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PHYLOGENETIC EVIDENCE FOR A MAJOR REVERSAL OF LIFE-HISTORY EVOLUTION IN PLETHODONTID SALAMANDERS

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Abstract.—The transition from aquatic to terrestrial eggs is a key evolutionary change that has allowed vertebrates to successfully colonize and exploit the land. Although most amphibians retain the primitive biphasic life cycle (eggs deposited in water that hatch into free-living aquatic larvae), direct development of terrestrial eggs has evolved repeatedly and may have been critical to the evolutionary success of several amphibian groups. We provide the first conclusive evidence for evolutionary reversal of direct development in vertebrates. The family Plethodontidae (lungless salamanders) contains the majority of salamander species, including major radiations of direct developers. We reconstruct the higher level phylogenetic relationships of plethodontid salamanders using molecular and morphological data and use this phylogeny to examine the evolution of direct development. We show that the predominantly biphasic desmognathines, previously considered the sister group of other plethodontids, are nested inside a group of direct-developing species (Plethodontini) and have re-evolved the aquatic larval stage. Rather than being an evolutionary dead end, the reversal from direct developing to biphasic life history may have helped communities in eastern North America to achieve the highest local diversity of salamander species in the world.

Key words.—Amphibians, ancestral states, direct development, larvae, life history, phylogeny, Plethodontidae.

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Direct development is a reproductive mode in which embryos develop without a free-living aquatic stage, allowing eggs to be deposited on land without the need for free-standing water (Duellman and Trueb 1986; Pough et al. 2001). The evolution of direct development was a key transition in vertebrate history because it allowed colonization of the land and diversification in terrestrial habitats without dependence on aquatic habitats for reproduction (Pough et al. 2001). The evolution of direct development in vertebrates obviously has had major consequences for their evolution and ecology and has been important in shaping terrestrial environments throughout the world (e.g., Huntly 1991; Begon et al. 1996).

Studies of life-history evolution in living amphibians can offer many unique insights into this critical transition, for at least two reasons. First, amphibians are unusual among vertebrates in that many retain a biphasic life cycle (aquatic larvae, terrestrial adults) that is intermediate between the fully aquatic life cycle of fishes and basal chordates and the terrestrial life cycle of amniotes (Duellman and Trueb 1986; Pough et al. 2001). Second, there appear to have been many evolutionary transitions from this biphasic life cycle to direct development in amphibians (Wake 1989; Hanken 1999). Thus, amphibians retain a key intermediate step in the evolution of direct development, and the critical transition has been replicated many times, facilitating studies of this ancient shift among closely related living taxa.

Direct development appears to have independently and repeatedly evolved in all three of the major groups of living amphibians (Duellman and Trueb 1986; Wake 1989; Hanken 1999), the anurans (frogs and toads), gymnophionans (caecilians), and caudates (salamanders). In an intriguing parallel to the broader pattern in vertebrates, many groups of direct-developing amphibians have been extremely successful, at least in terms of species richness. For example, the most speciose genus of vertebrates (*Eleutherodactylus*) consists of more than 500 species of exclusively direct-developing frogs (Hanken 1999; Frost 2002; Amphibiaweb 2003), and as many as 19 species of *Eleutherodactylus* may be found sympatrically at a given site (e.g., Lynch and Burrowes 1990). Similarly, the majority of salamander species belong to a single clade (Bolitoglossini) that consists entirely of direct-developing species (Wake 1966; Wake and Hanken 1996; Frost 2002; Amphibiaweb 2003); this clade is nested within the Plethodontidae, one of the most recently derived salamander families (Larson and Dimmick 1993; Wiens et al. 2004). Direct-developing plethodontids have been highly successful by almost any definition, can occur at extremely high densities (e.g., Ovaska and Gregory 1989; Welsh and Lind 1992), and may comprise the bulk of vertebrate biomass in some North American ecosystems (Burton and Likens 1975; Ducey et al. 2001).

We examine the evolution of direct development among the major groups of plethodontid salamanders using a phylogenetic approach. Based on the traditional hypothesis of plethodontid relationships, direct development is thought to have evolved multiple times within Plethodontidae (Wake and Hanken 1996). Plethodontid salamanders have been the focus of many studies in ecology and evolution (see Bruce et al. 2000 and references therein), but few studies have addressed the higher level relationships of the group. Lombard and Wake (1986) provided a hypothesis for higher level relationships based on 30 morphological characters. Although their hypothesis has been widely used in comparative evolutionary studies of plethodontids (e.g., Wake and Hanken

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1996; Jockusch 1997), it was not the most parsimonious hypothesis for the data then available and is not strongly supported.

Traditionally, Plethodontidae has been divided into the subfamilies Plethodontinae (containing tribes Bolitoglossini, Hemidactyliini, and Plethodontini) and Desmognathinae (Wake 1966; Lombard and Wake 1986). The generally accepted phylogenetic hypothesis for the group (Lombard and Wake 1986) posits a basal split between Desmognathinae and Plethodontinae; within Plethodontinae, Hemidactyliini is thought to be sister to (Bolitoglossini + Plethodontini). Although a biphasic life cycle is thought to be ancestral for Plethodontidae (Wake 1966; Collazo and Marks 1994; Wake and Hanken 1996), direct development occurs in the majority of plethodontids, including all Plethodontini and Bolitoglossini and three of the 21 species of desmognathines (Wake 1966; Titus and Larson 1996; Wake and Hanken 1996; Marks and Collazo 1998; Amphibiaweb 2003). The remaining desmognathines and all members of Hemidactyliini have free-living aquatic larvae.

In this study, we analyze the higher level phylogenetic relationships of plethodontids using character data from morphology and other nonmolecular characters, two mitochondrial genes, and one nuclear gene. We then use this phylogeny to reconstruct evolutionary changes in direct development. We find that the desmognathines, previously considered the sister group of other plethodontids, are nested inside a group of direct-developing species (Plethodontini). This result strongly suggests that direct development was lost and the aquatic larval stage re-evolved within desmognathines. The possibility of such a reversal has been suggested but only weakly supported in previous studies of hemiphractine hylid frogs and desmognathine plethodontid salamanders (Duellman and Hillis 1987; Duellman et al. 1988; Titus and Larson 1996), and reversal of direct development in plethodontids was deemed unlikely (from a developmental standpoint) by Wake and Hanken (1996). We provide the first rigorous evidence that direct development has been lost in a group of vertebrates. Rather than limiting evolutionary potential, we suggest that the loss of direct development may have important consequences for ecology and evolution. Our study adds direct development to the growing list of seemingly important and adaptive features that have been lost or reversed over evolutionary time scales (e.g., Wiens 2001a; Porter and Crandall 2003).

Materials and Methods

Taxon Selection and Sampling

DNA sequence and nonmolecular (primarily morphological) data were obtained for 31 species of Plethodontidae, including multiple divergent lineages within Bolitoglossini, Hemidactyliini, Plethodontini (Plethodontinae), and Desmognathinae. Taxa were chosen based on previous studies of relationships (Wake 1966; Wake and Elias 1983; Lombard and Wake 1986; Titus and Larson 1996) and within-group analyses currently in progress (P. T. Chippindale, R. M. Bonett, and J. J. Wiens, unpubl. data). Thirteen representatives of eight of the nine remaining families of salamanders (all but Hynobiidae) were included as outgroups to ensure correct placement of the root of the plethodontid tree.

Our sampling of plethodontids clearly was not exhaustive relative to the number of species and genera within the family. Most of the diversity of species and genera of plethodontids is within the neotropical Bolitoglossini, but these species form a monophyletic group (supergenus *Bolitoglossa*; Wake 1966), according to previous authors and our own data. We sampled the presumed basal lineages of neotropical bolitoglossines (Wake and Elias 1983) plus *Batrachoseps* (supergenus *Batrachoseps*) from western North America. We included almost all genera of nonbolitoglossine plethodontid salamanders (with the exception of two monotypic genera, *Typhlotriton* and *Haideotriton*, which are deeply nested within Hemidactyliini; Bonett and Chippindale 2004; P. T. Chippindale, R. M. Bonett, A. S. Baldwin, and J. J. Wiens, unpubl. data). Furthermore, we sampled multiple representatives of all speciose genera (i.e., *Aneides*, *Desmognathus*, *Eurycea*, *Plethodon*).

Morphological Data and Analysis

Morphological data were obtained from cleared-and-stained skeletal preparations and from the literature. Many osteological characters originally were described by Wake (1966) and were confirmed and coded based on our own observations. Additional morphological data were obtained from the literature, including data from histological studies of vertebral (Edwards 1976), auditory (Lombard 1977), cloacal (Sever 1991, 1994), and tongue morphology (Lombard 1977, Lombard and Wake 1986). Specimens examined are listed in online Appendix 1, characters are listed and described in online Appendix 2, and the matrix of characters used for phylogenetic analysis is given in online Appendix 3 (appendices are available online only at http://dx.doi.org/10.1554/04-185.1.s1). For many of these characters from the literature, data were not available for every species included in our analyses. In some of these cases, character states that were known for some species of a genus were generalized to congeners, as long as no intrageneric variation had been reported. If intrageneric variation was reported, species for which data were unavailable were coded as unknown. In one case, morphological data for one species were combined with molecular data for a closely related congener (morphology: *Taricha torosa*; DNA: *T. rivularis*). Anatomical terminology generally follows Duellman and Trueb (1986).

Most characters involved qualitative differences that were easily described using binary coding (e.g., presence vs. absence of a structure). Therefore, qualitative coding of morphological variation generally was used. Polymorphisms in binary characters were analyzed using the frequency step-matrix approach (Wiens 1995, 1999). In the few cases where an individual exhibited different states on different sides (asymmetry), each side was counted separately (as one-half of an individual) in calculations of the frequency for that species. This convention makes sense biologically in that individuals that exhibit bilateral variation presumably have intermediate conditions for whatever genetic and/or ontogenetic mechanisms control the expression of the trait. Many characters in which polymorphism was not actually observed

were also coded using the frequency approach; this has no impact on the results but should facilitate future analyses if polymorphisms are discovered subsequently. Multistate characters involving variation along a single axis (e.g., length of a structure or number of structures) were ordered. Other multistate characters generally were unordered. One quantitative meristic character (vertebral number) was analyzed using step-matrix gap weighting, with between-character scaling (Wiens 2001b). The maximum number of steps for all step-matrix characters was 100. Thus, step-matrix characters were weighted by 0.01 relative to other characters in phylogenetic analyses to maintain equivalent weights (i.e., the cost of a transition between fixed character states is the same for all morphological and molecular characters).

All parsimony analyses were conducted using heuristic searches with 100 random-taxon-addition replicates in PAUP* version 4.0b10 (Swofford 2001). Nonparametric bootstrap values were calculated using 1000 pseudoreplicates with 10 random-taxon-addition replicates per pseudoreplicated data matrix. Bootstrap values greater than 70% were considered strongly supported, following Hillis and Bull (1993; but see their caveats).

Our sample of ingroup and outgroup taxa included several paedomorphic species (or more specifically, neotenic, those reproducing in a gilled, aquatic larval stage; Gould 1977; but for discussion of terminology see McKinney and McNamara 1991; Hanken 1999). The morphology of sexually mature individuals of paedomorphic species may not be comparable to the adult morphology of transforming species, and inclusion of these taxa as adults may have a misleading impact on phylogenetic analyses (e.g., Wiens et al. 2004). Therefore, paedomorphic taxa were coded as unknown for characters involving adult morphology (following Wiens et al. 2004) and were excluded from analyses of morphological data alone. The paedomorphic taxa were *Cryptobranchus alleganiensis*, *Pseudobranchus axanthus*, *Siren intermedia*, *Dicamptodon copei*, *Necturus maculosus*, *Amphiuma means*, *Amphiuma pholeter*, *Eurycea neotenes*, and *Eurycea tonkawae*.

The nonmolecular dataset includes two nonmorphological (chromosomal and developmental) characters. Because the overwhelming majority of nonmolecular characters are morphological, we will refer to this dataset as ''morphology'' for the remainder of this paper.

Molecular Data

Sequences were obtained for portions of the mitochondrial cytochrome *b* (cyt *b*) and NADH dehydrogenase subunit 4 (ND4) genes and the nuclear recombination-activating gene 1 (RAG-1). Specimens examined for molecular data are listed in Appendix 4 (see appendices available online only), together with GenBank accession numbers for the corresponding sequences. Genomic DNA was extracted from frozen or ethanol-preserved specimens using standard protocols. Sequences were amplified by polymerase chain reaction (PCR) in MJ Research (Waltham, MA) thermocyclers using high-fidelity polymerases and primers from the literature (cyt *b*, Moritz et al. 1992; ND4, Arevalo et al. 1994; RAG-1, Greenhalgh et al. 1993; Ventakesh et al. 2001), or numerous taxon-specific primers designed for this study by P. T. Chippindale, primarily for RAG-1. Sequences of these primers may be obtained from P. T. Chippindale.

PCR products were purified by agarose gel extraction and cycle-sequenced using either ABI BigDye 3.0/3.1 chemistry on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA), or bidirectionally with a minimum of two cloned PCR products per amplification using Thermosequenase chemistry (United States Biochemical Corp., Lincoln, NE) on a LiCor 4200L automated sequencer (LiCor, Lincoln, NE). Sequences were aligned and edited using Sequencher 3.1.1 (Gene Codes Corp., Ann Arbor, MI). Alignments were unambiguous, with only rare, single-codon indels in ND4 for a few taxa.

Molecular and Combined-Data Phylogenetic Analyses

Trees were reconstructed using maximum parsimony and Bayesian methods. Parsimony analyses were conducted using heuristic searches with 100 random-taxon-addition replicates in PAUP* version 4.0b10 (Swofford 2001). Morphological, mitochondrial (cyt *b* and ND4), and nuclear (RAG-1) data were analyzed both separately and combined.

Bayesian analyses were performed using MrBayes version 3.0 (Huelsenbeck and Ronquist 2001), with 4,050,000 generations per analysis, discarding the first 50,000 generations as burn-in (stationarity of likelihoods was reached before this point in all analyses). Models of sequence evolution (HKY + I + Γ for cyt *b* and ND4; GTR + I + Γ for RAG-1) were determined by likelihood-ratio tests using ModelTest version 3.06 (Posada and Crandall 1998) and applied to separate, unlinked partitions (individual genes) in the combined Bayesian analyses. Analyses used four chains and uniform priors (i.e., specific values of model parameters were not defined a priori).

We do not favor Bayesian methods over maximum likelihood on statistical or philosophical grounds. We used Bayesian analysis because it is the only method for which currently available software packages allow effective tree searching with application of separate models and model parameters to different data partitions (i.e., use of maximum likelihood would have required us to apply identical model parameters to all genes, nuclear and mitochondrial, even though they clearly are evolving under very different rates and substitution patterns).

All trees based on analyses that included molecular data were rooted with representatives of the putative basal-most salamander lineages Cryptobranchoidea (Cryptobranchidae and Hynobiidae) and Sirenidae (Larson and Dimmick 1993; Wiens et al. 2004). Likelihoods of parsimony and Bayesian trees based on mitochondrial DNA alone, RAG-1 alone, and combined mitochondrial DNA and RAG-1 data were tested against likelihoods of topologies constrained for: (1) basal split between Desmognathinae and Plethodontinae; (2) monophyly of Plethodontini; and (3) the traditional tree: (Desmognathinae (Hemidactyliini (Plethodontini, Bolitoglossini))), using the method of Shimodaira and Hasegawa (1999; see also Goldman et al. 2000) implemented in PAUP* (Swofford 2001).

Progress in phylogenetics requires a methodology for reconciling conflicts between datasets. We favor the following

approach, described and justified by Wiens (1998). First, we perform separate analyses of different datasets to identify areas of phylogenetic congruence and conflict. Next, we combine the relevant data to generate a preferred hypothesis, but consider areas of conflict between trees that are strongly supported by each dataset to be ambiguously resolved in the combined-data tree, until the misleading signal is identified or a majority of unlinked datasets favors one hypothesis over another. We found almost no areas of strongly supported conflict in the separate analyses of the mitochondrial DNA, nuclear DNA, and morphological datasets (and none relevant to our key arguments). We performed combined analyses of the mitochondrial DNA data and the RAG-1 plus mitochondrial DNA data using parsimony and Bayesian methods, and combined analysis of the molecular and morphological data using parsimony analysis only. Although it is possible to combine morphological and molecular data for Bayesian analysis (e.g., Nylander et al. 2004), a combined Bayesian analysis would have required us to ignore frequency information from the many polymorphic characters (current methods for Bayesian analysis of morphology do not incorporate frequency information). Trees based on the combined molecular and morphological data using parsimony were very similar to trees based on the combined molecular data using Bayesian analysis.

Ancestral State Reconstruction

The evolution of direct development was mapped onto the reconstructed phylogenies using parsimony and likelihood methods. Topologies of both the tree based on combined molecular and morphological data (parsimony) and that based on combined nuclear and mitochondrial DNA data (Bayesian) were employed (i.e., the trees that maximized use of the available data). Both trees gave similar results.

Parsimony mapping was performed using the equivocal cycling option in MacClade version 4.02 (Maddison and Maddison 2001) to find all equally parsimonious reconstructions. Given recent evidence that the putative sister group to Plethodontidae (Amphiumidae) may exhibit direct development (Gunzburger 2003), separate ancestral state reconstructions were conducted with amphiumids coded either as larval or direct-developing.

Discrete version 4.01 (Pagel 1999a) was used to reconstruct the likelihood that ancestors at selected nodes exhibited direct development versus free-living aquatic larvae, using the local option (Pagel 1999b). Uncertainty in the topologies was limited, especially based on the Bayesian analysis of the molecular data, and the two trees were congruent in most major respects. Furthermore, areas of weak support were almost exclusively confined to regions of the tree that had little relevance to our estimation of ancestral states for desmognathines. However, the placement of *Hemidactylium* differed and was weakly supported in analyses of both the combined molecular and combined molecular and morphological data. Thus, we also reconstructed ancestral states for a tree (occurring at low frequency in the Bayesian analyses of the combined molecular data) in which this biphasic taxon is sister to all other plethodontids to examine the effects of this unusual arrangement on ancestral state estimates.

Branch length information is critical for likelihood reconstructions of ancestral states. Branch lengths were estimated for these topologies via maximum likelihood analysis of the RAG-1 sequences using PAUP*, with the best-fitting model for RAG-1 determined by Modeltest. Branch lengths were derived only from RAG-1 data due to apparent saturation at all three codon positions for the mitochondrial genes. Saturation was assessed by examination of transition:transversion plots using DAMBE (Xia and Xie 2001). Branch lengths of zero (when rounded off by PAUP* to six decimal places) were input as 10^{-10} for analysis by Discrete. Sirenids were removed from the tree to provide a root along the branch connecting cryptobranchids to other salamander families, because Discrete requires lengths of branches bifurcating from a basal node. Alternate rooting strategies yielded nearly identical likelihood values. Rates of transition between the two life-history states (α, aquatic larvae to direct development; β, direct development to aquatic larvae) were estimated using the ''run independent test'' command. These values were used to determine ancestral state probabilities for the ancestor of Desmognathinae, ancestor of Desmognathinae + Plethodontini, and ancestor of Plethodontidae. Other values for α and β also were tested: $\alpha = \beta$ (rate determined by Discrete); and ratios of α:β ranging from 1:1 to 1:0.00001. Alternate codings for amphiumids were employed as in the parsimony reconstructions. Likelihood-ratio tests (Mooers and Schluter 1999) showed no significant differences between the one-rate and more parameter-rich two-rate models in any of these analyses; thus, we favor ancestral state reconstructions based on the one-rate models as our best estimates of ancestral state probabilities.

Timing of Plethodontid Divergences

To estimate ages of several key clades in Plethodontidae, we used penalized likelihood (PL; Sanderson 2002) implemented in the Unix version of r8s version 1.6 (Sanderson 2003). This semiparametric approach allows differential rates of evolution across the tree, while including a rate-smoothing parameter that limits rapid rate shifts among nearby branches. We used the parsimony topology derived from combined analysis of all data, with likelihood-based branch lengths estimated for RAG-1 (see Ancestral State Reconstruction, above). Sirenids were removed to provide a basal node with associated branch lengths separating Cryptobranchoidea from the remaining salamander families (Salamandroidea). The TN (truncated Newton) algorithm was implemented, and smoothing parameters were chosen by cross-validated assessment (Sanderson 2002) spanning values from 10^0 to 10^4 in exponential increments of 0.5. In each case, the optimal smoothing value was 316.23. To test for multiple optima, five random sets of starting conditions (divergence times) were employed. Equality of optimization values occurred across these starting points. This indicates that optima of the penalized likelihood functions, and resulting age estimates, likely were reached (see Sanderson 2002, 2003). Confidence limits were estimated (where possible) using the method of Cutler (2000).

Fossil-based calibration points were used to constrain minimum ages of several nodes: ancestral salamandroid (114 million years; Evans and Milner 1996); ancestor of Am-

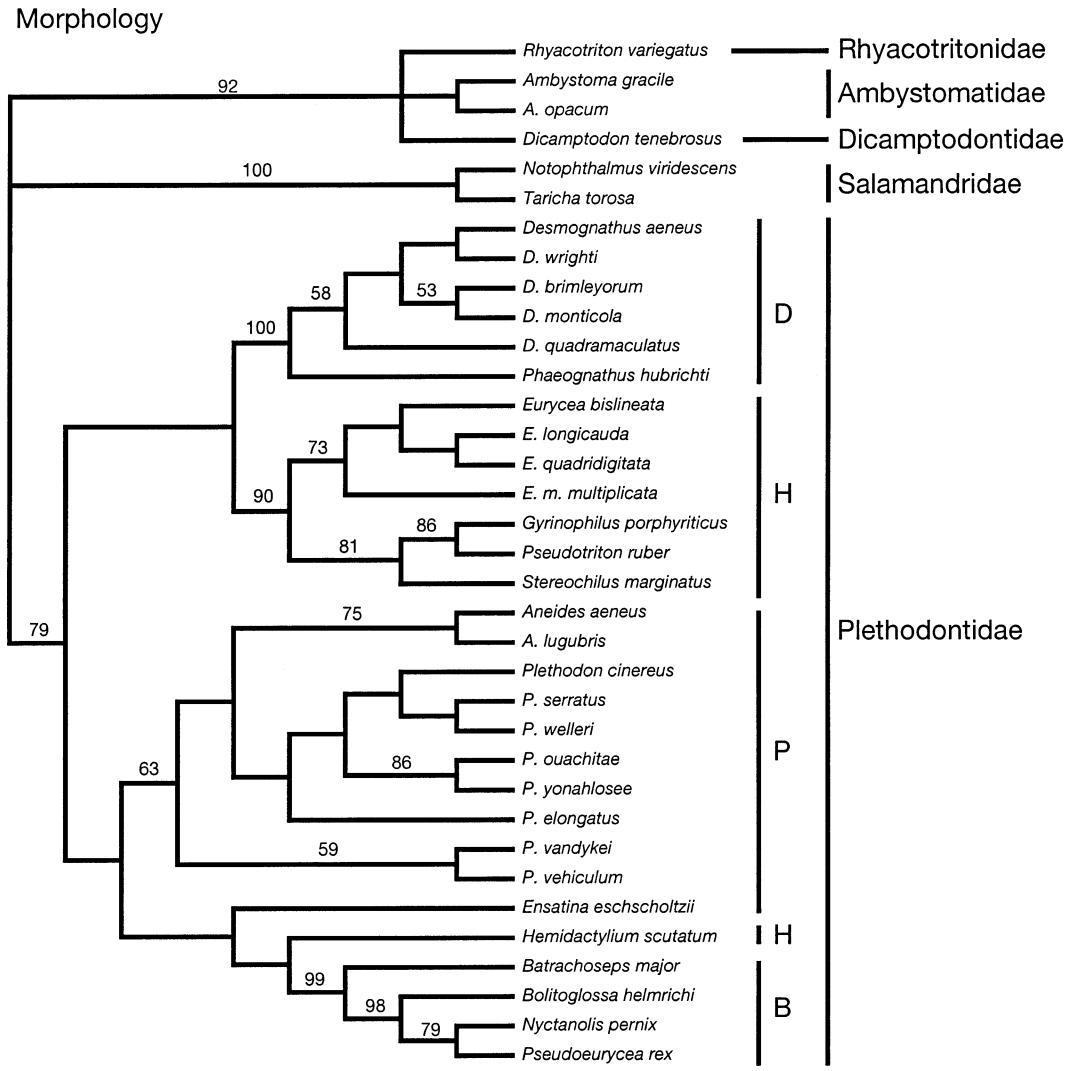

FIG. 1. Strict consensus of two shortest trees of plethodontid relationships based on parsimony analysis of nonmolecular (primarily morphological) data, with nonparametric bootstrap values from separate analyses shown at nodes (values <50% not shown). D, Desmognathinae; P, Plethodontini; H, Hemidactyliini; and B, Bolitoglossini. Tree length = 417.5500; consistency index (all characters informative) = 0.3137; retention index = 0.6503.

phiumidae + Plethodontidae (66 million years; Estes 1981); ancestor of Dicamptodontidae + Ambystomatidae (58 million years; Naylor and Fox 1993); split between *Taricha* and *Notophthalmus* (30 million years; Milner 2000); split between *Aneides* and *Ensatina* (7 million years; Tihen and Wake 1981); ancestor of plethodontine/desmognathine clade (7 million years; Tihen and Wake 1981); and ancestral bolitoglossine (5 million years; Clark 1985). Absolute dates are not available for divergence times of major salamander taxa, but PL requires fixation of the age of the root node. Thus, we employed two extremes for basal divergence between Cryptobranchoidea and Salamandroidea: (1) 160 million years ago, proposed by Milner (2000); note that a fossil cryptobranchid of this age recently was discovered (Gao and Shubin 2003); and (2) 250 million years ago, a reasonable maximum age for extant salamander lineages (e.g., Evans and Milner 1996).

RESULTS

We sampled 31 species of plethodontids (including all subfamilies and tribes) plus numerous outgroups, and reconstructed their phylogenetic relationships using 123 morphological characters (all 123 characters parsimony informative, pi), 1525 bp of the nuclear recombination-activating gene 1 (RAG-1; 551 pi), and 1473 bp of mitochondrial sequences from two genes, cyt *b* (783 bp, 409 pi) and ND4 (690 bp, 410 pi). Of the combined 3121 characters, 1493 were parsimony informative.

Parsimony analysis of the morphological data alone with paedomorphic taxa excluded (Fig. 1) is consistent with many aspects of the traditional taxonomy (e.g., monophyly of Desmognathinae, Bolitoglossini, and of most members of the tribes Hemidactyliini and Plethodontini). However, this analysis does not place desmognathines as sister to other plethodontids nor does it recover a monophyletic Plethodontinae,

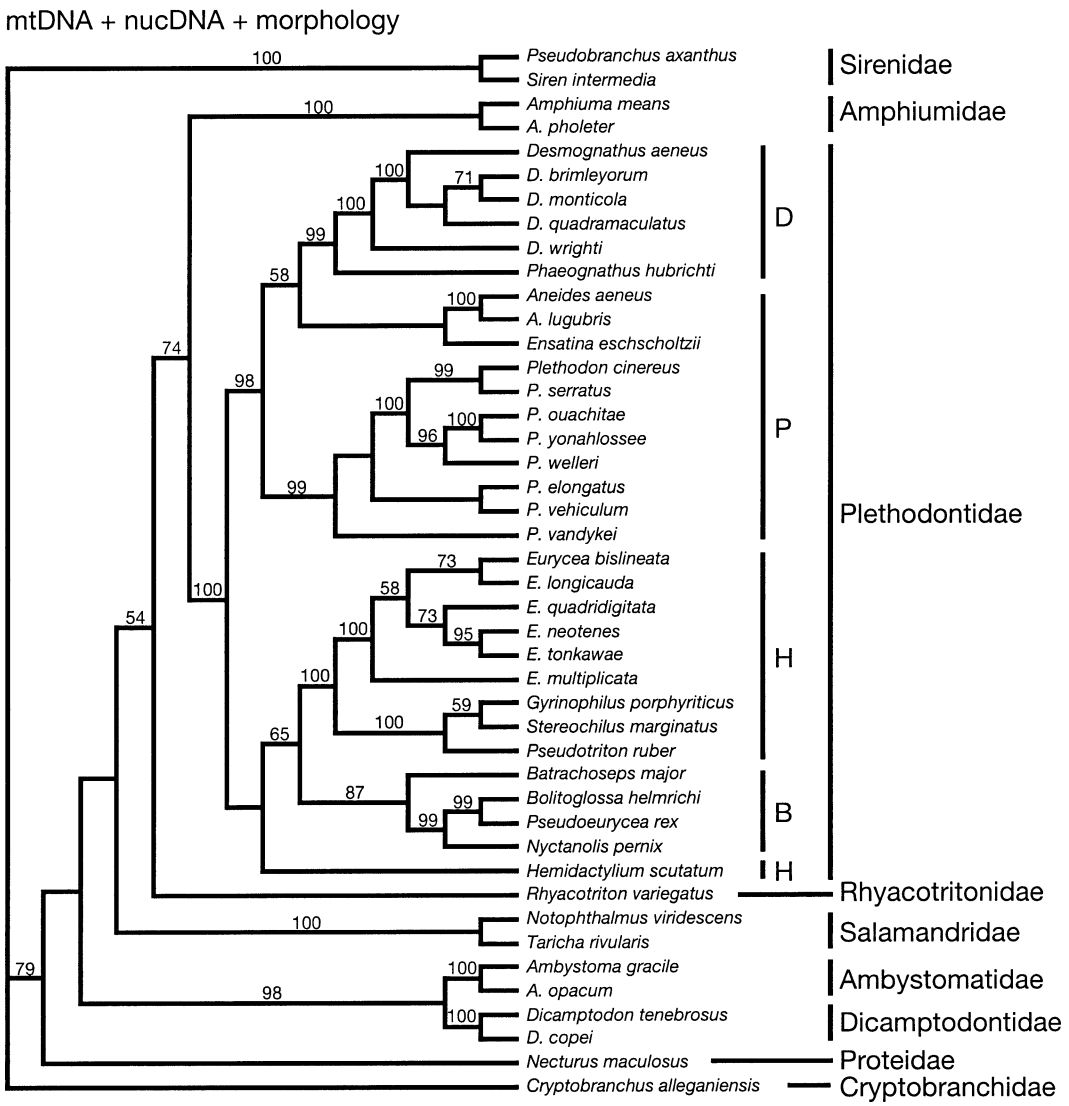

FIG. 2. Single shortest tree of plethodontid and other salamander family relationships based on parsimony analysis of combined nonmolecular, nuclear, and mitochondrial sequence data, with nonparametric bootstrap values from separate analyses shown at nodes (values <50% not shown). D, Desmognathinae; P, Plethodontini; H, Hemidactyliini; and B, Bolitoglossini. Tree length = 10,490.4000; consistency index (excluding uninformative characters) = 0.2643; retention index = 0.4758.

and support for the placement of Desmognathinae is weak based on the morphological data.

In contrast, the molecular and combined molecular and morphological data strongly support a radically different phylogeny, in which the desmognathines are nested inside the direct-developing plethodontines (Figs. 2, 3). Placement of desmognathines within the Plethodontini is corroborated by parsimony and Bayesian analyses of the separate nuclear and mitochondrial genes (results not shown; the only exception is Bayesian analysis of mitochondrial genes alone, in which Desmognathinae is sister to Plethodontini). Monophyly of Desmognathinae + Plethodontini is very strongly supported in analyses of the combined molecular data (parsimony bootstrap = 99%; Bayesian posterior probability = 100%) and parsimony analysis of the combined molecular and morphological data (bootstrap = 98%), with Desmognathinae nested inside Plethodontini in each case. The exact position of desmognathines within the Desmognathinae + Plethodontini clade is relatively weakly supported in the combined-data parsimony analysis (58% bootstrap for desmognathines sister to the plethodontine genera *Aneides* + *Ensatina* vs. 100% posterior probability for a sister relationship with *Aneides* in the Bayesian analysis). However, additional analyses (not shown) indicate that this is due to the unique combination of morphological character states exhibited by *Ensatina*. For example, removal of *Ensatina* from the combined morphological and molecular datasets results in 89% bootstrap support for a sister-group relationship between *Aneides* and desmognathines and 100% support for monophyly of Desmognathinae + Plethodontini.

Alternative topologies (e.g., basal placement of desmognathines within Plethodontidae or monophyly of Plethodontini excluding desmognathines) are highly significantly rejected by the combined and separately analyzed molecular

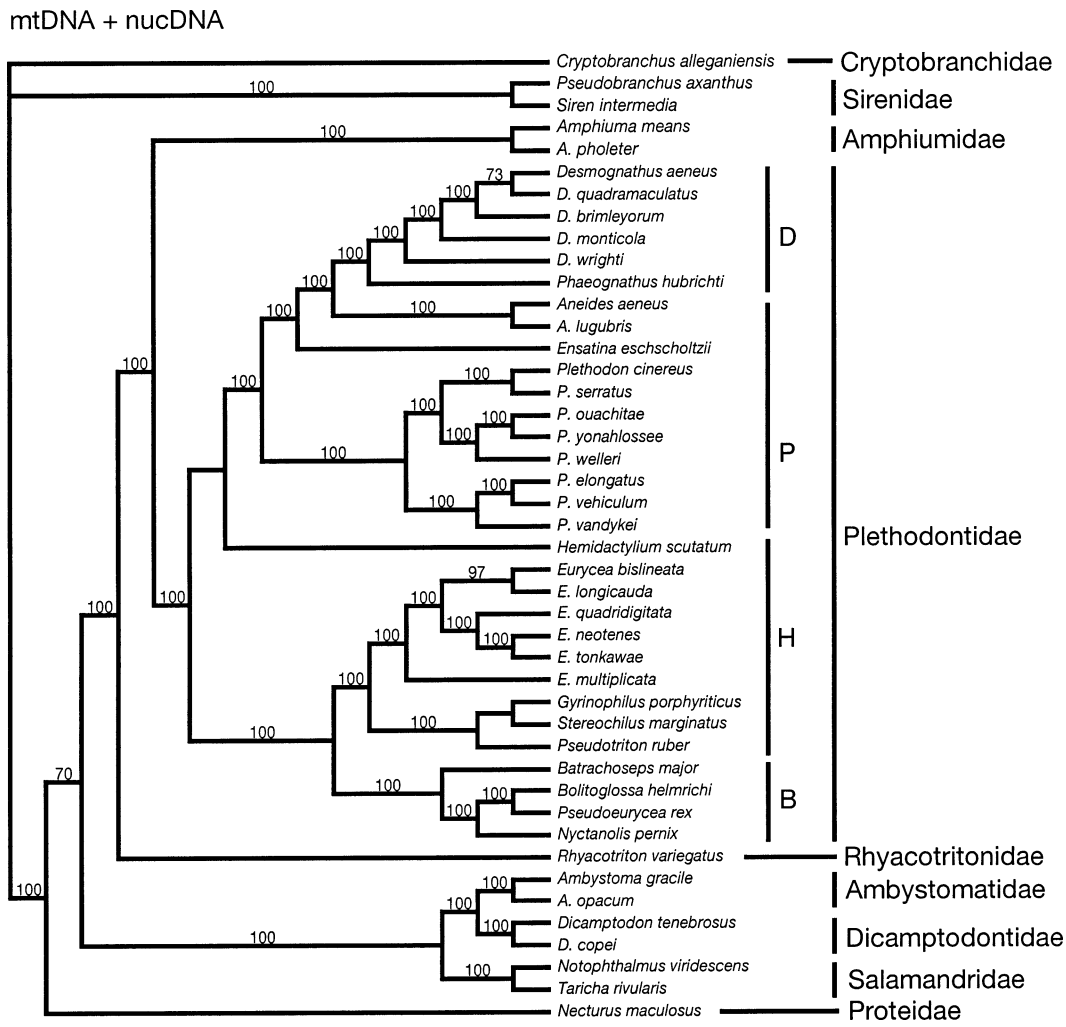

FIG. 3. Bayesian tree (4×10^6 generations) of plethodontid and other salamander family relationships based on combined molecular data, with posterior probabilities shown at nodes (values <50% not shown). D, Desmognathinae; P, Plethodontini; H, Hemidactyliini; and B, Bolitoglossini.

data ($P < 0.001$ for Shimodaira-Hasegawa tests of all subsets and combinations of the sequence data). Given that the morphological support for the traditional placement of desmognathines is weak and the new placement is strongly supported by the molecular and combined data, the available evidence favors the placement of desmognathines shown in Figures 2 and 3. The results of the parsimony and Bayesian analyses are not identical, but they agree in nearly all major respects and indicate that desmognathines are deeply nested within Plethodontini.

Although our molecular results may seem surprising to some, we reiterate that the traditional placement of desmognathines and the monophyly of Plethodontini are not supported by analysis of the morphological data. In fact, basal placement of desmognathines within Plethodontidae was based on only a single morphological character (presence of three larval epibranchials), and monophyly of Plethodontini was supported by only two (Lombard and Wake 1986). The conflicts between our molecular results, the morphological results, and traditional taxonomy may simply be an artifact of the limited morphological character support for relationships among these clades.

Mapping life-history mode onto this new phylogenetic hypothesis strongly suggests that direct development was lost in desmognathines and that the aquatic larval stage reevolved. Parsimony-based ancestral state reconstructions on the combined-data (molecular plus morphology; parsimony) tree (Fig. 4) indicate that the primitive condition for desmognathines is direct development; the same result was obtained via parsimony mapping on the combined-molecular-data Bayesian tree (results not shown). Likelihood-based estimates of ancestral states on the combined-data tree show greater than 99% probability that the ancestral desmognathine was a direct-developer (Table 1, Fig. 4), using estimated one-rate likelihood values for character-state changes (α, larval to direct-developing; β direct-developing to larval). Similar values were obtained using two-rate transition ratios and 1:1 likelihood estimates of transition probabilities. Results of all reconstructions provide strong support for a direct-developing ancestor of desmognathines under any but the high-

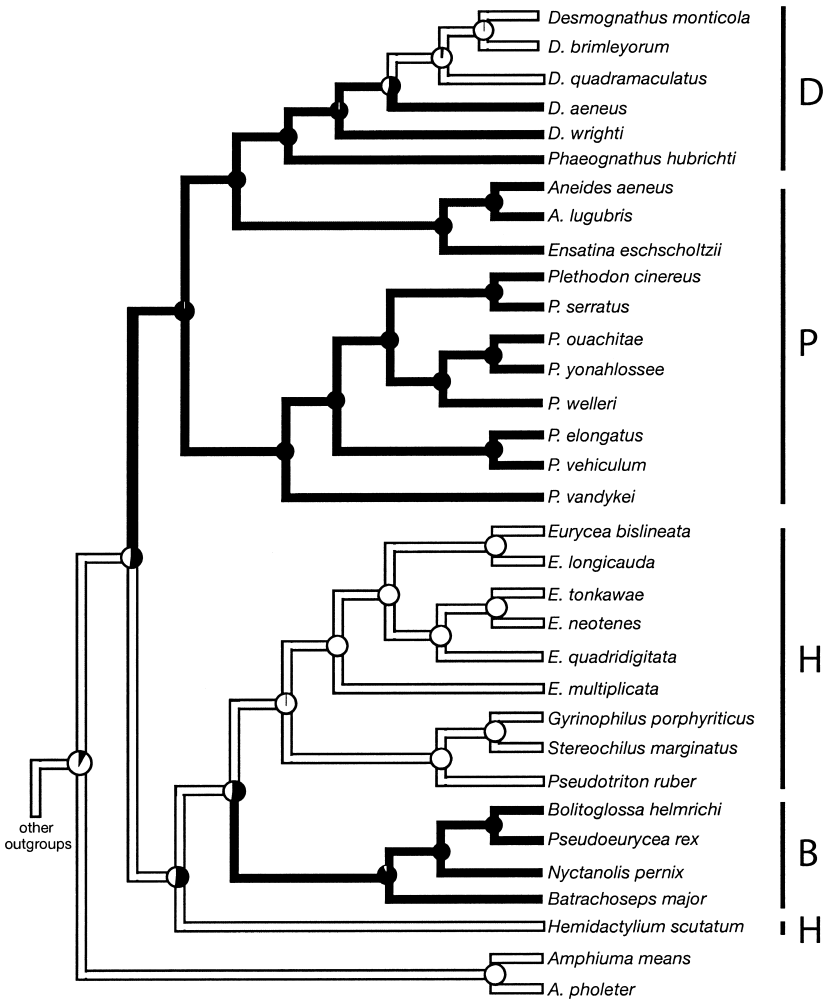

FIG. 4. Phylogeny of plethodontid salamanders, showing parsimony and maximum likelihood-based reconstructions of ancestral developmental modes. Topology is that of the single most parsimonious tree based on 123 nonmolecular and 2998 mitochondrial and nuclear sequence characters. D, Desmognathinae; P, Plethodontini; H, Hemidactyliini; and B, Bolitoglossini. Branch shading reflects the single most parsimonious reconstruction for ancestral developmental mode with amphiumids coded as biphasic; light branches represent free-living aquatic larvae and dark branches represent direct development. Pie charts at nodes indicate likelihood-based probability of biphasic life cycle (white) versus direct development (black), using likelihood estimates of transition rates (fixed as equal) between biphasic and direct-developing life histories.

est α:β ratios (Tables 1–3) and varying levels of support for the condition of other selected nodes (Tables 1, 2). The probability that desmognathines were primitively direct developers (and thus lost this trait secondarily) falls below 95% only when the transition from direct development to aquatic larvae is assumed to be about 4000 times less likely than the opposite change (combined-data parsimony topology), or about 5000 times less likely (combined-molecular-data Bayesian topology). The probability falls below 75% when this ratio is about 26,000 or 32,000, and below 50% only when the ratio is about 77,000 or 91,000 (combined-data parsimony and combined-molecular-data Bayesian topologies, respectively; Table 3). Even under the different (and implausible) topology seen in a small subset of the Bayesian analyses (*Hemidactylium* sister to all other plethodontids; other relationships very similar), the probability that the ancestral desmognathine was direct-developing falls below 95% only when the ratio is about 100 and below 50% when it is about 2000. Although it is possible that direct development was not the ancestral state in desmognathines, this hypothesis seems extremely unlikely given the new phylogenetic results.

Analyses of clade ages using PL (Table 4) strongly suggest that the reversal to aquatic larvae and subsequent radiation of biphasic species occurred relatively late in the history of desmognathines. The PL analyses also suggest that diversification of biphasic *Desmognathus* and eastern *Plethodon* (the two most speciose groups in the Appalachian Mountains of eastern North America) may have occurred over a similar time frame.

DISCUSSION

Evolution of Life Histories in Plethodontidae

Direct development has been a highly successful life-history strategy for plethodontids and other vertebrates, allowing adaptive radiation on land and freedom from the need for

TABLE 1. Likelihood-based probabilities that ancestors of selected plethodontid subgroups were direct-developing (based on parsimony analysis of the combined molecular and morphological data), using varying transition rates between developmental modes and alternate developmental states for amphiumids. Des, ancestor of Desmognathinae; D + P, ancestor of Desmognathinae + Plethodontini (*Aneides*, *Ensatina*, *Plethodon*); PL, ancestor of Plethodontidae; α, rate of transition from free-living aquatic larvae to direct development; β, rate of transition from direct development to free-living aquatic larvae.

	Probability (%) ancestral state is direct development, assuming *Amphiuma* has free-living larvae			Probability (%) ancestral state is direct development, assuming *Amphiuma* has direct development		
Transition rates	Des	D + P	PL	Des	D + P	PL
$\alpha = \beta$	99.818	97.962	51.855	99.703	98.666	78.306
Two-rate model[1]	100.000	99.702	88.756	99.983	99.843	95.451
$\alpha = 1$ $\beta = 1$	100.000	99.101	25.823	99.998	99.686	74.074
$\alpha = 1$ $\beta = 0.1$	99.986	98.804	0.004	99.986	98.837	3.099
$\alpha = 1$ $\beta = 0.01$	99.872	98.689	0.000	99.872	98.690	0.045
$\alpha = 1$ $\beta = 0.001$	98.749	97.579	0.000	98.749	97.579	0.000
$\alpha = 1$ $\beta = 0.0001$	88.764	87.713	0.000	88.765	87.713	0.000
$\alpha = 1$ $\beta = 0.00001$	44.138	43.615	0.000	44.138	43.615	0.000

[1] Two-rate model for *Amphiuma* larval or direct-developing, respectively: $\alpha = 1.91934$, $\beta = 7.59463$; $\alpha = 2.41116$, $\beta = 7.38332$.

aquatic habitats for reproduction (Wake 1966; Duellman and Trueb 1986; Pough et al. 2001). Our results show that direct development has been lost in desmognathines and free-living larvae have re-evolved. To our knowledge, this is the first study to strongly support this hypothesis in vertebrates. A previous study, focusing on relationships within desmognathines (Titus and Larson 1996), also suggested the possibility that the biphasic life cycle may have re-evolved within desmognathines. However, that analysis was inconclusive, at least partly because desmognathines were not suspected to be nested within a group of direct-developing species. Our resolution of the phylogenetic placement of desmognathines now strongly supports the hypothesis of loss of direct development. Some studies of marsupial treefrogs (Hylidae, Hemiphractinae) also have suggested the possibility that direct development was lost and the tadpole stage re-evolved (Duellman and Hillis 1987; Elinson 1987; Duellman et al. 1988), but this hypothesis remains to be verified with rigorous phylogenetic analysis and ancestral state reconstructions.

The loss of direct development appears to have had important and surprising consequences for salamander ecology, evolution, and diversity. Despite the loss of a trait that was critical for diversification of terrestrial vertebrates, desmognathines nevertheless appear to be highly successful. Although we show that they originated relatively recently (Table 4), they include at least 20 species (Amphibiaweb 2003), with up to seven species existing in sympatry in some areas (Bruce 1991). Some species are extremely abundant, reaching densities of up to 6.9 individuals/m^2 in some populations (Tilley 1980). They also occur in a wide variety of habitats,

TABLE 2. Likelihood-based probabilities that ancestors of selected plethodontid subgroups were direct-developing (based on Bayesian analysis of the combined molecular data), using varying transition rates between developmental modes and alternate developmental states for amphiumids. Des, ancestor of Desmognathinae; D + P, ancestor of Desmognathinae + Plethodontini (*Aneides*, *Ensatina*, *Plethodon*); PL, ancestor of Plethodontidae; α, rate of transition from free-living aquatic larvae to direct development; β, rate of transition from direct development to free-living aquatic larvae.

	Probability (%) ancestral state is direct development, assuming *Amphiuma* has free-living larvae			Probability (%) ancestral state is direct development, assuming that *Amphiuma* has direct development		
Transition rates	Des	D + P	PL	Des	D + P	PL
$\alpha = \beta$	99.682	98.330	54.334	99.515	98.712	77.471
Two-rate model[1]	99.953	99.644	86.576	99.925	99.740	92.341
$\alpha = 1$ $\beta = 1$	99.994	99.419	24.189	99.994	99.788	72.418
$\alpha = 1$ $\beta = 0.1$	99.984	99.239	0.331	99.985	99.258	2.742
$\alpha = 1$ $\beta = 0.01$	99.892	99.146	0.000	99.891	99.150	0.028
$\alpha = 1$ $\beta = 0.001$	98.976	98.237	0.000	98.976	98.237	0.000
$\alpha = 1$ $\beta = 0.0001$	90.663	89.986	0.000	90.663	89.986	0.000
$\alpha = 1$ $\beta = 0.00001$	49.276	48.908	0.000	49.275	48.908	0.000

[1] Two-rate model for *Amphiuma* larval or direct-developing, respectively: $\alpha = 2.87813$, $\beta = 8.95165$; $\alpha = 3.57695$, $\beta = 8.35414$.

TABLE 3. Likelihood-based probabilities that the ancestral desmognathine was direct-developing, under varying transition rates between developmental modes. In each case, α was fixed at 1.0 and β was varied to find the minimum transition ratio for a given probability level of reversal from direct development to biphasic life cycle. Values for parsimony (pars; all data) and Bayesian (Bayes; combined molecular data) topologies are shown.

	Probability ancestral state is direct development, assuming *Amphiuma* has free-living larvae			Probability ancestral state is direct development, assuming *Amphiuma* has direct development		
	95%	75%	50%	95%	75%	50%
β, pars	0.000250	0.000038	0.000013	0.00022	0.000038	0.000013
β, Bayes	0.000200	0.000031	0.000011	0.00018	0.000031	0.000011

both aquatic and terrestrial (Titus and Larson 1996; Petranka 1998). As additional evidence of their success, some desmognathines (e.g., *Desmognathus quadramaculatus*, *D. fuscus*) prey on other salamander species (Hairston 1986; Jaeger et al. 1998; Petranka 1998), and aquatic-breeding *Desmognathus* have been shown to exclude terrestrial plethodontids from Appalachian streamside habitats (Fauth 1998; Jaeger et al. 1998; Grover 2000; Grover and Wilbur 2002). Thus, reinvasion of aquatic habitats seems to have enabled desmognathines to exploit a key niche (or adaptive zone) in a region densely packed with plethodontid species.

Direct-developing plethodontines exhibit very high species richness in some areas and are geographically widespread. For example, up to 11 species of direct-developing plethodontids may occur in sympatry in some localities in Central America (García-París et al. 2000), and up to five species of the direct-developing genus *Plethodon* may be syntopic in the southern Appalachians (Highton 1995). Furthermore, direct-developing plethodontids exist in eastern North America, western North America, Europe, Middle America (Central America plus Mexico), and South America, whereas biphasic plethodontids are found only in eastern North America (Wake 1966; Duellman and Trueb 1986). Yet, despite their success, almost none of these direct-developing species have invaded mountain stream habitats, where desmognathines predominate (the sole exception is one Mexican bolitoglossine species [likely now extinct] with a very limited geographic distribution; Wake and Campbell 2001). Even in the areas where desmognathines are absent but direct-developing plethodontids are diverse (montane regions in western North America and the neotropics), almost no plethodontids have exploited stream habitats (Wake 1966; Petranka 1998; García-París et al. 2000).

Why have other direct-developing plethodontids not undergone the same transition as desmognathines and exploited aquatic habitats? Embryological evidence suggests that direct-developing desmognathines and their plethodontine ancestors retain the larval hyobranchial apparatus in the egg (Dent 1942; Alberch 1987; Wake and Hanken 1996; Marks and Collazo 1998), a key feature for aquatic respiration and feeding. The retention of the larval hyobranchial apparatus may have greatly facilitated the re-invasion of aquatic habitats by larval desmognathines. Conversely, the extreme reduction of these structures in larval bolitoglossines (Wake 1966; Alberch 1987; Wake and Hanken 1996) may explain their failure to exploit this habitat, despite their impressive evolutionary radiation. Thus, re-invasion of aquatic habitats by larval desmognathines may only have required a change in hatching times, whereas re-evolution of the biphasic life cycle in bolitoglossines would have required de novo re-evolution of major larval structures. In a potentially similar example, some direct-developing marsupial frogs (Hylidae, Hemiphractinae) also may have lost direct development and regained aquatic larvae, particularly in lineages that retain

TABLE 4. Estimated ages of key nodes in the phylogeny of Plethodontidae, based on penalized likelihood (PL) applied to the combined-data parsimony tree, using branch lengths derived from RAG-1. Values associated with basal calibration correspond to estimates derived from calibration of the basal split between Cryptobranchoidea and Salamandroidea at 160 million years ago vs. 250 million years ago (minimum ages for internal nodes were identical for both analyses). ''Larson timing'' refers to clade ages estimated by Larson et al. (2003) for the specified nodes, based on molecular clocks. PLETH, ancestral node for family Plethodontidae; D + P, ancestral node for Plethodontini inclusive of Desmognathinae; DESMOG, ancestral node for Desmognathinae; DBMQA, ancestral node for *Desmognathus brimleyorum, D. monticola, D. quadramaculatus*, and *D. aeneus*; DBMQ, ancestral node for *Desmognathus brimleyorum, D. monticola*, and *D. quadramaculatus* (i.e., biphasic radiation); and EASTPL, ancestral node for radiation of eastern *Plethodon*. Confidence intervals are given parenthetically where their calculation was possible. Estimates for ages of Desmognathinae and contained clades are congruent for the PL and clock-based methods, but PL suggests that radiation of eastern *Plethodon* was more recent and may have overlapped with that of biphasic desmognathines.

Basal calibration/ Larson timing	Taxon					
	PLETH	D + P	DESMOG	DBMQA	DBMQ[1]	EASTPL
	Clade age (million years)					
160 million years	49.7 (42.5–57.9)	39.3 (32.3–47.7)	28.1 (21.8–35.8)	10.4	9.9	9.1
250 million years	84.8 (44.1–137.3)	56.1 (45.2–69.7)	46.7	14.7	13.8	16.6
Larson et. al. (2003)	—	—	~40	~15	~10	~27

[1] Larson et al. (2003) placed the biphasic species *D. quadramaculatus* outside the clade containing *D. brimleyorum* and *D. montanus*, but included most other biphasic *Desmognathus* in the latter group.

the typical larval morphology inside the egg (Wassersug and Duellman 1984; Duellman and Hillis 1987; Duellman et al. 1988). In contrast, a closely related (J. J. Wiens, unpubl. data) group of direct-developing frogs (Leptodactylidae, *Eleutherodactylus*) occurs sympatrically with all hemiphractines and is far more geographically widespread and species rich (>500 species vs. ~70 hemiphractines; Frost 2002), yet has never re-evolved the aquatic tadpole stage. *Eleutherodactylus* species studied thus far do not appear to develop the typical anuran larval morphology while in the egg (Hanken 1999). These comparisons suggest that the loss of larval structures in the egg in *Eleutherodactylus* and bolitoglossine plethodontids prevents reacquisition of free-living larvae in these clades.

Our analyses suggest that additional reversals from direct-developing to biphasic life history may have occurred in other plethodontid lineages (Tables 1, 2; Fig. 4). Thus, this phenomenon may be even more widespread. However, evidence for a direct-developing ancestor of hemidactyliines plus bolitoglossines, or even a direct-developing ancestral plethodontid (see also Gunzburger 2003), remains equivocal and requires further analysis.

The two global hotspots of salamander biodiversity are the Appalachian Mountains of eastern North America and the highlands of Middle America (Duellman and Trueb 1986; Amphibiaweb 2003). Although Middle America has higher regional plethodontid diversity than the Appalachians (~168 species vs. ~57; Campbell 1999; Duellman and Sweet 1999), the local diversity of Appalachian salamanders appears to be greater, with a maximum of 19 species in the southern Appalachians versus 11 in lower Central America (Petranka 1998; García-París et al. 2000). The larger number of species in Appalachian communities may be explained (at least in part) by their use of both aquatic and terrestrial habitats, whereas neotropical salamanders are almost entirely terrestrial or arboreal (Wake 1966, 1987; Petranka 1998; García-París et al. 2000). The reacquisition of the biphasic life cycle in desmognathines may have contributed to the ecological diversity and local species richness of Appalachian communities. We speculate that competition with terrestrial plethodontids in diverse Appalachian salamander communities could have exerted selective pressure on desmognathines to re-evolve the aquatic phase of their life cycle, and their retention of larval features in the egg facilitated this change developmentally. In support of this latter hypothesis, studies of Appalachian salamander communities indicate that competition may be intense in terrestrial habitats (reviewed by Jaeger and Forester 1993; Maerz and Madison 2000), favoring exploitation of aquatic resources. Our estimates of clade ages (and those of Larson et al. 2003) are consistent with the hypothesis that competition with terrestrial, direct-developing plethodontines may have promoted a major reversal to aquatic larvae in desmognathines.

Evolution in Reverse

Recent phylogenetic studies have shown that many apparently important and adaptive phenotypes may be lost (e.g., sexually selected male traits; Wiens 2001a; behavioral and morphological adaptations for arboreality; Ober 2003) and that complex features may be regained (e.g., well-developed hindlimbs in snakes, Tchernov et al. 2000; wings in stick insects, Whiting et al. 2003). Numerous other cases of reversal and reacquisition of major traits are reviewed by Porter and Crandall (2003). Our study provides three interesting insights on this topic. First, we show that an important life-history trait that was critical for the success of terrestrial vertebrates has reversed. Such a reversal was considered unlikely by previous workers (e.g., Wake and Hanken 1996) and a similarly critical life-history transition (oviparity to viviparity) also has been considered irreversible by some authors (e.g., Lee and Shine 1998). Second, our results indicate that such reversals might actually promote diversification and that they may have important implications for patterns of species richness and community structure. Finally, our results suggest that the ability to undergo such reversals and reacquisitions may be tied to differences in underlying developmental patterns among clades (i.e., the ability to re-evolve the aquatic larval stage appears to have been lost in some lineages of amphibians in which key embryonic larval structures do not develop, including some salamanders and possibly frogs).

Taxonomic Implications

Our new phylogenetic hypothesis also has obvious implications for the higher-level classification of plethodontids. Although we do not wish to engage in a detailed discussion of taxonomy, it is clear that consideration of desmognathines as a subfamily is inappropriate if they are nested inside of plethodontines. Given our phylogeny, the simplest solution to this problem is to: (1) elevate the tribes Hemidactyliini and Bolitoglossini to the rank of subfamilies (Hemidactylinae and Bolitoglossinae), and (2) make the subfamily Plethodontinae equivalent to the former tribe Plethodontini, while recognizing *Desmognathus* and *Phaeognathus* as the supergenus *Desmognathus* within Plethodontinae (use of the rank supergenus follows standard usage in other plethodontid clades; e.g., Wake 1966, 1993). The placement of *Hemidactylium* currently is uncertain, but based on the available data it seems unlikely to be allied with other members of the tribe Hemidactyliini (*Eurycea* [including *Typhlomolge* and *Typhlotriton*; Chippindale et al. 2000; Bonett and Chippindale 2004], *Gyrinophilus*, *Haideotriton*, *Pseudotriton*, *Stereochilus*) or nested within any of the subfamilial clades recognized here. Therefore, we favor recognition of the subfamily Spelerpinae (Cope 1859) for these remaining members of the Hemidactyliini (for discussion and taxonomic history see Frost 2002) and apply the name Hemidactylinae only to *Hemidactylium*. This classification is consistent with our phylogeny, yet involves minimal changes to the traditional taxonomy.

Conclusions

Plethodontid salamanders have been the focus of numerous studies in ecology and evolutionary biology (Bruce et al. 2000 and references therein). Much of this research has centered on understanding life-history evolution and the origin of direct development (e.g., Tilley 1977; Tilley and Bernardo 1993; Wake and Hanken 1996; Hanken 1999; Ryan and Bruce

2000). By showing that a major group is secondarily biphasic, our results suggest a new paradigm for research in life-history evolution. We also provide a new phylogenetic framework for studies of plethodontid biology. More generally, we provide strong evidence that life history can reverse one of its most important evolutionary transitions. Rather than being an evolutionary dead end, we suggest that such reversals may have major consequences for ecology, evolution, and biodiversity.

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