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A multigenic perspective on phylogenetic relationships in the largest family of salamanders, the Plethodontidae

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ABSTRACT

Despite several recent studies, the phylogeny of plethodontid salamanders is not yet fully resolved and the phylogenetic positions of several key genera, especially *Aneides, Hemidactylium, Hydromantes* and *Karsenia*, are contentious. Here we present a combined dataset of complete mitochondrial genomes and three nuclear loci for 20 species (16 genera) of plethodontids, representing all major clades in the family. The combined dataset without mitochondrial third codon positions provides a fully resolved, statistically well-supported tree. In this topology two major clades are recovered. A northern clade includes *Aneides, Desmognathus, Ensatina, Hydromantes, Karsenia, Phaeognathus* and *Plethodon*, with *Plethodon* being the sister taxon to the rest of the clade. *Hydromantes* and *Karsenia* are sister taxa, and *Aneides* is recovered as the sister taxon to *Ensatina*. *Desmognathus* + *Phaeognathus* form the sister taxon to *Aneides* + *Ensatina*. An eastern/southern clade comprises two subclades. One subclade, the spelerpines (*Eurycea, Gyrinophilus, Pseudotriton, Stereochilus, Urspelerpes*) is the sister taxon to a subclade comprising *Hemidactylium*, *Batrachoseps* and the tropical plethodontids (represented by *Bolitoglossa, Nototriton* and *Thorius*). In this topology *Hemidactylium* is well-supported as the sister taxon to *Batrachoseps*. Only when mitochondrial third codon positions are included using maximum likelihood analysis is *Hemidactylium* recovered as the sister taxon to *Batrachoseps* + tropical genera. Hypothesis testing of alternative topologies supports these conclusions. On the basis of these results we propose a conservative taxonomy for Plethodontidae.

1. Introduction

Resolving phylogenies for very old (more than 50 million years [myr] old) and large clades is challenging. Lineage origins can be concentrated in particular short spans of time, producing short internodes and ambiguous resolution, especially when the nodes are old. In this paper we examine the evolutionary history of such a clade, the salamander family Plethodontidae, the largest of the ten families of salamanders, which contains approximately two-thirds of the roughly 610 living species forming the order Caudata.

The phylogenetic analysis of the Plethodontidae by Wake (1966) led to a taxonomic revision that recognized two subfamilies, a very large Plethodontinae and a much smaller Desmognathinae. The Plethodontinae included three tribes, Bolitoglossini (with three supergenera, *Batrachoseps* including only *Batrachoseps*, *Bolitoglossa* including all of the tropical genera, and *Hydromantes* including European and American species), Hemidactyliini (*Hemidactylium*, which was not clearly associated with the remainder of the genera in the group, currently assigned as spelerpines, see below), and Plethodontini (*Aneides, Ensatina, Plethodon*). This taxonomy, based on analysis of comparative osteology and life history, was almost universally accepted until the publication of large molecular (e.g., Mueller et al., 2004) and combined molecular and morphological (e.g., Chippindale et al., 2004) datasets. Analyses of these databases showed that taxonomic revision was necessary. In particular, one of the two subfamilies, Desmognathinae, was deeply nested within the other, Plethodontinae. Organization of the Plethodontinae into three clades, corresponding mainly to the existing Bolitoglossini, Hemidactyliini, and Plethodontini, was generally supported, but with some important exceptions. *Hydromantes*, placed by Wake (1966) in the Bolitoglossini, was nested within the Plethodontini (Mueller et al., 2004), and *Hemidactylium* was recovered as the sister taxon of *Batrachoseps* (ML and BI analysis of complete mitochondrial [mt] genomes by Mueller et al., 2004) or as the sister taxon to all other plethodontids (MP analysis of data of Mueller et al., 2004, by Macey, 2005; topology found in MP analysis including third codon positions but not without them by

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Mueller et al., 2004). Chippindale et al. (2004) analyzed a combined dataset (two mt genes plus one nuclear gene, RAG1, and morphological data from the literature), producing a largely resolved phylogeny, except for the uncertain position of *Hemidactylium*. They proposed a new taxonomy with four subfamilies: Bolitoglossinae (which included the former Bolitoglossini minus *Hydromantes*, a taxon not included in their analysis but from results of Mueller et al., 2004, by implication placed in their Plethodontinae), Hemidactyliinae (for *Hemidactylium* only), Plethodontinae (which included the former Plethodontini plus Desmognathinae), and Spelerpinae (the former Hemidactyliini minus *Hemidactylium*). This taxonomy was widely adopted (e.g., Bruce, 2007; Frost, 2010).

Until the discovery of the first Asian plethodontid, *Karsenia* (Min et al., 2005), the family was known mainly from the New World (more than 97% of species), although eight species occur in the western Mediterranean area. Min et al. (2005) produced a RAG1 dataset; ML analysis showed that *Karsenia* and *Hydromantes* were embedded within the Plethodontinae. They also showed that *Hemidactylium* was not a member of any other clade (but *Batrachoseps* was not included in their dataset), and while they made no taxonomic revision, they implicitly supported the taxonomy of Chippindale et al. (2004). Vieites et al. (2007) presented a dataset based on three nuclear genes (RAG1, POMC and BDNF) and including all relevant genera (but only one tropical plethodontid, a group that includes more than 260 species). They recovered *Hydromantes* and *Karsenia* as sister taxa, but with low statistical support, and found this clade to be the sister taxon to the remaining members of the Plethodontinae. *Aneides* was recovered as the sister taxon to *Desmognathus/Phaeognathus*, with *Ensatina* as the sister taxon to the combined three-taxon clade, and *Plethodon* as the sister taxon to the combined four-taxon clade. *Bolitoglossa* and *Batrachoseps* were sister taxa, and *Hemidactylium* was recovered as the sister taxon to *Bolitoglossa* and *Batrachoseps*. Spelerpinae was the sister taxon to *Hemidactylium* plus *Batrachoseps*/*Bolitoglossa*. The identification of two well-supported major clades led to a taxonomy with two subfamilies, Plethodontinae and Hemidactyliinae, the latter including all members of the subfamilies Bolitoglossinae (which was not well-supported in the analysis), Hemidactyliinae, and Spelerpinae of Chippindale et al. (2004). However, phylogenetic relationships within the major clades were not fully resolved. The recent discovery of a new spelerpine lineage (*Urspelerpes*) in southeastern United States led to an analysis of relationships based on a combined nuclear and mitochondrial gene sequence database (RAG1, CytB; Camp et al., 2009). *Urspelerpes* was recovered as the sister taxon to *Eurycea*. These two genera were the sister taxon of the remaining spelerpine genera (*Gyrinophilus, Pseudotriton, Stereochilus*), all of which they placed in a tribe Spelerpini, recovered as the sister taxon to the bolitoglossines (two tropical genera, *Bolitoglossa* and *Nyctanolis*, sister taxa in this study, were in turn the sister taxon to *Batrachoseps*). *Hemidactylium* was included in a basal polytomy.

Kozak et al. (2009; see also Kozak and Wiens, 2010) studied North American plethodontids and presented a combined analysis of three nuclear genes (*RAG1*, *POMC*, *BDNF*) and three mitochondrial genes (*ND4*, *Cytb*, *ND2*), using previously published data but with the addition of many new sequences of *RAG1* and of mitochondrial sequences from eastern North American species. While many taxa lack data for one or more of the genes, this is the most taxon-rich sampling of the Plethodontidae published to date. Based on a maximum likelihood analysis they presented a well-supported tree with two major clades. In the first, *Plethodon* is the sister taxon to all remaining plethodontines (including *Karsenia* and *Hydromantes*). The second clade finds *Hemidactylium* as the sister taxon to *Batrachoseps* + the topical plethodontids, and in turn these three taxa form the sister taxon to the spelerpines. The position of *Hemidactylium* is resolved with high support (ML bootstrap 96), and they use the taxonomy of Chippindale et al. (2004): four subfamilies, three of which (Bolitoglossinae, Hemidactyliinae, Spelerpinae) are included in a single major clade.

The taxonomy of Wake (1966) was based on morphological and life historical traits. Wake's subfamily Plethodontinae was characterized by larval and embryonic characters of the hyobranchial apparatus thought to be derived, but otherwise on traits thought to be ancestral relative to desmognathines. The subfamily Desmognathinae was based on synapomorphies associated with head movement, mouth opening, and locomotion (especially vertebral characters). The three plethodontine tribes differed in life history (hemidactyiines having larvae, absent in the other tribes) and in features of the hyobranchial apparatus and tongue function. The morphological traits were elaborated further by Lombard and Wake (1986), Schwenk and Wake (1993), and generally validated by Chippindale et al. (2004), although analyses differed in some important respects (parsimony support for desmognathines as the sister to other plethodontids was weak, as was the grouping of *Hemidactylium* with other then-recognized hemidactyliines, now spelerpines).

We undertook this study to obtain a robust, resolved phylogeny, with particular focus on the position of the contentious *Hemidactylium* and structure within the Plethodontinae. Our taxon sampling includes all relevant taxa, including three tropical bolitoglossines. Our dataset includes complete mitochondrial genomes and sequences from three nuclear genes (*RAG1*, *POMC*, *BDNF*) for all taxa. We add the complete mt genome of *Karsenia*, the closest relative of the Plethodontidae, and we use the nuclear sequences presented in Vieites et al. (2007), extended by the addition of nuclear genes for *Nototriton* and *Thorius*. Methodological approaches have differed in previous publications, so we investigate implications of different analytical methods, such as maximum likelihood, Bayesian, and parsimony approaches. For complete mitochondrial genomes, there are issues concerning inclusion or exclusion of third codon positions. There are also issues concerning concatenation of mitochondrial and nuclear gene sequences. We obtain a well resolved phylogeny and use it as the foundation for a new formal taxonomy of the family.

2. Materials and methods

2.1. Taxon sampling

Complete mitochondrial genomes for representatives of 17 plethodontid genera are available in Genbank from a previous study (Mueller et al., 2004), well representing plethodontid diversity. We added a new mitochondrial genome for the geographically remote and phylogenetically divergent *Karsenia*. We used nuclear data from Vieites et al. (2007) and added two additional tropical genera (*Nototriton, Thorius*). Our dataset includes 20 plethodontid species representing 16 genera. In many cases we sequenced nuclear genes from the same individuals used by Mueller et al. (2004, see Appendix 1 for voucher information). We included as outgroups representatives of five salamander families, with full datasets for each: Ambystomatidae, Amphiumidae, Cryptobranchidae, Rhyacotritonidae and Salamandridae. Accordingly, we have avoided potential problems related to incomplete data (Lemmon et al., 2009).

2.2. Laboratory protocols

Genomic DNA was extracted from tissue samples that were frozen or preserved in ethanol using a standard salt extraction method (Bruford et al., 1992). For the PCR amplification of the mitochondrial genome of *Karsenia koreana*, we used the set of primers and conditions listed in Zhang and Wake (2009) to amplify overlapping fragments of the mitochondrion. Some fragments

were amplified with specifically designed primers: KARS_DRV_-NAD1_L (CCTATTATAATAATTGGCCTTCCACCC), PLETHOD_ DRV_ COX1_H (GCCAATATCTTTRTGRTTTGTTGA), KARS_DRV_ COX1_L (GCAGGAGGAGGAGATCCAGTA), PLETHOD_DRV_COX2_H (TTCTATAATTGGTGAGGCTGCRTCTTG), KARS_DRV_NAD3_L (GTATATGAATGAATGCAAGGAGGT), KARS_DRV_NAD4_H (CCCTAGCTTTAA TAATACGGCAGC), KARS_DRV_NAD4_L (TGCCTAATAACAATACACCTATTACCC), KARS_DRV_NAD5_H (AGTTGATGAGTTGATGGTTAAGGG). Prior to fragment amplification, we performed long PCRs of two ca. 8 kb fragments of the mitochondrion to avoid potential amplification of mitochondrial pseudogenes from the nuclear genome. Primers for *RAG1*, *BDNF* and *POMC* are the same reported in Vieites et al. (2007). PCRs were performed using the following conditions: an initial denaturation at 94 °C for 3 min, 35–39 cycles at 94 °C for 30 s, annealing at 48–60 °C for 45 s–1 min, extension at 72 °C for 2–3 min and final extension of 5 min at 72 °C. PCR products were loaded onto 1% agarose gels, stained with GelStar gel stain (Cambrex), and visualized in a Dark reader transilluminator (Clare Chemical). If results were satisfactory, products were purified using 2 μL, from a 1:4 dilution of ExoSapIt (Amersham) per 5 μL of PCR product prior to cycle sequencing. A 10 μL sequencing reaction included 2 μL of template, 2.5 μL of sequencing buffer, 0.8 μL of 10 pmol primer, 0.4 μL of BigDye Terminator version 3.1 Sequencing Standard (Applied Biosystems) and 4.2 μL of water. The sequence reaction was 35 cycles of 10 s at 96 °C, 10 s at 50 °C and 4 min at 60 °C. Cycle sequencing products were purified by ethanol precipitation. Sequence data collection and visualization were performed on an ABI 3730xl automated sequencer (Applied Biosystems). Sequences are deposited in GenBank (see Supplementary appendix for accession numbers).

2.3. Phylogenetic inference

Nuclear gene sequences were aligned considering amino acid properties. In the case of mitochondrial genomes, alignments were done gene by gene; global alignments are not possible because of gene re-arrangements and complexity of the genomes (Mueller and Boore, 2005). We used the Wisconsin Sequence Analysis Package (GCG, version 10.3) to align each gene, using default parameters for extension costs and gap creation, adjusted to preserve reading frames and tRNAs secondary structures. The alignments were refined with Gblocks (Castresana, 2000) to extract regions of defined sequence conservation. All loci were concatenated for the different analyses. The D-loop, 11 tRNAs, and ambiguously alignable positions (such as tRNA loops, beginnings and ends of protein-coding genes, rRNA regions with indels, or amino acid positions present in less than 25% of the samples) were excluded, yielding a combined dataset of 15,946 base pairs.

Phylogenies for the complete mitochondrial genomes, nuclear genes and combined datasets were inferred by both maximum likelihood (ML) and Bayesian inference (BI) approaches. Non-parametric bootstrap ML analysis was performed with RaxML version 7.0.3 (Stamatakis et al., 2005). One thousand bootstrap repetitions were run for each dataset and the 50% majority-rule consensus trees were calculated. Bayesian inference of phylogeny was performed with several partitioning strategies, as follows: (1) to assess which partitioning option best fits the mitochondrial data we partitioned by gene, using 14 partitions: tRNAs; ATPases; Cytochrome B (*CytB*); Cytochrome Oxidase I (*COX1*), *COX2* and *COX3*; NADH dehydrogenase subunits 1–6; 16s rRNA and 12s rRNA; (2) by codon and gene, which included 39 partitions; (3) a concatenated analysis modeling the whole dataset as a single partition. Previous studies showed that the partition by codon and gene strategy has the highest likelihood in plethodontid mitochondrial (e.g. Mueller et al., 2004) and nuclear data (Vieites et al., 2007). We ran Bayesian analyses to obtain the marginal likelihoods and compared those using Bayes factors (Huelsenbeck and Imennov, 2002). These analyses (not shown) for the mitochondrial dataset provided the best likelihoods for the second partitioning strategy (−171,586), followed by partition by gene (−175,585) and the unpartitioned dataset (−177,614). Bayes factors (not shown) suggest that partitioning by codon and gene is statistically better than the other two, because Bayes factor values exceeded by far the value of 40 (see Mott and Vieites, 2009). Hence, we used a codon and gene partitioning strategy for the mitochondrial and nuclear data.

Separate evolutionary models and parameters that best fit each partition were selected for the first, second and third codon positions of each protein-coding gene, for each ribosomal gene, and a concatenation of tRNAs, using PAUP* 4.0b10 (Swofford, 2003) and the Akaike Information Criterion implemented in MrModeltest version 2.25 (Nylander et al., 2004). The selected models were set as priors in Bayesian analyses. The model parameters were used as priors in the Bayesian analyses in the program MrBayes, version 3.1.2 (Ronquist and Huelsenbeck, 2003). We ran two independent analyses consisting of four Markov chains that ran for 40 million generations, sampled every 1000 generations, for each gene and partition strategy with a random starting tree, default priors, and the option "*prset ratepr*" set as "variable" to ensure that branch lengths were estimated separately for each partition. The temperature was optimized between 0.03 and 0.05 after several preliminary test runs for one million generations for each dataset. Stationarity and convergence between runs were assessed using the "*sump*" command in MrBayes, and with trace plots generated in TRACER V1.4 (Rambaut and Drummond, 2007). We also checked for stationarity and compared runs with the online application AWTY (Wilgenbusch et al., 2004), which produced similar results. Most runs became stationary after 15 to 30 million generations, so after discarding corresponding generations, the remaining trees from both analyses for each dataset were combined and a 50% majority-rule consensus tree was calculated.

Saturation of third codon positions in the relatively rapidly evolving mitochondrial genomes is a potential bias in phylogenetic inference (e.g. Springer et al., 2001). At the depths of time associated with the present study saturation has been shown to occur in salamanders (Zhang and Wake, 2009), suggesting that third codon positions should be excluded from analyses. In previous analyses of plethodontid mitochondrial genome data the inclusion or exclusion of third codon positions yielded different topologies under maximum parsimony (Mueller et al., 2004; Macey, 2005), and significantly changed support values under maximum likelihood phylogenetic inference (Mueller et al., 2004).

We performed analyses with and without third codon positions to assess their potential impact in inferring plethodontid phylogenies. Our final analysis included nuclear data only, mitochondrial data only with and without third codon positions and a combination of mitochondrial genomes with and without third codon positions and nuclear genes.

In parallel to the previous concatenated strategies, we employed a non-concatenated approach for phylogenetic inference implemented in the software BEST v2.3.1 (Liu, 2008). This method accounts for the stochastic variation expected for individual gene trees from multiple unlinked loci, sampled from a single species history after a coalescent process (Liu and Pearl, 2007; Edwards et al., 2007). In our analyses, each nuclear locus was considered a partition and assigned its own evolutionary model, the same ones as for the concatenated dataset. For each BEST analysis, two independent Markov chain Monte Carlo (MCMC) runs were initiated at different starting seeds and allowed to proceed for half billion generations (5×10^8), sampling every 1×10^5 steps. This method uses an inverse gamma probability distribution with a mean defined by two parameters, -α and β-, and equal to $\beta/(\alpha - 1)$ (when

$\alpha > 1$). Because priors on the inverse gamma distribution may have an effect on phylogenetic inference, we performed four different analyses with different priors for the effective population size parameter theta (Liu et al., 2008), following a similar strategy as in Leaché (2009). Thus, Theta values ranged from very small ($\Theta = 0.00015$; $\alpha = 3$, $\beta = 0.0003$), small ($\Theta = 0.0015$; $\alpha = 3$, $\beta = 0.003$), medium ($\Theta = 0.015$; $\alpha = 3$, $\beta = 0.03$), to large ($\Theta = 0.15$; $\alpha = 3$, $\beta = 0.3$), where small values of Θ prior are expected to reduce its influence on the estimated species trees (Liu et al., 2008). The gene mutation prior was set to (0.5, 1.5) in all analyses. Convergence and stationarity were assessed as in previous analyses. The harmonic means of these analyses after burn in were compared using Bayes factors as before.

2.4. Hypothesis testing in relation to the topological positions of Hemidactylium and Karsenia

Previous phylogenetic hypotheses based on mitochondrial genomes placed the genus *Hemidactylium* as the sister taxon to all plethodontids (MP, Macey, 2005), the sister taxon to *Batrachoseps* (ML and Bayesian, Mueller et al., 2004) (both studies lacked *Karsenia*), with an uncertain position based on partial mitochondrial data plus one nuclear locus, *RAG1* (Chippindale et al., 2004, who did not have either *Karsenia* or *Hydromantes*), or as sister to (*Batrachoseps* + tropical species) + spelerpines (Kozak et al., 2009, who used the nuclear data of Vieites et al., 2007, for *Karsenia* and *Hydromantes,* and mitochondrial data of *Hydromantes* from Mueller et al., 2004). The recently discovered genus *Karsenia* (Min et al., 2005) was placed as the sister taxon to *Hydromantes* with moderate support using a nuclear dataset (Vieites et al., 2007). Based on previous phylogenetic hypotheses and the new results obtained by analyzing our complete mitochondrial genome plus multilocus nuclear data, we tested several alternative positions of these genera on a maximum likelihood framework. These included treating the genus *Hemidactylium* as the sister taxon to all plethodontids (sensu Macey, 2005); *Hemidactylium* as the sister taxon to (*Batrachoseps* + Bolitoglossini) plus Spelerpini (Kozak et al., 2009); *Hemidactylium* as the sister taxon to *Batrachoseps* plus Bolitoglossini; *Hemidactylium* as the sister taxon to Bolitoglossini; *Karsenia* as the sister taxon to *Aneides*; *Karsenia* as the sister taxon to *Aneides* plus *Hydromantes*; and a plethodontine polytomy (for taxonomic terminology see the revised taxonomy section below). Alternative tree topologies were created and uploaded to PAUP*. These topologies were based on our preferred topology (see below), using some alternative branching arrangements. For tests we used CONSEL (Shimodaira and Hasegawa, 2001) and PAUP* (Swofford, 2003).

Using the combined mitochondrial plus nuclear dataset with and without third codon positions, we calculated the likelihood for the different tree topologies to be tested, and generated a log file for the site-wise log-likelihoods of alternative trees under a GTR + I + C model. This log file was then run in CONSEL to estimate the probability values for each alternative topology using both the Kishino–Hasegawa test (KH) (Kishino and Hasegawa, 1989) and the approximately unbiased test (AU) (Shimodaira, 2002), and the Bayesian posterior probabilities of each topology.

3. Results

3.1. Basic data

Fragment length varied among taxa due to missing nucleotides at the beginning of the sequences and different lengths of variable regions, corresponding mainly to loops in the secondary structures of the ribosomal rRNA molecules and insertions/deletions in most of mitochondrial genes. The mitochondrial genome of *K. koreana* has the typical plethodontid gene arrangement with no gene duplications or re-arrangements as in some species of plethodontids. In the nuclear dataset, only one amino acid insertion was observed in *POMC*.

3.2. Phylogenetic analyses

Our different analytical approaches provided a series of phylogenetic trees that were in general congruent, but with some exceptions, as detailed in the following sections.

3.2.1. Mitochondrial genomes without 3rd codon positions, plus nuclear loci (Fig. 1A)

Both ML and Bayesian analyses provided congruent topologies with high statistical support for each node. Only one node, that supporting the split between *Aneides* + *Ensatina* and *Desmognathus* + *Phaeognathus*, was supported in Bayesian analysis (BPP = 0.95) but not in ML analysis. This phylogenetic hypothesis supported Amphiumidae as the sister taxon to the Plethodontidae, and the Rhyacotritonidae as the sister taxon to the combined amphiumid–plethodontid clade. Within the Plethodontidae two main clades are recovered with high statistical support (BPP 1, MLBS 100): a northern clade (across North America and extending to Korea and western Europe), which includes *Aneides, Desmognathus, Ensatina, Hydromantes, Karsenia, Phaeognathus* and *Plethodon*, and an eastern/southern clade (eastern North America, Central and South America, with *Batrachoseps* in western North America, mainly California), which includes *Batrachoseps, Bolitoglossa, Eurycea, Gyrinophilus, Hemidactylium, Nototriton, Pseudoeurycea, Stereochilus,* and *Thorius*. In the northern clade *Plethodon* is the sister taxon to the remaining taxa, and *Karsenia* and *Hydromantes* form a clade that is the sister taxon to a clade comprising the remaining genera. *Desmognathus* and *Phaeognathus* (the desmognathines) form a clade that is the sister taxon to *Ensatina* + *Aneides*. The eastern/southern clade includes two subclades, one combining *Hemidactylium* + *Batrachoseps* (from eastern and western North America, respectively) with the tropical genera (*Bolitoglossa, Nototriton, Thorius*, i.e., the bolitoglossines). The other subclade includes genera from the southeastern United States: *Eurycea, Gyrinophilus, Pseudotriton* and *Stereochilus* (the spelerpines). All these relationships are well-supported (MLBS 70 or higher, BPP 0.95 or higher). We treat this as the reference topology against which all others are compared in this paper.

3.2.2. Mitochondrial genomes including 3rd codon positions, plus nuclear loci (Fig. 1B and C)

Topologies based on the combined dataset including the third codon positions of mitochondrial genes differ depending on the analytical method. The ML analysis (Fig. 1B) differs from the reference topology in finding *Aneides* as the sister taxon to *Karsenia* + *Hydromantes* (MLBS 72), and in finding *Hemidactylium* to be the sister taxon to *Batrachoseps* and the bolitoglossines (MLBS 88). Bayesian analysis (Fig. 1C) places *Hemidactylium* as the sister taxon to *Batrachoseps* (BPP 0.95), as in the reference phylogeny, and differs only in finding *Aneides* in a polytomy with *Ensatina* and *Desmognathus* + *Phaeognathus,* in contrast to being well resolved as the sister taxon to *Ensatina.*

3.2.3. Mitochondrial genomes including 3rd codon positions (Fig. 2A)

Identical topologies (with different support levels for some nodes) obtained for BI and ML analyses differ from the reference topology in the position of *Aneides* (grouped with *Karsenia* + *Hydromantes* rather than *Ensatina*). *Hemidactylium* is the sister taxon to *Batrachoseps* (BPP 1, but with low support in ML, MLBS 51) and there is no support for *Karsenia* + *Hydromantes*. The topology for the eastern/southern clade is identical to that of the reference topology, but with no support in BI at the base of this clade.

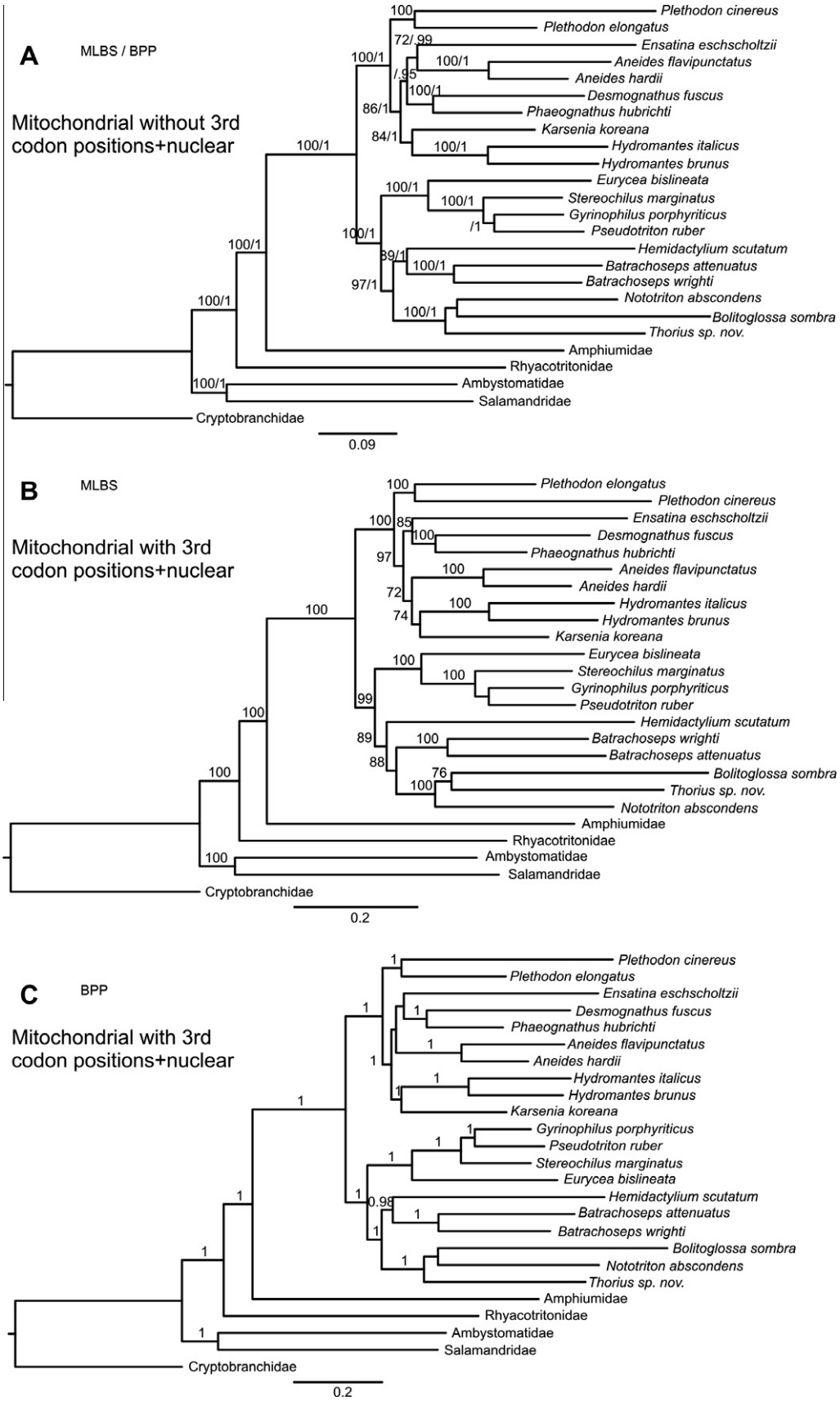

Fig. 1. Phylogenetic hypotheses based on a combined dataset (A) complete mitochondrial genomes without third codon positions plus nuclear genes, (B) complete mitochondrial genomes with third codon positions and three nuclear genes under Maximum likelihood, and (C) complete mitochondrial genomes with third codon positions and three nuclear genes under Bayesian phylogenetic inference. Values above branches denote maximum likelihood bootstrap support (MLBS) and Bayesian posterior probabilities (BPP).

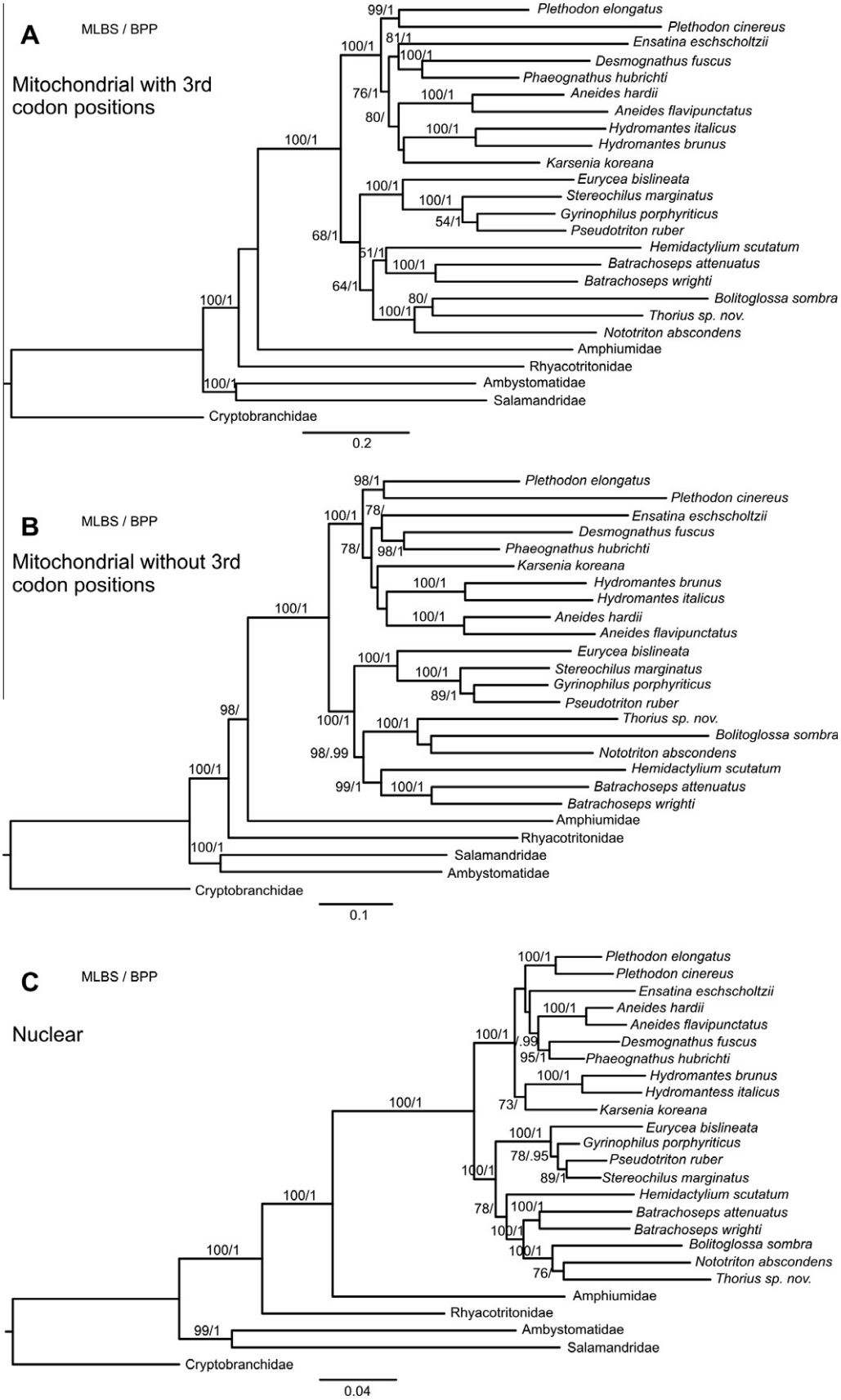

Fig. 2. Phylogenetic hypotheses based on (A) complete mitochondrial genomes with third codon positions included, (B) complete mitochondrial genomes with third codon positions, and (C) three nuclear genes, under Maximum likelihood and Bayesian phylogenetic inference. Values above branches denote maximum likelihood bootstrap support (MLBS) and Bayesian posterior probabilities (BPP).

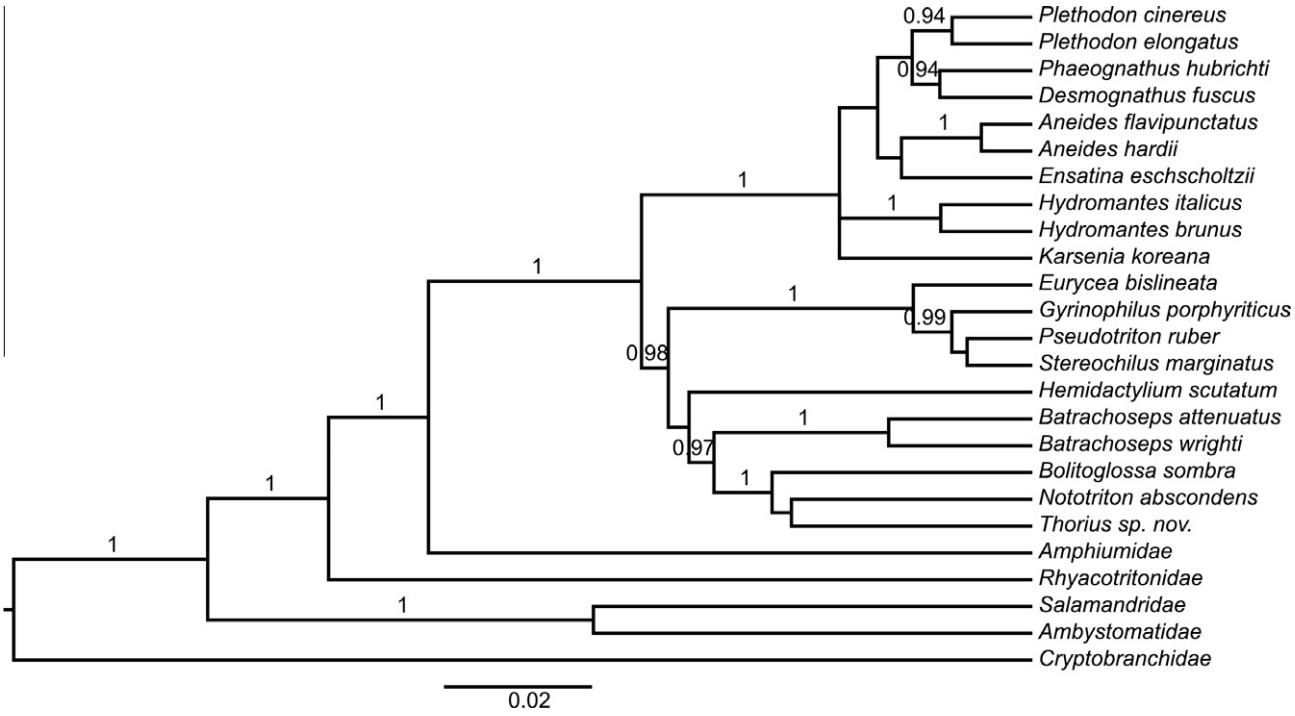

Fig. 3. Phylogenetic hypothesis based on a three nuclear gene dataset analyzed using BEST. Values above branches denote Bayesian posterior probabilities (BPP).

3.2.4. Mitochondrial genomes without 3rd codon positions (Fig. 2B)

Both methodological approaches provide the same topology. In comparison with the preceding analysis, the topologies are very similar in general but the support levels have increased substantially. The topology of the eastern/southern clade is identical to the reference topology, and all nodes but one receive high statistical support (BPP > 0.99, MLBS > 89). In the northern clade *Karsenia* is the sister taxon to *Hydromantes* + *Aneides*, but with low support.

3.2.5. Concatenated nuclear loci (Fig. 2C)

The topology differs from the reference topology in recovering *Hemidactylium* as the sister taxon to *Batrachoseps* + bolitoglossines with high support. Furthermore, the *Karsenia* + *Hydromantes* clade is the sister taxon to the remaining genera in the northern clade, but with weak statistical support. Elsewhere in the northern clade, desmognathines form the sister taxon to *Aneides*, and this combined clade is the sister taxon to *Ensatina*, but with low support, and *Plethodon* is the sister taxon to all of these.

3.2.6. Non-concatenated nuclear loci (Fig. 3)

The BEST analyses were run for a half billion generations to achieve convergence and stable results in all runs. The analyses with highest Theta priors (Θ = 0.15 and Θ = 0.015) converged very rapidly after a few million generations, while the analyses with smaller Theta priors (Θ = 0.0015 and Θ = 0.00015) required much longer runs to converge. The best performance as judged by Bayes factors (not shown for complete analysis but see Fig. 3) was the combination of α = 3 and β = 0.003. The resulting topology contains a number of unresolved nodes. The northern clade is basically unresolved, with a polytomy at the base. The eastern/southern clade has better resolution, and differs from the reference topology in recovering *Batrachoseps* + bolitoglossines with high support, but the position of *Hemidactylium* as the sister taxon to the previous clade does not reach significance (BPP = 0.85). This result is similar to that obtained with analysis of the concatenated nuclear sequences (Fig. 2C).

3.3. Hypothesis testing

Hypothesis testing was done in a maximum likelihood framework using concatenated datasets of both combined data with and without mitochondrial third codon positions. Results of the program CONSEL are summarized in Table 1. In both AU and KH tests the preferred topology has very high probability values (AU 0.936, KH 0.941), which are also much higher than any of the other topologies tested. Several topologies were rejected by those tests, including the position of *Hemidactylium* as the sister taxon to all plethodontids or to the Bolitoglossini, the position of *Karsenia* as the sister taxon to *Aneides*, or a basal polytomy in the eastern/southern clade. Other positions of *Hemidactylium* received much lower probabilities than the preferred topology, but still cannot be rejected. These include *Hemidactylium* as the sister taxon to *Batrachoseps* + Bolitoglossini and Spelerpini, or as the sister taxon to *Batrachoseps* + Bolitoglossini. The position of *Karsenia* as the sister taxon to *Aneides* + *Hydromantes* cannot be rejected either. However, the Bayesian posterior probabilities for all tested topologies provided by CONSEL suggest that only the preferred topology has high probability.

When the input data are the combined dataset with third codon positions, the best topology is still the preferred topology (AU 0.576, KH 0.515), but other topologies receive higher support than before, especially *Karsenia* as the sister taxon to *Aneides* + *Hydromantes*, or *Hemidactylium* as the sister taxon to *Batrachoseps* + Bolitoglossini. According to the Bayesian results provided by CONSEL, the preferred topology has the highest posterior probability but no topology reaches a high BPP.

4. Discussion

4.1. Tree structure

Our goal is to produce a robust phylogenetic hypothesis for plethodontid salamanders based on a large and complete dataset including both mitochondrial and nuclear sequences for the main clades, in order to resolve controversies regarding plethodontid

Table 1
Statistical confidence (*P*-values) for alternative branching hypotheses based on combined nuclear and mitochondrial data. AU refers to approximately unbiased test; KH refers to Kishino–Hasegawa test; BPP refers to Bayesian posterior probability. The last column summarizes the rejection (+) or failure to reject (−) the different topologies for the AU and KH tests respectively.

Dataset	Alternative topology tested	Δln *L*	*P*-value			Rejection
			AU test	KH test	BPP	
Mitochondrial + nuclear without 3rd codon positions	Best ML (reference topology)	0	0.936	0.941	1	− −
	Hemidactylium sister to plethodontids	−62.6	0.023	0.021	0.000	++
	Hemidactylium sister to (*Batrachoseps* + Bolitoglossini) + Spelerpini	−28.8	0.169	0.083	0.000	− −
	Hemidactylium sister to *Batrachoseps* + Bolitoglossini	−22.9	0.122	0.059	0.000	− −
	Hemidactylium sister to Bolitoglossini	−37.1	0.001	0.002	0.000	++
	Karsenia sister to *Aneides* (no *Hydromantes*)	−62.1	0.001	0.001	0.000	++
	Karsenia sister to *Aneides* + *Hydromantes*	−24.3	0.152	0.131	0.000	− −
	Plethodontinae polytomy	−179.9	1E−04	0	0.000	++
Mitochondrial + nuclear	Best ML (Reference topology)	0	0.576	0.515	0.682	− −
	Hemidactylium sister to plethodontids	−10.8	0.394	0.37	0.000	− −
	Hemidactylium sister to (*Batrachoseps* + Bolitoglossini)+Spelerpini	−17.3	0.190	0.236	0.000	− −
	Hemidactylium sister to *Batrachoseps* + Bolitoglossini	−4.6	0.485	0.382	0.007	− −
	Hemidactylium sister to Bolitoglossini	−26.0	0.013	0.015	0.000	++
	Karsenia sister to *Aneides* (no *Hydromantes*)	−64.2	5E−04	0.002	0.000	++
	Karsenia sister to *Aneides* + *Hydromantes*	−0.8	0.531	0.485	0.311	− −
	Plethodontinae polytomy	−219.4	0.015	0	0.000	++

phylogenetic relationships, and to produce a new conservative taxonomy that is likely to prove stable through time.

Mitochondrial genomes contribute ca. 80% of the data in our analyses. The reference topology (the one obtained from combined nuclear + mitochondrial but excluding mitochondrial third codon positions) is similar to the mitochondrial (minus third codon positions) topology, but adding the nuclear data resolves the polytomy observed in the northern clade (Fig. 1A vs. 2B), increasing the overall support values across the tree as well. Tree topology based on the nuclear dataset alone and considering only well-supported clades differs from the reference topology principally in the position of *Hemidactylium* (Figs. 1A vs. Fig. 2C), which has the topology of the ML analysis of the combined dataset including third codon positions (Fig. 1B). Thus, despite its numerical superiority, the mt dataset does not govern the reference topology, except for the position of *Hemidactylium*. Increasing the amount of nuclear data might eventually produce the placement for that genus found in the nuclear-only tree (Fig. 2C), which has the traditional topology of sister–taxon relationship of *Batrachoseps* to the tropical genera (the bolitoglossines in the strict sense of the present study).

Inclusion or exclusion of third codon positions for analyses restricted to complete mt genomes affects only levels of ML support, lowering them when third codons are included, except for one node (*Aneides*, *Hydromantes, Karsenia*) that is supported only when third codons are included (Fig. 2A, 2B). In the combined nuclear and mt genome dataset using BI, the topologies are almost identical, but the position of *Aneides* is not resolved when third codons are included. The ML analysis without third codon positions produces the same topology, but when third codon positions are excluded there is one important difference: *Hemidactylium* becomes the sister taxon to *Batrachoseps* + Bolitoglossini. At the phylogenetic level of this analysis (deeper divergences date variously from Cretaceous to Paleocene: Mueller, 2006; Mueller et al., 2004; Roelants et al., 2007; Schwartz and Mueller, 2010; Vieites et al., 2007; Zhang and Wake, 2009) third positions are saturated for mitochondrial genes (Zhang and Wake, 2009), under which conditions extensive homoplasy is expected because of saturation (e.g. Springer et al., 2001). Although third codon positions may provide useful phylogenetic signal at recent timescales (e.g. when dealing with divergences of a few millions of years, when little saturation has occurred), at the scale of our study they add noise that can mislead phylogenetic inference. Plethodontids are old, and most genera that diverged more than 20 mya show saturation at mt 3rd codon positions (Mueller et al., 2004), but not at other mt positions or in the nuclear data. Although we cannot reject the possibility that inclusion of third codon positions may add some useful phylogenetic information, the conflicting results with and without them that are likely related to full saturation, as well as conflicts with the nuclear dataset, caution against their use in our dataset. In order to fully test this hypothesis, at least an equal amount of independent nuclear data will be needed to contrast with topologies including and excluding mt genome third codon positions.

The problematic nodes in the plethodontid phylogeny are short and some cannot be resolved with available datasets, even by adding substantial amounts of nuclear data to the complete mt genomes. Within our northern clade, *Plethodon* is resolved as sister to the remaining taxa, but because these taxa likely arose in a short time interval (cf. Vieites et al., 2007), a combination of the short internodes and likely extinction of some relevant lineages leads to some uncertain resolution. Our use of non-concatenated coalescence phylogenetic approaches with nuclear data, such as the one implemented in BEST, did not resolve some phylogenetic relationships. Possibly this irresolution is not the result of insufficient data but may be a true reflection of real (i.e., "hard") polytomies. The development of next generation sequencing technologies holds promise of providing large amount of nuclear genomic data (e.g. thousands of novel independent loci) that should illuminate these controversies.

Results of our tests of alternative topologies show that the reference phylogeny is the most likely hypothesis regardless of which dataset is used (e.g., combined mt and nuclear data both with and without third codon positions). In the paragraphs below we discuss specific issues starting from the base of the plethodontid clade.

4.1.1. Two main clades

Whereas Mueller et al. (2004) recovered two major clades in the plethodontidae (equivalent to the subfamilies Plethodontinae and Hemidactyliinae of Vieites et al., 2007), Macey (2005, using exactly the same dataset) did not. Instead, he recovered *Hemidactylium* as the sister taxon to the remaining genera, with an unresolved (in terms of statistical support) polytomy of the plethodontines, the spelerpines and his bolitoglossines (*Batrachoseps* and the tropical genera). Chippindale et al. (2004) recovered two main clades, a well-supported Plethodontinae and a second clade without basal

statistical support. Kozak et al. (2009) recovered two main clades with statistical support. Our results strongly support division of the Plethodontidae into two major clades, the Hemidactyliinae (with about 320 species) and the Plethodontinae (with about 100 species).

4.1.2. Position of Karsenia in relation to Hydromantes

The most likely position of *Karsenia* in the analysis of the nuclear dataset of Vieites et al. (2007) was as the sister taxon to *Hydromantes*, but this was not strongly supported. The addition of the complete mt genomes, now including *Karsenia*, to the nuclear dataset again finds this relationship, now with high statistical support. *Karsenia* is most likely the sister taxon of *Hydromantes*; however, a clade of *Aneides, Karsenia* and *Hydromantes* cannot be rejected (Table 1).

4.1.3. Position of Aneides and Ensatina

Aneides and *Plethodon* were long considered to be sister taxa, with *Ensatina* as the sister taxon to *Aneides* + *Plethodon* (Maxson and Wake, 1981; Wake, 1966). While the three genera are all within our northern clade, their relationships to each other remain somewhat unsettled. Mueller et al. (2004) found *Plethodon* well-supported as the sister taxon to the other genera. Topologically *Ensatina* was the sister taxon to the desmognathines and *Aneides* the sister taxon to *Hydromantes* (*Karsenia* was not yet known). However, there was no statistical support and thus the sister clade of *Plethodon* was essentially a polytomy. The reanalysis of the same dataset by Macey yielded an unresolved polytomy including *Plethodon.* Our reference topology produces a largely resolved tree, with *Ensatina* and *Aneides* as sister taxa, but alternative topologies show (Figs. 1 and 2) show *Aneides* either with *Karsenia* + *Hydromantes* or with *Ensatina* + desmognathines. *Plethodon* is always the sister taxon to all other members of the clade (see also Kozak et al., 2009, where *Plethodon* is sister to the other taxa, whose relationships are unresolved).

4.1.4. Position of Hemidactylium

The main point of contention in the analyses of the same dataset by Mueller et al. (2004) and Macey (2005) was the position of *Hemidactylium*, which was well-supported as the sister taxon to *Batrachoseps* in the former (BI, ML and MP excluding third codons, but not including third codon positions) and well-supported as the sister taxon to all other plethodontids in the latter (MP). Our reference topology is identical to that of Mueller et al., but with higher statistical support. An alternative placement, using a combined mt and nuclear dataset including 3rd codons (Fig. 1B), has *Hemidactylium* as the sister taxon to *Batrachoseps* + bolitoglossines. These were the only alternative placements found in our analyses. In our nuclear dataset *Hemidactylium* occurs in this alternative placement, with statistical support. Vieites et al. (2007) recovered a similar topology from their nuclear dataset, but without statistical support. Perhaps addition of two more tropical species explains the increase of statistical support. Kozak et al. (2009) recovered *Hemidactylium* as the sister taxon to the tropical species, with statistical support. In none of our analyses is *Hemidactylium* found in a more basal position, neither as the sister taxon to the spelerpines plus *Batrachoseps* and our bolitoglossines, nor as the sister taxon to all other plethodontids (Table 1, see Hypothesis testing, above).

4.1.5. Position of Batrachoseps

Batrachoseps once was thought to be closely related to *Plethodon* (Dunn, 1926; Noble, 1931, who wrote that *Batrachoseps* "–has been derived from *Plethodon*–", p. 482), but since the work of Wake (1966) it has been associated with the tropical salamanders, and *Hydromantes*. While *Hydromantes* is well-supported as a member of our northern clade, *Batrachoseps* is always embedded in our southern/eastern clade, where it is either the sister taxon to *Hemidactylium* (reference topology, with high statistical support; Fig. 1A), or the sister taxon to the bolitoglossines (mt + nuclear including 3rd codons, with equivalent statistical support; Fig. 1B; nuclear Figs. 2C and 3).

4.2. History of the Plethodontidae

Our reference topology, as well as all other topologies from our different analyses, recovers long terminal branches and short basal internodes, suggesting that major cladistic events occurred in a relatively short span of time a long time ago (e.g. early Tertiary: Vieites et al., 2007). Some ambiguity is likely inevitable in such a scenario, and in our analysis this can be exemplified by the shifting relationships of *Aneides* and *Hemidactylium*. The reference phylogenetic hypothesis, the concatenation of the complete mitochondrial genomes plus three nuclear genes, is fully resolved. Amphiumidae and Rhyacotritonidae, both exclusively North American, are successive outgroups to the Plethodontidae (see also Zhang and Wake, 2009), and it is most parsimonious to conclude that plethodontids originated in North America and later spread to the Old World and the New World tropics. Two well-supported clades of plethodontids are recovered, the plethodontines and the hemidactyliines. The plethodontines include species generally from north of Mexico (*Aneides* and *Ensatina* occur in northern Baja California) across the northern hemisphere to Korea and the western Mediterranean (Italy/southeastern France). *Karsenia* is the sister taxon of *Hydromantes*. *Aneides* and *Ensatina* are sister taxa and together form the sister taxon to *Desmognathus*/*Phaeognathus* (the desmognathines). The *Karsenia*/*Hydromantes* clade is the sister taxon to the *Aneides*/*Ensatina*/desmognathine clade, and *Plethodon* is sister to them all.

The Hemidactyliinae contains two main clades, located in eastern North America and tropical regions of Central and northern South America, with one genus (*Batrachoseps*) along the west coast of North America, from Oregon to Baja California. One of these major clades, the spelerpines, is found in all analyses and is widely recognized (e.g., Camp et al., 2009). The other remains somewhat problematic. Our reference topology places *Hemidactylium* as the sister taxon to *Batrachoseps*, and these genera together form the sister taxon to the tropical genera (former supergenus *Bolitoglossa*; a clade well-supported by morphology (Wake, 1966; Lombard and Wake, 1986) and molecular data (Wiens et al., 2007), containing about 264 species and not considered in detail here). This topology is in conflict with the morphological data (Lombard and Wake, 1986), which finds *Batrachoseps* as the sister taxon to the tropical genera. The fact that *Batrachoseps* and the bolitoglossines share a unique synapomorphy, 13 pairs of chromosomes with all other plethodontids having 14, is consistent with such a sister–taxon relationship.

The phylogenetic hypothesis of Wake (1966) assumed that larvae were ancestral for plethodontids and that in particular the larvae of the desmognathines displayed more ancestral features than did those of his subfamily Plethodontinae, which were thought to have synapomorphic traits such as reduction in the numbers of larval and embryonic epibranchials. Molecular data force the conclusion that larvae have re-evolved in lineages such as desmognathines that are deeply nested within otherwise terrestrial clades. The first hints that this might be so came from partial mitochondrial gene sequences (Titus and Larson, 1996) that showed that even within *Desmognathus* itself the inferred ancestral condition was absence of a larval stage. Newer evidence (Chippindale et al., 2004; Mueller et al., 2004; Kozak et al., 2005; this paper) is consistent and conclusive. *Phaeognathus*, the sister taxon of *Desmognathus*, displays direct development, as do sequential outgroups to the desmognathines. Furthermore, spelerpines

(most having stream larvae) are deeply nested, raising the possibility that stream larvae, so long associated with plethodontid origins (Bruce, 2005, 2007), might have been independently derived in desmognathines and spelerpines. In the first major clade, the plethodontines, parsimony argues in favor of an ancestral condition of direct development. For the second clade, the hemidactyliines, it is equally parsimonious for the ancestral condition to have been either larval or direct development. The spelerpines all develop from larvae. Their sister clade comprises *Hemidactylium*, *Batrachoseps,* and the bolitoglossines (that is, the entire tropical plethodontid clade). Both *Batrachoseps* and the bolitoglossines are direct developers. *Hemidactylium* is unique in having a short-lived pond-type larva. If the node ancestral to the spelerpines and their sister clade had larval development, two losses of larvae, or one loss and one gain of larvae, are equally parsimonious. If, on the other hand, direct development was ancestral, two gains of larvae occurred, or one gain and one loss. These alternatives are equally parsimonious. In the alternative topology (including 3rd codons), if the ancestral node had larval development, one loss occurred (tropical plethodontids + *Batrachoseps*). If the ancestral node had direct development, two gains or one gain and one loss of larvae are equally parsimonious. One must consider the once highly unlikely possibility that direct development may have been ancestral for Plethodontidae, which raises anew the controversy over the origin of lunglessness, long considered an adaptation to the development of stream-adapted larvae in montane settings (Wilder and Dunn, 1920; Beachy and Bruce, 1992). While Bruce et al. (1994) have shown that lung reduction and/or solidification reduce buoyancy and enhance rheotactic ability in salamander larvae, this does not mean that the initial loss of lungs in plethodontids was a rheotactic adaptation. The sister taxon of plethodontids, the amphiumids, has well developed lungs but they lack free-living larvae; eggs are laid on land and hatchlings might retain gill rudiments for up to two weeks (Gunzburger, 2003). Thus amphiumids show an approximation of direct development. The sister taxon of plethodontids + amphiumids is Rhyacotritonidae, which has tiny, solidified, functionless lungs and long-lived stream (or more appropriately seep) larvae. Accordingly, it remains unclear what the biology of ancestral plethodontids might have been, but there is no longer phylogenetic evidence that they had stream larvae.

Homoplasy is a dominant factor in salamander evolution (Wake, 1966, 1991, 2009; Wake and Larson, 1987; Mueller et al., 2004; Wiens et al., 2005). Our current understanding of phylogenetic relationships is relatively neutral with respect to major aspects of homoplasy, except regarding tongue evolution. Freely projectile tongues have independently evolved in *Hydromantes*, the spelerpines (*Stereochilus* is somewhat an exception in having a non-muscular attachment of the tongue to the front of the mouth, a further homoplastic reversion resembling the ancestral state), and the tropical bolitoglossines. Loosening of the anterior attachment of the tongue has occurred in *Batrachoseps* and to a lesser extent in *Hemidactylium* and *Ensatina* (Lombard and Wake, 1977). Spelerpines and tropical bolitoglossines are never sister taxa, and so the conclusion that there were three independent evolutions of complete tongue freedom (Lombard and Wake, 1986) is reinforced.

4.3. Clades and genera of plethodontid salamanders

At present nearly 420 species are assigned to the Plethodontidae (http://amphibiaweb.org/lists/index.shtml). These are arranged in 26–30 genera, varying among authors. Several of these are monotypic and hence monophyletic by default. At least one of these, *Haideotriton*, is controversial and lacks molecular data; Frost et al. (2006) place its lone species in *Eurycea*, but in the absence of molecular data we continue to recognize it (see also Crother, 2008). All other currently recognized genera have been studied to some extent using combinations of molecular (allozymes, mtDNA, nuclear DNA) and morphological (usually osteology) data. Evidence is strong for monophyly of *Aneides* (Vieites et al., 2007), *Batrachoseps* (Jockusch and Wake, 2002; Mueller et al., 2004; Vieites et al., 2007), *Bolitoglossa* (Wiens et al., 2007), *Chiropterotriton* (Wiens et al., 2007), *Cryptotriton* (at least Nuclear Central American species, Wiens et al., 2007; Rovito, unpublished), *Dendrotriton* (Rovito et al., submitted), *Desmognathus* (Kozak et al., 2005), *Eurycea* (Camp et al., 2009; Kozak et al., 2009), *Hydromantes* (Vieites et al., 2007), *Gyrinophilus* (Niemiller et al., 2008), *Ixalotriton* (Parra-Olea, 2002), *Nototriton* (Wiens et al., 2007), *Oedipina* (McCranie et al., 2008), *Plethodon* (Wiens et al., 2006; Kozak et al., 2006; Vieites et al., 2007), *Pseudotriton* (no molecular studies but morphological synapomorphies), and *Thorius* (Wiens et al., 2007; Parra-Olea, unpublished). Parra-Olea and Wake (2001; Parra-Olea, 2002) used mtDNA data to show that *Lineatriton* is deeply nested within *Pseudoeurycea*, despite great morphological disparities, and furthermore that *Lineatriton* haplotypes are diphyletic with respect to *Pseudoeurycea*. The likelihood that *Pseudoeurycea* is paraphyletic is near certainty, not only because *Ixalotriton* is also nested using mtDNA data, but also because the generotype of *Lineatriton* (*lineolus*) is within the same subclade that contains the generotype of *Pseudoeurycea* (*leprosa*). The monotypic *Parvimolge* is also associated with what might be called the *Pseudoeurycea* complex (Parra-Olea, 2002). These facts led Frost et al. (2006) to designate both *Ixalotriton* and *Lineatriton* junior synonyms of *Pseudoeurycea*. That genus contains between 45 and 50 species, several of which have been unavailable for study, so subdivision of *Pseudoeurycea* is premature. However, Parra-Olea et al. (2010) reaffirmed the deeply nested position of *Lineatriton* and treated it as a synonym of *Pseudoeurycea.* But the base of their tree is essentially a polytomy and like them we retain *Ixalotriton* pending data from nuclear sequences.

Several plethodontid genera are large. When robust phylogenies are obtained for substantial numbers of species in such genera, we consider recognition of subgenera a useful way to provide some phylogenetic information and at the same time to maintain taxonomic stability. Subgenera currently are recognized for *Batrachoseps, Bolitoglossa, Hydromantes* and *Oedipina* (see below). *Plethodon* long has been considered a candidate for subdivision, based on both morphological (especially vertebral shape – Highton, 1962; Wake, 1966) and molecular data. Early molecular studies found *Plethodon* paraphyletic with respect to *Aneides* (e.g., Larson et al., 1981). Wiens et al. (2006) sampled nearly all species of *Plethodon*, and they found the genus monophyletic. The species fell into two well-supported clades, equivalent to the eastern and western *Plethodon* of Wake (1966). With less dense taxon sampling but increased amounts of sequence, we obtain similar results (in accord also with Vieites et al., 2007). Accordingly, we designate the western clade as *Hightonia*, New Taxon, with the rank of subgenus and with the type species *Ambystoma vehiculum* Cooper, 1869 (currently *Plethodon vehiculum*). Other species assigned to this taxon are: *P. asupak, P. dunni, P. elongatus, P. idahoensis, P. larselli, P. neomexicanus, P. stormi*, and *P. vandykei*. The taxon is diagnosed relative to the subgenus *Plethodon* by having relatively broader and stouter vertebrae (as described by Highton, 1962; Wake, 1963, 1966; Tihen and Wake, 1981). The name honors Richard Highton, premier student of *Plethodon* for more than 50 years.

4.4. Revised taxonomy

While the reference phylogeny is our preferred topology, we also obtain a fully resolved tree from the concatenation of mitochondrial and nuclear genes including third codon positions. In

the latter topology, the positions of *Aneides* and *Hemidactylium* have shifted, *Aneides* being recovered as the sister taxon to *Karsenia/Hydromantes* and *Hemidactylium* as the sister taxon to *Batrachoseps* and the tropical genera. Because both topologies are fully resolved with statistical support, which alternative to use depends on how one views inclusion or exclusion of third codon positions. Given the great inferred age of the clade (the split between the two major clades is estimated at about 94 myr by Vieites et al., 2007, using nuclear genes, and about 128 myr by Schwartz and Mueller, 2010, using the complete mitochondrial dataset of Mueller et al., 2004, and some different calibration points), it seems reasonable to assume that third positions are fully saturated (see also Zhang and Wake, 2009), and accordingly we prefer our reference topology. However, in making our taxonomic revision (see below) we have chosen to be conservative and to use a taxonomy compatible with both alternatives (e.g., with or without third codon positions) for our combined datasets.

We offer a new, formal taxonomy of the Family Plethodontidae, guided but not governed by principles enunciated by Dubois (2008) and Dubois and Raffaëlli (2009). For details regarding type species, etc., refer to the Amphibian Species of the World website (http://research.amnh.org/vz/herpetology/amphibia/). Our taxonomy takes into account the fact that alternative analytical treatments of the combined nuclear and mitochondrial DNA dataset provide alternative topologies with respect to certain taxa. Our conservative taxonomy recognizes five tribes within Plethodontinae and three within Hemidactyliinae. Wake (1966) used supergenera for *Hydromantes*, *Batrachoseps*, and *Bolitoglossa* (the last including all tropical genera), but it is more in accord with the International Rules of Zoological Nomenclature (1999) to use infrafamilial categories. Both *Hydromantes* and *Batrachoseps* are diverse genera with clear subdivisions. However, each is supported by many morphological synapomorphies (Lombard and Wake, 1986; Wake, 1966) and they are always recovered as monophyletic in molecular studies. Accordingly, placing the species in subgenera retains more phylogenetic information than does recognizing more genera. An alternative to the classification below for the Plethodontinae would be a tribe Ensatinini that includes *Ensatina* and *Aneides*, should the reference topology gain more support by the addition of more nuclear data. An alternative classification for the Hemidactyliinae, should the alternative topology gain more support, would be to include *Batrachoseps* in an expanded Bolitoglossini.

Family Plethodontidae Gray, 1850
  Subfamily Plethodontinae Gray, 1850
Tribe Aneidini new taxon
  Genus *Aneides* Baird, 1851 (six species)
Tribe Desmognathini Gray, 1850
  Genus *Desmognathus* Baird, 1850 (21 species)
  Genus *Phaeognathus* Highton, 1961 (one species)
Tribe Ensatinini Gray, 1850
  Genus *Ensatina* Gray, 1850 (one species)
Tribe Hydromantini new taxon
  Genus *Hydromantes* Gistel, 1843 (11 species)
    Subgenus *Atylodes* Gistel, 1868
    Subgenus *Hydromantes* Gistel, 1843
    Subgenus *Speleomantes* Dubois, 1984
  Genus *Karsenia* Min, Yang, Bonnet, Vieites, Brandon and Wake, 2005 (one species)
Tribe Plethodontini Gray, 1850
  Genus *Plethodon* Tschudi, 1838 (55 species)
    Subgenus *Hightonia* New Taxon
    Subgenus *Plethodon* Tschudi,1838
    Subfamily Hemidactyliinae Hallowell 1856
Tribe Batrachosepini new taxon
  Genus *Batrachoseps* Bonaparte, 1839 (19 species)
    Subgenus *Batrachoseps* Bonaparte, 1839
    Subgenus *Plethopsis* Bishop, 1937
Tribe Bolitoglossini Hallowell, 1856
  Genus *Bolitoglossa* Duméril, Bibron and Duméril, 1854 (117 species)
    Subgenus *Bolitoglossa* Duméril, Bibron and Duméril, 1854
    Subgenus *Eladinea* Miranda-Ribeiro, 1937
    Subgenus *Magnadigita* Taylor, 1944
    Subgenus *Mayamandra* Parra-Olea, García-París and Wake, 2004
    Subgenus *Nanotriton* Parra-Olea, García-París and Wake, 2004
    Subgenus *Oaxakia* Parra-Olea, García-París and Wake, 2004
    Subgenus *Pachymandra* Parra-Olea, García-París and Wake, 2004
  Genus *Bradytriton* Wake and Elias, 1983 (one species)
  Genus *Chiropterotriton* Taylor, 1944 (12 species)
  Genus *Cryptotriton* García-París and Wake, 2000 (seven species)
  Genus *Dendrotriton* Wake and Elias, 1983 (eight species)
  Genus *Ixalotriton* Wake and Johnson, 1989 (two species)
  Genus *Nototriton* Wake and Elias, 1983 (14 species)
  Genus *Nyctanolis* Elias and Wake, 1983 (one species)
  Genus *Oedipina* Keferstein, 1868 (29 species)
    Subgenus *Oedipina* Keferstein, 1868
    Subgenus *Oeditriton* McCranie, Vieites and Wake, 2008
    Subgenus *Oedopinola* Hilton, 1946
  Genus *Parvimolge* Taylor, 1944 (one species)
  Genus *Pseudoeurycea* Taylor, 1944 (49 species)
  Genus *Thorius* Cope, 1869 (23 species)
Tribe Hemidactyliini Hallowell 1856
  Genus *Hemidactylium* Tschudi, 1838 (one species)
Tribe Spelerpini Cope, 1859
  Genus *Eurycea* Rafinesque, 1822 (26 species)
  Genus *Gyrinophilus* Cope, 1869 (four species)
  Genus *Haideotriton* Carr, 1939 (one species)
  Genus *Pseudotriton* Tschudi, 1838 (two species)
  Genus *Stereochilus* Cope, 1869 (one species)
  Genus *Urspelerpes* Camp, Peterman, Milanovich, Lamb, Maerz and Wake, 2009 (one species)

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.03.012.

References

Beachy, C.K., Bruce, R.C., 1992. Lunglessness in plethodontid salamanders is consistent with the hypothesis of a mountain stream origin: a response to Ruben and Boucot. Am. Nat. 139, 839–847.

Bruce, R.C., 2005. Did *Desmognathus* salamanders reinvent the larval stage? Herpetol. Rev. 36, 107–112.
Bruce, R.C., 2007. Out of the frying pan into the fire: an ecological perspective on evolutionary reversal in life history in plethodontid salamanders (Amphibia: Plethodontidae). Evol. Ecol. 21, 703–726.
Bruce, R.C., Beachy, C.K., Lenzo, P.G., Pronych, S.P., Wassersug, R.I., 1994. Effects of lung reduction on rheotactic performance in amphibian larvae. J. Exp. Zool. 268, 377–380.
Bruford, M., Hanotte, O., Brookfield, J., Burke, T., 1992. Single locus and multilocus DNA fingerprint. In: Hoelzel, A. (Ed.), Molecular Genetic Analysis in Conservation. IRL Press, Oxford, pp. 225–270.
Camp, C.D., Peterman, W.E., Milanovich, J.R., Lamb, T., Maerz, J.C., Wake, D.B., 2009. A new genus and species of lungless salamander (family Plethodontidae) from the Appalachian highlands of the south-eastern United States. J. Zool. 279, 86–94.
Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol. Biol. Evol. 17, 540–552.
Chippindale, P.T., Bonett, R.M., Baldwin, A.S., Wiens, J.J., 2004. Phylogenetic evidence for a major reversal of life-history evolution in plethodontid salamanders. Evolution 5, 2809–2822.
Crother, B.I. (Ed.), 2008. Scientific and Standard English Names of Amphibians and Reptiles of North America North of Mexico, with Comments Regarding Confidence in Our Understanding, sixth ed. Herp. Circ., pp. 1–84 (37).
Dubois, A., 2008. Phylogenetic hypotheses, taxa and nomina in zoology. Zootaxa 1950, 51–86.
Dubois, A., Raffaëlli, J., 2009. A new ergotaxonomy of the family Salamandridae Goldfuss, 1820 (Amphibia, Urodela). Alytes 26, 1–85.
Dunn, E.R., 1926. Salamanders of the Family Plethodontidae. Smith College, Northampton, MA.
Edwards, S.V., Liu, L., Pearl, D.K., 2007. High resolution species trees without concatenation. Proc. Natl. Acad. Sci. USA 104, 5936–5941.
Frost, D.R., 2010. Amphibian Species of the World: An Online Reference. Ver. 5.4. Electronic Database. <http://research.amnh.org/vz/herpetology/amphibia/> (accessed 08.04.10).
Frost, D.R., Grant, T., Faivovich, J., Bain, R.H., Haas, A., Haddad, C.F.B., de Sá, R.O., Channing, A., Wilkinson, M., Donnellan, S.C., Raxworthy, C.J., Campbell, J.A., Blotto, B.L., Moler, P., Drewes, R.C., Nussbaum, R.A., Lynch, J.D., Green, D.M., Wheeler, W.C., 2006. The amphibian tree of life. Bull. Am. Mus. Nat. Hist. 297, 1–370.
Gunzburger, M.S., 2003. Evaluation of the hatching trigger and larval ecology of the salamander *Amphiuma means*. Herpetologica 59, 459–468.
Highton, R., 1962. Revision of North American salamanders of the genus *Plethodon*. Bull. Florida St. Mus. 6, 235–367.
Huelsenbeck, J.P., Imennov, N.S., 2002. Geographic origin of human mitochondrial DNA: accommodating phylogenetic uncertainty and model comparison. Syst. Biol. 51, 155–165.
Jockusch, E.L., Wake, D.B., 2002. Falling apart and merging: diversification of slender salamanders (Plethodontidae: *Batrachoseps*) in the American West. Biol. J. Linn. Soc. 76, 361–391.
Kishino, H., Hasegawa, M., 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching order in Hominoidea. J. Mol. Evol. 29, 170–179.
Kozak, K.H., Wiens, J.J., 2010. Niche conservatism drives elevational diversity patterns in Appalachian salamanders. Am. Nat. 176, 40–54.
Kozak, K.H., Larson, A., Bonett, R.M., Harmon, L.J., 2005. Phylogenetic analysis of ecomorphological divergence, community structure, and diversification rates in dusky salamanders (Plethodontidae: *Desmognathus*). Evolution 59, 2000–2016.
Kozak, K.H., Mendyk, R.W., Wiens, J.J., 2009. Can parallel diversification occur in sympatry? Repeated patterns of body-size evolution in coexisting clades of North American salamanders. Evolution 63, 1769–1784.
Kozak, K.H., Weisrock, D.W., Larson, A., 2006. Rapid lineage accumulation in a non-adaptive radiation: phylogenetic analysis of diversification rates in eastern North American woodland salamanders (Plethodontidae: *Plethodon*). Proc. R. Soc. B 273, 539–546.
Larson, A., Wake, D.B., Maxson, L.R., Highton, R., 1981. A molecular phylogenetic perspective on the origins of morphological novelties in the salamanders of the tribe Plethodontini (Amphibia, Plethodontidae). Evolution 35, 405–422.
Leaché, A.D., 2009. Species tree discordance traces to phylogeographic clade boundaries in North American fence lizards (*Sceloporus*). Syst. Biol. 58, 547–559.
Lemmon, A.R., Brown, J.M., Stanger-Hall, K., Moriarty Lemmon, E., 2009. The effect of ambiguous data on phylogenetic estimates obtained by maximum likelihood and Bayesian inference. Syst. Biol. 58, 130–145.
Liu, L., 2008. BEST: Bayesian estimation of species trees under the coalescent model. Bioinformatics 24, 2542–2543.
Liu, L., Pearl, D.K., 2007. Species trees from gene trees: reconstructing Bayesian posterior distributions of a species phylogeny using estimated gene tree distributions. Syst. Biol. 56, 504–514.
Liu, L., Pearl, D.K., Brumfield, R.T., Edwards, S.V., 2008. Estimating species trees using multiple-allele DNA sequence data. Evolution 62, 2080–2091.
Lombard, R.E., Wake, D.B., 1977. Tongue evolution in the lungless salamanders, Family Plethodontidae. II. Function and evolutionary diversity. J. Morphol. 153, 39–80.
Lombard, R.E., Wake, D.B., 1986. Tongue evolution in the lungless salamanders, Family Plethodontidae. IV. Phylogeny of plethodontid salamanders and the evolution of feeding dynamics. Syst. Zool. 35, 532–551.
Macey, J.R., 2005. Plethodontid salamander mitochondrial genomics: a parsimony evaluation of character conflict and implications for historical biogeography. Cladistics 21, 194–202.
Maxson, L.R., Wake, D.B., 1981. Albumin evolution and its phylogenetic implications in the plethodontid salamander genera *Pseudoeurycea* and *Chiropterotriton*. Herpetologica 37, 109–117.
McCranie, J.R., Vieites, D.R., Wake, D.B., 2008. Description of a new divergent lineage and three new species of Honduran salamanders of the genus *Oedipina* (Caudata, Plethodontidae). Zootaxa 1930, 1–17.
Min, M.S., Yang, S.Y., Bonett, R.M., Vieites, D.R., Brandon, R.A., Wake, D.B., 2005. Discovery of the first Asian plethodontid salamander. Nature 435, 87–90.
Mott, T., Vieites, D.R., 2009. Molecular phylogenetics reveals extreme morphological homoplasy in Brazilian worm lizards challenging current taxonomy. Mol. Phylogenet. Evol. 51, 190–200.
Mueller, R.L., 2006. Evolutionary rates, divergence dates, and the performance of mitochondrial genes in Bayesian phylogenetic analysis. Syst. Biol. 55, 289–300.
Mueller, R.L., Boore, J.L., 2005. Molecular mechanisms of extensive mitochondrial gene rearrangement in plethodontid salamanders. Mol. Biol. Evol. 22, 2104–2112.
Mueller, R.L., Macey, J.R., Jaekel, M., Wake, D.B., Boore, J.L., 2004. Morphological homoplasy, life history evolution, and historical biogeography of plethodontid salamanders inferred from complete mitochondrial genomes. Proc. Natl. Acad. Sci. USA 101, 13820–13825.
Niemiller, M.L., Fitzpatrick, B.M., Miller, B.T., 2008. Recent divergence with gene flow in Tennessee cave salamanders (Plethodontidae: *Gyrinophilus*) inferred from gene genealogies. Mol. Ecol. 17, 2258–2275.
Noble, G.K., 1931. The Biology of the Amphibia. McGraw-Hill, New York, NY, p. 577.
Nylander, J.A.A., Ronquist, F., Huelsenbeck, J.P., Nieves Aldrey, J.L., 2004. Bayesian phylogenetic analysis of combined data. Syst. Biol. 53, 47–67.
Parra-Olea, G., 2002. Molecular phylogenetic relationships of neotropical salamanders of the genus *Pseudoeurycea*. Mol. Phylogenet. Evol. 22, 234–246.
Parra-Olea, G., Wake, D.B., 2001. Extreme morphological and ecological homoplasy in tropical salamanders. Proc. Natl. Acad. Sci. USA 98, 7888–7891.
Parra-Olea, G., Rovito, S.M., Márquez-Valdelamar, L., Cruz, G., Murrieta-Galindo, R., Wake, D.B., 2010. A new species of *Pseudoeurycea* from the cloud forest in Veracruz, México. Zootaxa 2725, 57–68.
Rambaut, A., Drummond, A.J., 2007. Tracer v1.4. Program Distributed by the Authors. <http://beast.bio.ed.ac.uk/Tracer>.
Roelants, K., Gower, D.J., Wilkinson, M., Loader, S.P., Biju, S.D., Guillaume, K., Moriau, L., Bossuyt, F., 2007. Global pattern of diversification in the history of modern amphibians. Proc. Natl. Acad. Sci. USA 104, 887–892.
Ronquist, F., Huelsenbeck, J.P., 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
Schwartz, R.S., Mueller, R.L., 2010. Branch length estimation and divergence dating: estimates of error in Bayesian and maximum likelihood frameworks. BMC Evol. Biol. 10, 5.
Schwenk, K., Wake, D.B., 1993. Prey processing in *Leurognathus marmoratus* and the evolution of form and function in desmognathine salamanders (Plethodontidae). Biol. J. Linn. Soc. 49, 141–162.
Shimodaira, H., 2002. An approximately unbiased test of phylogenetic tree selection. Syst. Biol. 51, 492–508.
Shimodaira, H., Hasegawa, M., 2001. CONSEL: for assessing the confidence of phylogenetic tree selection. Bioinformatics 17, 1246–1247.
Springer, M.S., DeBry, R.W., Douady, C., Amrine, H.M., Madsen, O., de Jong, W.W., Stanhope, M.J., 2001. Mitochondrial versus nuclear gene sequences in deep-level mammalian phylogeny reconstruction. Mol. Biol. Evol. 18, 132–143.
Stamatakis, A., Ludwig, T., Meier, H., 2005. RAxML-III: a fast program for maximum likelihood-based inference of large phylogenetic trees. Bioinformatics 21, 456–463.
Swofford, D.L., 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, USA.
Tihen, J.A., Wake, D.B., 1981. Vertebrae of plethodontid salamanders from the Lower Miocene of Montana. J. Herpetol. 15, 35–40.
Titus, T.A., Larson, A., 1996. Molecular phylogenetics of desmognathine salamanders (Caudata: Plethodontidae): a reevaluation of evolution in ecology, life history, and morphology. Syst. Biol. 45, 451–472.
Vieites, D.R., Min, M.S., Wake, D.B., 2007. Rapid diversification and dispersal during periods of global warming by plethodontid salamanders. Proc. Natl. Acad. Sci. USA 104, 19903–19907.
Wake, D.B., 1966. Comparative osteology and evolution of the lungless salamanders, Family Plethodontidae. Mem. South. Calif. Acad. Sci. 4, 1–111.
Wake, D.B., 1991. Homoplasy: the result of natural selection, or evidence of design limitations? Am. Nat. 138, 543–567.
Wake, D.B., 2009. What salamanders have taught us about evolution. Annu. Rev. Ecol. Evol. Syst. 40, 333–352.
Wake, D.B., 1963. Comparative osteology of the plethodontid salamander genus *Aneides*. J. Morphol. 113, 77–118.
Wake, D.B., Larson, A., 1987. Multidimensional analysis of an evolving lineage. Science 238, 42–48.
Wiens, J.J., Bonett, R.M., Chippindale, P.T., 2005. Ontogeny discombobulates phylogeny: paedomorphosis and higher-level salamander relationships. Syst. Biol. 54, 91–110.
Wiens, J.J., Engstrom, T.N., Chippindale, P.T., 2006. Rapid diversification, incomplete isolation, and the "speciation clock" in North American salamanders (genus

Plethodon): testing the hybrid swarm hypothesis of rapid radiation. Evolution 60, 2585–2603.
Wiens, J.J., Parra-Olea, G., García-París, M., Wake, D.B., 2007. Phylogenetic history underlies biodiversity patterns in tropical salamanders. Proc. R. Soc. B 274, 919–928.
Wilder, I.W., Dunn, E.R., 1920. The correlation of lunglessness in salamanders with a mountain brook habitat. Copeia 1920, 63–68.
Wilgenbusch, J.C., Warren, D.L., Swofford, D.L., 2004. AWTY: A System for Graphical Exploration of MCMC Convergence in Bayesian Phylogenetic Inference. <http://ceb.csit.fsu.edu/awty>.
Zhang, P., Wake, D.B., 2009. Higher-level salamander relationships and divergence dates inferred from complete mitochondrial genomes. Mol. Phylogenet. Evol. 53, 492–508.