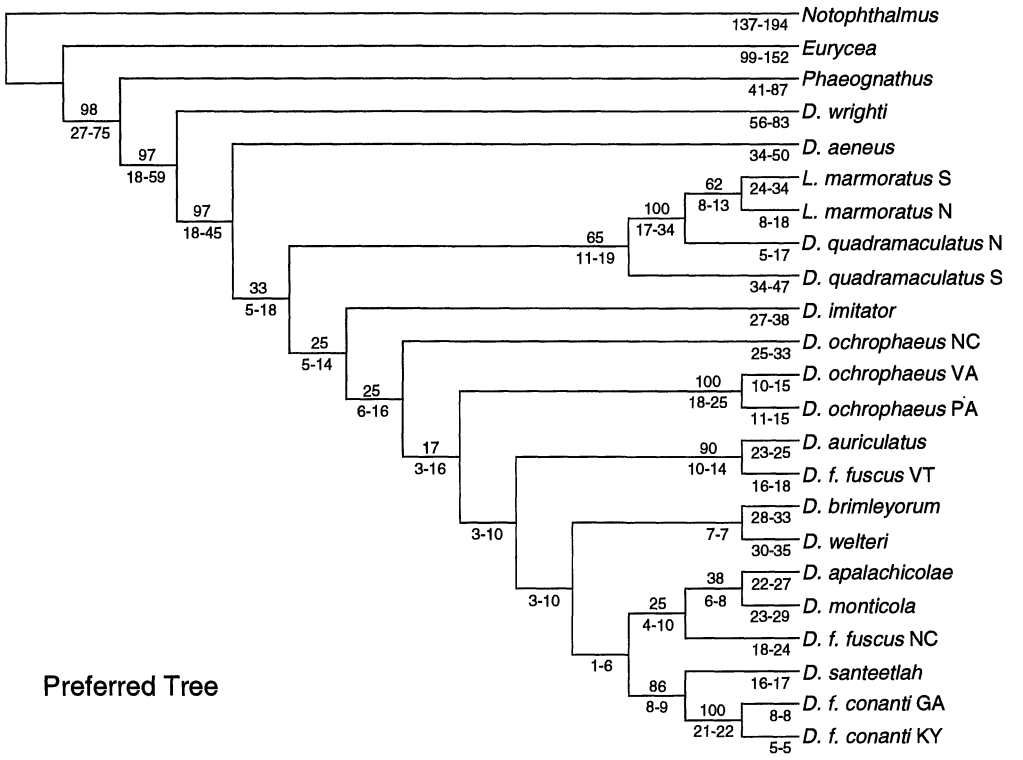

FIGURE 7. One of four equally most-parsimonious trees for desmognathines plus outgroups based on simultaneous analysis of the molecular and nonmolecular data. Numbers below each branch are minimum and maximum numbers of character changes. Number above each branch is the bootstrap value based on 1,000 pseudoreplications. Branches without bootstrap values did not appear in the resampling analysis.

arose independently in *Leurognathus* and *D. quadramaculatus*, we consider the monotypic *Leurognathus* Moore, 1899 a junior synonym of *Desmognathus* Baird, 1849 (new combination).

The cost to parsimony of placing the *D. f. fuscus* and *D. f. conanti* sequences in a clade was not significant. However, a sister-group relationship between *D. santeetlah* and *D. f. conanti* was supported by a high bootstrap value in both the molecular and combined analyses (81% and 86%, respectively), suggesting that *D. fuscus* may be polyphyletic. In addition, the *D. f. conanti–D. santeetlah* clade supported by the mtDNA data is congruent with studies of geographic variation in allozymes (Tilley and Schwerdtfeger, 1981; Karlin and Guttman, 1986) and morphometry (Tilley, 1981). One *D. f. conanti* sample was from a population within 30 km of the type locality in western Kentucky. In addition, the Kentucky and Georgia *D. f. conanti* sequences represent the extreme eastern and western limits of the range but differed by only 1.1% sequence divergence, and their monophyly was strongly supported (Fig. 7). These points indicate that the sister-group relationship between *D. f. conanti* and *D. santeetlah* is not the result of sampling error in the mtDNA analysis, and congruence between the mtDNA tree and trees derived from nuclear markers indicate no conflict between the mtDNA genealogy and the species phylogeny. Therefore, *Desmognathus fuscus conanti* Rossman is elevated to species status as *Desmognathus conanti* Rossman.

Our taxonomic rearrangement leaves two genera within the plethodontid subfamily Desmognathinae, the monotypic genus *Phaeognathus* and the diverse genus

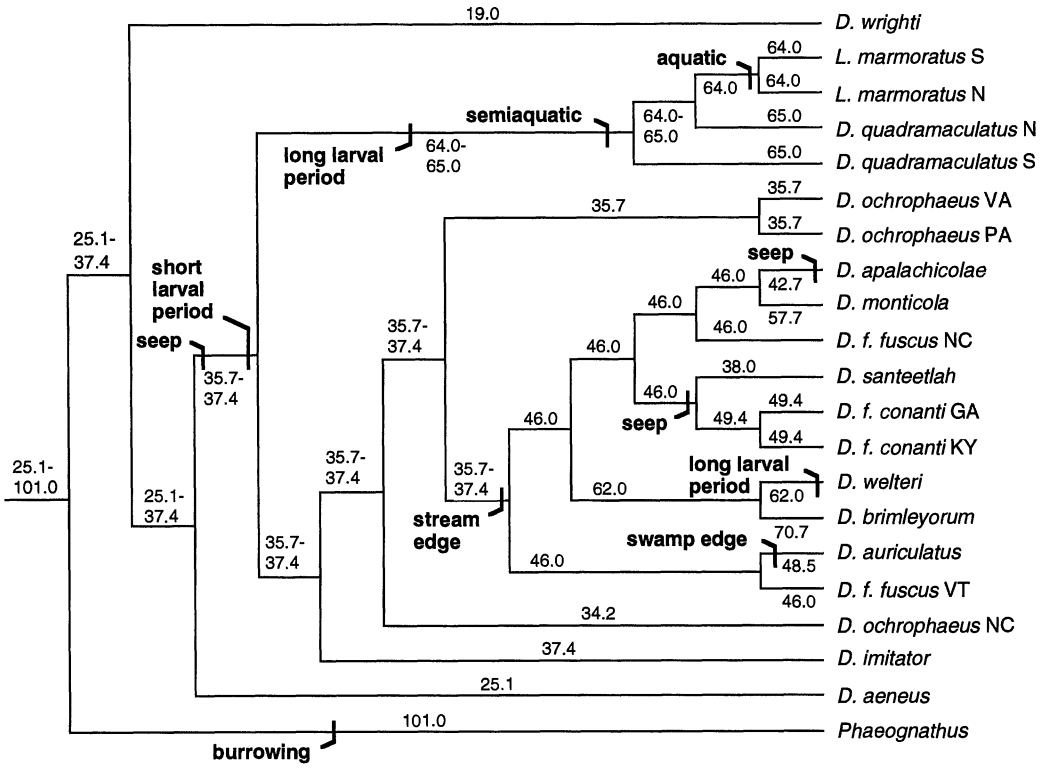

FIGURE 8. Optimization of body size (mean snout–vent length in millimeters) using linear parsimony (maximum and minimum values shown for all internal nodes), larval period, and selective regime on the preferred tree (Fig. 7).

Desmognathus. This arrangement has the advantage of retaining the familiar name *Desmognathus* for all species traditionally recognized as such and changing the generic name of only one species, *Desmognathus marmoratus*. An alternative scheme is to give new generic names to both *D. aeneus* and *D. wrighti* and to combine *D. marmoratus* and *D. quadramaculatus* under the generic name *Leurognathus*, leaving the remaining species in the genus *Desmognathus*. The subfamily Desmognathinae would then be divided into five genera, all of which have diagnostic characters and distinct life histories. This arrangement would highlight the major adaptive discontinuities within the Desmognathinae. The constricted genus *Desmognathus* would be more compact in terms of characters, distribution, and life history, and ecological experiments could be directed toward more meaningful comparisons. This revision might be desirable if further phylogenetic studies corroborate the relationships in Figure 7. Until such results are available, we prefer to minimize confusion by retaining as much as possible the generic names by which these salamanders are commonly known.

DISCUSSION

Evolutionary History of Metamorphosis

Our finding that the strictly terrestrial desmognathines form the three deepest branches in desmognathine phylogeny is unexpected and contradicts all previous hypotheses of desmognathine evolution. Our phylogenetic analysis does not support the notion that the ancestral desmognathines occupied a semiaquatic mountain-stream habitat resembling that of *D.*

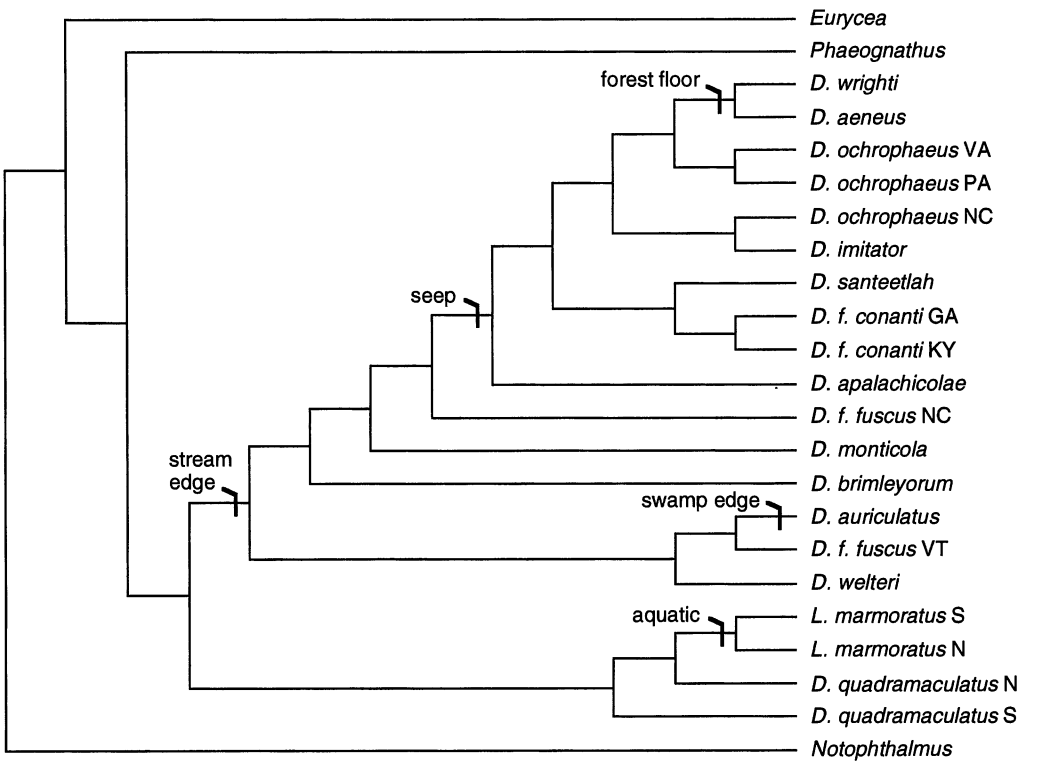

FIGURE 9. Most-parsimonious cladogram congruent with the character state tree in Figure 1 representing the evolution from a semiaquatic ancestor to a terrestrial habit in *Desmognathus* and an aquatic habit in *Leurognathus*. This tree required a significant cost in steps relative to the preferred tree (Fig. 7).

quadramaculatus (Dunn, 1926). Moreover, a tree consistent with a character-state tree exhibiting an ordered transformation from primitively semiaquatic to completely terrestrial species (Fig. 10) is rejected by our data. Parsimony suggests alternatively that absence of an aquatic larval stage may be ancestral for desmognathines and that the semiaquatic habit characteristic of *D. quadramaculatus* arose along the lineage immediately ancestral to *D. quadramaculatus* and *D. marmoratus*. Our results do, however, corroborate the hypothesis of Dunn (1926) that the highly aquatic habit of *D. marmoratus* was derived from the semiaquatic habit of the ancestor of *D. quadramaculatus*. Phylogenetic testing of the hypothesis that direct development is the ancestral state for desmognathines depends on resolution of relationships within desmognathines, in particular the position of *D. aeneus*, and on relationships among plethodontid outgroups. Branches supporting *P. hubrichti* and *D. wrighti* as the first and second divergence events within the subfamily are supported by bootstrap values of >90% in our analyses. However, the position of *D. aeneus* as the next lineage to diverge is not strongly supported. Figure 10 illustrates possible optimizations of direct development for the Plethodontidae using a tree corresponding to the "working hypothesis" of Lombard and Wake (1986). If future data corroborate the position of *D. aeneus* in our most-parsimonious tree, then direct development is inferred to have evolved either twice in the Plethodontidae, once in the most recent common ancestor to the Desmognathinae and again on the branch immediately ancestral to Plethodontini + Bolitoglossini, or once in the most recent common ancestor to the Plethodontidae,

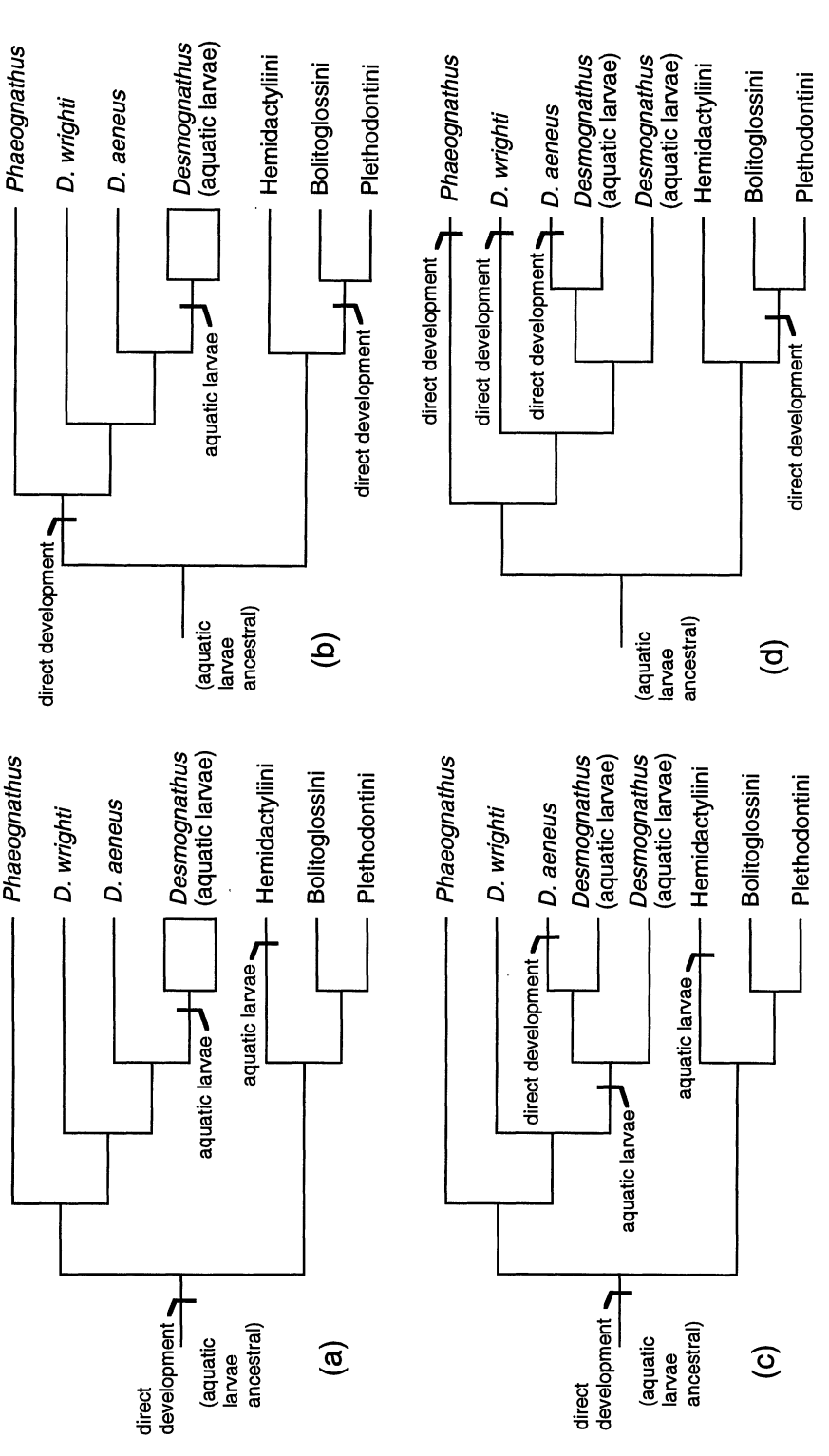

FIGURE 10. Alternative reconstructions for the evolution of direct development in the Plethodontidae. (a) Three basal desmognathine lineages showing direct development; ACCTRAN optimization. (b) Three basal desmognathine lineages showing direct development; DELTRAN optimization. (c) Two basal desmognathine lineages showing direct development; ACCTRAN optimization. (d) Two basal desmognathine lineages showing direct development; DELTRAN optimization.

depending on optimization method. However, if the position of *D. aeneus* changes so that it is separated on the desmognathine tree by even a single lineage with aquatic larvae, then direct development is inferred to have arisen either three times independently in the direct-developing desmognathines and again in the ancestor of Plethodontini + Bolitoglossini or in the most recent common ancestor of the Plethodontidae and again in *D. aeneus*, depending on optimization method.

Evaluation of these alternative evolutionary hypotheses of desmognathine metamorphosis is complicated by the fact that *D. aeneus*, *D. wrighti*, and *P. hubrichti* all represent ancient, unbranched lineages. The remaining clade of desmognathine salamanders shows that substantial evolutionary changes in ecology and life history are possible on the evolutionary time scale of these lineages. Furthermore, although these three species share direct development, their life histories are not identical. *Phaeognathus hubrichti* is the most terrestrial of these three species; it is a large animal with large eggs and presumably does not use standing water even for breeding. *Desmognathus aeneus* and *D. wrighti* are very small animals with small eggs, and they do use aquatic sites for breeding and guarding eggs. Only the lack of an aquatic larval stage is common to the life histories of all three species. For *D. aeneus* and *D. wrighti*, parallel loss of the aquatic larva may constitute a correlate of parallel evolution of miniaturization (required by both of the alternative hypotheses being discussed) in a selective regime featuring strong predation. Comparative developmental studies on desmognathines may indicate nonhomology of direct development of *D. aeneus*, *D. wrighti*, and *P. hubrichti* (Wagner, 1989). If these lineages are corroborated as the three most basal splits within desmognathines and their ontogenies can be interpreted as different states within a transformation series involving length of the larval period, direct development would appear ancestral for the Desmognathinae. If direct development occurs by shortening the larval stage and retaining it entirely within the egg, then reversal to a free-living aquatic larval stage seems plausible.

Evolutionary Radiation of Metamorphosing Desmognathines

The clade containing the metamorphosing desmognathines (all except *Phaeognathus, D. aeneus,* and *D. wrighti*; Fig. 5) appears to have undergone a rapid evolutionary radiation following its origin. Adaptive radiation used in the strict sense of Simpson (1953:223) denotes an almost simultaneous divergence of numerous branches from the same ancestral lineage into different adaptive zones. A prediction based on this model is that phylogenetic reconstruction for such lineages is unlikely to produce a well-resolved dichotomous tree. Particularly for radiations that occurred in the distant evolutionary past, the major lineages will trace to a single point, represented by a star phylogeny. A major problem in phylogenetic reconstruction is to determine when lack of phylogenetic resolution represents either insufficient information or the presence of characters whose rate of evolution is poorly matched to the phylogenetic question being asked and when it represents an evolutionary radiation where phylogenetic branching events occurred nearly simultaneously and for which even well-chosen characters are unlikely to produce a strictly dichotomous tree.

The relationships among the major lineages of metamorphosing desmognathines are the least well-resolved part of our phylogenetic tree. Several observations suggest that this area of the tree contains short internodes resulting from rapid cladogenesis and that incomplete phylogenetic resolution is not just an artifact of insufficient variation in the molecular sequences being studied. Sequences show 4–9% divergence among these lineages, and there are 159 phylogenetically informative sites at this phylogenetic level, indicating that the variation needed potentially to resolve internodes is present. Another possible cause for low resolution is evolutionary saturation of molecular characters, but three dif-

ferent observations suggest that saturation is not a problem here. First, the two deepest divergences within desmognathines, events older than the branching events in question, are well resolved as indicated by our statistical analyses. Second, scatter plots of transitions and transversions against maximum likelihood estimates of sequence divergence within desmognathines (Fig. 3) indicate an increase in both classes of substitutions at the highest levels of sequence divergence observed here. Third, uncorrected sequence divergence among metamorphosing taxa is always <10%, below the level at which global saturation is expected for these genes (Mindell and Honeycutt, 1990). Furthermore, an independent analysis of nuclear DNA-encoded allozyme markers also indicated that many of these species are connected by short internal branches (Karlin and Guttman, 1986). An ancient and rapid radiation of desmognathine lineages into seepage and stream-edge habitats therefore is suggested.

Evolutionary Increases in Body Size and Use of Aquatic Habitats

Inferred evolutionary changes in body size suggest either (1) the appearance of small size in a common ancestor of all desmognathines excluding *Phaeognathus* or (2) parallel occurrence of miniaturization in *D. aeneus* and *D. wrighti* (Fig. 9). The first interpretation contradicts the commonly held view that desmognathines have undergone primarily a phylogenetic decrease in size (reviewed by Hairston, 1987). However, size increase is the more common pattern in other organisms (LaBarbera, 1986; McKinney, 1990). Evolution of an extremely small body can have dramatic effects on morphology, life history, and behavior (Hanken and Wake, 1993). Thus, attributes that seem to be correlated with miniaturization in *D. aeneus* and *D. wrighti*, such as male biting behavior during courtship (Promislow, 1987) and living on the forest floor, may have been derived in parallel.

The desmognathine phylogeny (Fig. 9) suggests at least three independent derivations of large size from smaller ancestors: in the *D. marmoratus–D. quadramaculatus* lineage, the *D. welteri–D. brimleyorum* lineage, and the *D. monticola* lineage. New hypotheses are needed to explain these size increases. The inferred lengthening of larval stages in some lineages challenges Organ's (1961b) suggestion that higher mortality in aquatic environments has selected for shorter larval periods in desmognathines; our results suggest that the small terrestrial *Desmognathus* may have evolved before their large predatory congeners, which were formerly considered a cause of the evolutionary origin of the smaller species.

Hairston (1986) demonstrated that experimental removal of smaller species negatively affects larger predators. The apparent importance of smaller salamanders as prey items could have selected for large body size in the *D. quadramaculatus–D. marmoratus* lineage as a means of facilitating use of this resource. Southerland (1986b) demonstrated that *D. monticola* is both prey of and a competitor with *D. quadramaculatus* and hypothesized that these factors led to a phylogenetic decrease in size in the *D. monticola* lineage that was constrained by the effects of desiccation. However, *D. monticola* represents an increase in size (Fig. 9), apparently as a result of rapid postmetamorphic growth (Bruce, 1989). Alternative explanations consistent with the evolution of large size in *D. monticola* include ameliorating the effects of desiccation, reducing the risk of predation by *D. quadramaculatus*, or increased efficiency of predation on *D. ochrophaeus*. Competition with *D. quadramaculatus* would have been an outcome of this size increase (Hairston, 1986; Southerland, 1986b) and may have constrained the final size attained by *D. monticola*. These hypotheses should be given high priority for further testing with ecological experiments.

Our analysis corroborates a recent hypothesis that long larval period is a derived attribute of the common ancestor of *D. quadramaculatus* and *D. marmoratus* (Bruce, 1991) and has evolved independently in *D. welteri*. This increase in larval

period could be adaptive. Sweet (1973) suggested that if larval survivorship is higher than that of juveniles, evolution would be in the direction of prolongation of the larval period. Appalachian mountain streams may have provided stable aquatic habitats that enhanced larval survivorship and favored a longer larval period, as predicted by the model of Wilbur and Collins (1973). Long larval periods could have evolved in response to saturation of available breeding sites and associated selection for delayed maturation (Bruce, 1990).

Our analysis suggests a revision of adaptive hypotheses for the origins of nine morphological characters that desmognathines share uniquely among plethodontid salamanders (Soler, 1950; Wake, 1966; Hinderstein, 1971a; reviewed by Schwenk and Wake, 1993): (1) heavily ossified and strongly articulated skull and mandible, (2) flat, wedgelike head profile, (3) stalked occipital condyles, (4) modified atlas, (5) modified anterior trunk vertebrae, (6) atlanto-mandibular ligaments, (7) enlarged dorsal spinal muscles, (8) enlarged quadrato-pectoralis muscles, and (9) hind limbs relatively larger than forelimbs. Characters 1, 2, 5, 6, 7, and 9 are associated with burrowing and wedging between rocks, and characters 3, 4, 6, and 8 are associated with feeding. The atlanto-mandibular ligaments (character 6) are associated with both activities. Schwenk and Wake (1993) demonstrated that the atlanto-mandibular ligaments are used in the unique "head tuck" behavior exhibited by desmognathines. This behavior is effective in producing a static-pressure bite during feeding and for moving the head during burrowing and wedging. Schwenk and Wake (1993) hypothesized that the atlanto-mandibular ligaments represent a key innovation that allowed the common ancestor of the Desmognathinae to reinvade stream habitats as metamorphosed adults. Evidence for this secondary invasion comes from functional data showing that despite feeding entirely underwater, adults of *D. marmoratus* exhibit tongue protrusion, a characteristic attribute of terrestrial feeding (Schwenk and Wake, 1988). Our phylogeny suggests that use of aquatic habitats by metamorphosed adults is derived within desmognathines and is not a characteristic of their most recent common ancestor. Occupation of the aquatic selective regime by metamorphosed adults occurred at a less inclusive level than proposed by Schwenk and Wake (1993). Tongue protrusion in aquatic adult *D. marmoratus* has been retained from a condition characteristic of the terrestrial adult desmognathine ancestor. A terrestrial adult stage should be considered when evaluating the ecological context and selective factors accompanying the origin of the atlanto-mandibular ligaments and associated morphological novelties.

All of these hypotheses regarding the evolution of ecology, life history, and morphology in desmognathines depend upon the principle of parsimony. Departure of these hypotheses from commonly held views on evolution within desmognathines may lead some to question whether parsimony is an appropriate criterion for analyzing these characters (see discussion by Larson and Losos, 1996); conclusions drawn using parsimony would be in error, for example, if there were strong orthogenetic tendencies in character evolution. Orthogenetic tendencies, in which developmental or metabolic constraints impart directionality to character variation, have been observed in the evolution of coat color in mammals (Jacobs et al., 1995) and could apply also to the evolution of life histories and correlated ecological and morphological attributes in salamanders. We favor the results of parsimony as currently the best framework for developing hypotheses of desmognathine evolution. Tests of the ecological hypotheses generated from our phylogenetic analysis will help to determine whether hypotheses of orthogenetic evolution of desmognathine life history are preferable.

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APPENDIX
MORPHOLOGICAL CHARACTERS

The following morphological characters were obtained from diverse sources as cited. Characters were verified and extended whenever possible to additional species by examining preserved specimens at the University of Kansas Museum of Natural History (Titus, 1992).

1. *Lower jaw commissure of adult males.*—The lower jaw commissure is either markedly sinuate (0) or not markedly sinuate (1) in adult males (Means, 1974; Tilley, 1981; Titus, 1992).
2. *Vomerine teeth.*—Vomerine teeth are either retained (0) or lost (1) in adult males (Dunn, 1916, 1917; King, 1936; Brown and Bishop, 1947; Bishop and Valentine, 1950; Rossman, 1958; Martof, 1962; Brandon, 1965; Valentine, 1974; Juterbock, 1984; Means and Karlin, 1989). Intraspecific variation has been reported for *Leurognathus marmoratus* (Martof, 1962) and *Desmognathus monticola* (Juterbock, 1984), so the character was coded as polymorphic for these species.
3. *Egg deposition.*—Eggs may be deposited in a grapelike cluster, not attached by individual stalks (0) or attached by individual stalks (1) (Hilton, 1909; Wilder, 1913; Pope, 1924, 1928; Bishop, 1941; Neill and Rose, 1949; Bishop and Valentine, 1950; Organ, 1961a, 1961b; Martof, 1962; S. G. Tilley, pers. comm.).
4. *Premaxillary fontanelle.*—A premaxillary fontanelle is absent (0), present and well developed (1), or present but reduced (2) (Martof, 1962; Brandon, 1965; Wake, 1966; Means, 1974).
5. *Tooth crown.*—The tooth crown is either sharp and recurved (0) or fungiform (1) in shape (Means, 1974; Caldwell and Trauth, 1979; Tilley, 1981).
6. *Melanophores on testes.*—Melanophores may be absent (0) or present (1) on testes (Dunn, 1917; Barbour, 1950; Martof, 1962; Brandon, 1965; Titus, 1992).
7. *Female ventral glands.*—Ventral glands may be present (0) or absent (1) in cloacae of females; *D. quadramaculatus* exhibits both conditions and was coded as polymorphic (Sever and Trauth, 1990).
8. *Female anterior dorsal glands.*—Anterior dorsal glands may be absent (0) or present (1) in cloacae of females (Sever and Trauth, 1990). Anterior and posterior dorsal glands are absent in *D. aeneus, D. wrighti, D. fuscus* from Ohio, and *D. ochrophaeus* from West Virginia, but other species have posterior, anterior, or both glands, indicating that the two gland groups are potentially independent and should be coded as two transformation series rather than as a single complex character. *Desmognathus fuscus* from Ohio are treated as *D. f. fuscus*, whereas *D. fuscus* from Alabama are within the range of *D. f. conanti*. *Desmognathus ochrophaeus* as characterized by Sever and Trauth (1990) includes localities in West Virginia, Tennessee, and North Carolina that were not sampled in this study. Allozymic data indicate that *D. ochrophaeus* may be a complex of species with additional forms that have not been characterized adequately (S. G. Tilley, pers. comm.). Rather than assume that the *D. ochrophaeus* in this study and those sampled by Sever and Trauth (1990) are conspecific, *D. ochrophaeus* populations were coded as unknown for this character.
9. *Female posterior dorsal glands.*—Posterior dorsal glands may be present (0) or absent (1) in the cloacae of females (Sever and Trauth, 1990). For the same reasons discussed for female anterior dorsal glands, *D. ochrophaeus* populations were coded as unknown for this character.
10. *Female cloacal tube.*—A cloacal tube may be present (0) or absent (1) in females (Sever and Trauth, 1990). Sever and Trauth (1990) reported the cloacal tube as 10–30% of the total cloacal length in all species having a cloacal tube except *P. hubrichti*. Sever (pers. comm.) suggested that *P. hubrichti* exhibits yet another state; the cloacal tube is present but is only 5% of the total length of the cloaca. Because variation among species in length of the cloacal tube is not well quantified, we have coded this character conservatively.
11. *Male anterior ventral glands.*—In males, anterior ventral glands of the cloaca may be enlarged, extending inferiorly to the inner edge of the cloacal orifice and posteriorly to the caudal one-fourth of the cloacal chamber (0), or they may extend only to the midpoint of the cloacal chamber (1) (Sever, 1983).
12. *Male cloacal dorsolateral recesses.*—In males, dorsolateral recesses may be absent (0), small and shifted ventrally and posteriorly (1), or well developed (2) (Sever, 1983).
13. *Internal nares.*—Internal nares may open medially (0) or laterally (1) (Wake, 1966).