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Diversification and biogeographical history of Neotropical plethodontid salamanders

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The Neotropical bolitoglossine salamanders represent an impressive adaptive radiation, comprising roughly 40% of global salamander species diversity. Despite decades of morphological studies and molecular work, a robust multilocus phylogenetic hypothesis based on DNA sequence data is lacking for the group. We estimated species trees based on multilocus nuclear and mitochondrial data for all major lineages within the bolitoglossines, and used our new phylogenetic hypothesis to test traditional biogeographical scenarios and hypotheses of morphological evolution in the group. In contrast to previous phylogenies, our results place all Central American endemic genera in a single clade and suggest that Central America played a critical role in the early biogeographical history of the group. The large, predominantly Mexican genus *Pseudoeurycea* is paraphyletic, and analyses of the nuclear data place two lineages of *Pseudoeurycea* as the sister group of *Bolitoglossa*. Our phylogeny reveals extensive homoplasy in morphological characters, which may be the result of truncation or alteration of a shared developmental trajectory. We used our phylogenetic results to revise the taxonomy of the genus *Pseudoeurycea*.

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INTRODUCTION

Salamanders (Amphibia: Caudata) are abundant in North Temperate regions worldwide. Seven of the ten families are restricted to temperate areas, and the family Salamandridae has only a few tropical species in South-East Asia. However, in the New World, salamanders of the family Plethodontidae have not only entered the tropics but have diversified extensively, especially in Middle America. The tropical plethodontids are classified in a single inclusive taxon, tribe Bolitoglossini (hereafter, bolitoglossines), which is found only in Mesoamerica (Mexico and Central America) and South America. Its closest relative is the tribe Batrachosepini, of western North America (Wake, 2012). Plethodontidae is the largest of the ten families of salamanders, with 446 species, 66.1% of all living salamanders (675 species; AmphibiaWeb, 2015). The extensive diversification of tropical plethodontids, currently totalling 291 species,

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accounts for 65.2% of the family and 43.1% of the order Caudata. These tropical species are classified in 12 genera, ranging in size from *Bolitoglossa* (130 species), the geographically most widespread (north-east Mexico to central Bolivia) to three monotypic genera (*Bradytriton*, *Nyctanolis*, *Parvimolge*); all 12 genera are found in Mesoamerica. The bolitoglossines form a remarkably distinctive, readily diagnosable clade, characterized by the following: 13 pairs of chromosomes (shared with Batrachosepini), direct terrestrial development with no larval stage (shared with Batrachosepini and all tribes, but not all species, of the subfamily Plethodontinae), and a complicated and uniquely structured tongue and feeding system (Lombard & Wake, 1986).

The unambiguous north temperate origin of bolitoglossines and southward advance to South America makes them an attractive group for biogeographical studies, given that both their origin and generalized route of geographical expansion are well understood. The bolitoglossines have diversified primarily in several focal areas, including southern Mexico, Nuclear Central America, and the highlands of Costa Rica and Panama, with a secondary and more recent diversification in South America (Wake & Lynch, 1976; Elmer *et al*., 2013). This biogeographical history implies a dispersal through northern Mexico to these diversification foci; although this history has long been accepted based on both the extant distributions of salamander families and species, as well as patterns of species diversity in Mesoamerica and South America, explicit parametric tests of the hypotheses concerning the route of dispersal and early areas of diversification are lacking.

Tropical plethodontids have been known since the early 19th century [the present-day *Bolitoglossa platydactyla* was described by Cuvier (in Gray, 1831)], but they were first considered comprehensively in the seminal work of Dunn (1926). He recognized only 30 species in a single genus (*Oedipus*), extending from north-eastern Mexico to central Bolivia, essentially the entire range of the bolitoglossines. Even then, the diversity in size and shape amongst the 30 species greatly exceeded that in any other genus, but Dunn was unable to find gaps and stated, 'The extremes are quite different but there are many connecting links'. Dunn's view prevailed until Taylor (1944) separated the then more numerous species into several genera. *Oedipus*, a preoccupied name, was replaced with *Bolitoglossa*, to which 14 species were assigned, as well as six more that were assigned to the genus tentatively. Taylor's genera largely stand to this day (his *Magnadigita* is now a subgenus of *Bolitoglossa*), but new discoveries have added several new genera and new analyses have led to subdivision of others. Since the revision by Taylor, the following genera have been named, either as the result of subdivision of Taylor's genera (*Cryptotriton*, *Dendrotriton*, *Nototriton*) or as a result of new discoveries (*Bradytriton*, *Ixalotriton*, *Nyctanolis*).

Dunn (1926) recognized four groups of species that 'if distinct enough and in the absence of annectant forms, rank as genera'. However, his group I, which he considered to be made up of 'primitive' species, includes species today placed in three different genera (*Bolitoglossa*, *Chiropterotriton*, *Pseudoeurycea*); three series of species were recognized, each containing species now placed in two or three genera. His group II included species today placed in *Cryptotriton*, *Nototriton*, *Parvimolge*, and *Thorius*, his group III included species now placed in *Bolitoglossa*, and his group IV included species now placed in *Pseudoeurycea* and *Oedipina*. These arrangements were an early preview of the extensive homoplasy that has become so evident in subsequent studies of tropical plethodontids.

What led to Dunn's confusion is the fact that tropical salamanders have undergone a significant adaptive radiation that has been proceeding during tens of millions of years. Taylor (1944) had hints of this, and knowledge progressively increased from Wake (1966) through Wake & Lynch (1976) to Wake (1987). Mitochondrial DNA sequencing and analysis began with García-París & Wake (2000) (*Oedipina*) and García-París, Parra-Olea & Wake (2000) (*Bolitoglossa*), culminating in Wiens *et al*. (2007), and led to hypotheses of phylogenetic relationship. Addition of a nuclear gene led to the understanding that there have been mini-adaptive radiations within the larger one [*Thorius*; Rovito *et al*. (2013)]. Molecular studies conducted to date have found all genera except *Pseudoeurycea* to be monophyletic. Parra-Olea (2002) showed that *Lineatriton*, *Ixalotriton*, and *Parvimolge* were potentially nested within *Pseudoeurycea* based on mtDNA sequence data, and Wiens *et al*. (2007) affirmed this finding. Using far more limited taxon sampling (one member of each of the four genera), Frost *et al*. (2006) reduced *Lineatriton* and *Ixalotriton* to junior synonyms of *Pseudoeurycea* and assigned all species to the latter taxon, but left *Parvimolge* (which they recovered as a sister taxon of *Bolitoglossa*) unchanged. None of these studies included both extensive taxon sampling and multiple, informative nuclear genes, and they were thus limited in their usefulness to resolve taxonomic issues.

Attempts to determine the timing of the entrance of salamanders into the American tropics started with Dunn (1926), who estimated that migration to the tropics from North America occurred from the Late Miocene to the Pliocene. More recent estimates were presented by Vieites, Min & Wake (2007), who used sequences of three nuclear genes to estimate the split between *Batrachoseps* and the bolitoglossines at roughly 73–75 Mya (Late Cretaceous). However only a single bolitoglossine was included. All clades identified in Wiens

et al. (2007) occur in Middle America, but only two enter South America. Nevertheless, Elmer *et al*. (2013), using mitochondrial and nuclear gene sequences, estimated the entrance of bolitoglossines into South America at 23.6 Mya (Early Miocene), thus giving a minimal date for the start of the entire Mesoamerican diversification.

The adaptive radiation of tropical plethodontids has resulted in extensive invasion of arboreal (Wake, 1987) and fossorial (Wake, 1966; Wake & Lynch, 1976) habitats, and even includes one aquatic species (Wake & Campbell, 2001). Size disparity amongst the species is great, and there are both relative giant and miniature species. Most adaptive radiations involve diversification of locomotion (e.g. vertebral, tail, limb, and digit specialization), feeding (e.g. jaw, teeth, and tongue specialization), and life history (egg deposition, larval stages, direct development, etc.). However, all of the bolitoglossines share a specialized feeding system and have direct development, making only limited specialization possible in these dimensions of their life history and morphology. A few species have lost maxillary teeth and some have enlarged jaw musculature. Most eggs are laid in terrestrial settings, but many species deposit eggs in bromeliads or other arboreal epiphytes. Even vertebral numbers are invariant in all but one genus (*Oedipina*). The greatest structural diversity occurs in size, tail length and usage, and in such locomotion-related features as limb and digit length and general structure, and especially in degree of development and extent of interdigital webbing of the manus and pes.

Because of the constraints imposed by the specialized tongue feeding mechanism and direct development, severe limitations are imposed on structural and functional evolution. This has led to very high degrees of homoplasy (Wake, 1966, 1987, 1991, 2009), which for so long impeded taxonomic resolution of the clade, starting with Dunn (1926). Our goals for this paper were to produce a robust phylogenetic hypothesis of the Bolitoglossini based on multilocus data and analyses, to frame hypotheses of the biogeographical history of the major clades, and to reassess the status of the adaptive radiation and the extent of homoplasy.

METHODS

DNA extraction and sequencing

We extracted genomic DNA from frozen or ethanol-preserved liver or tail tips from the Museum of Vertebrate Zoology, UC Berkeley (MVZ), or Instituto de Biología, UNAM (IBH), tissue collections using Qiagen DNeasy extraction kits (Qiagen, Valencia, CA, USA). We sequenced five mitochondrial fragments from two regions (L1, L2, A, C2, and E) using the primers and PCR protocols of Zhang *et al*. (2008). These fragments formed two contigs: the first (L1, L2, and A) contained the small and large subunit ribosomal RNA genes (*12S* and *16S*), NADH-ubiquinone oxidoreductase chain 1 *(ND1)*, and tRNAs V, L, I, and Q, whereas the second (C2 and E) contained the genes cytochrome *c* oxidase subunit I (*COI*) and *COII* and tRNAs S, D, and K. We also sequenced a fragment of the mitochondrial cytochrome *b* (*cytb*) gene using primers MVZ15 and MVZ16 (Moritz, Schneider & Wake, 1992) and fragments of the nuclear genes proopiomelanocortin (*POMC*) using primers POMC_DRV_F1/POMC_DRV_R1 (Vieites *et al*., 2007), recombination activating gene 1 (*RAG1*) using primers Amp-RAG1-F/Amp-RAG1-R1 and Amp-RAG1-F1/Amp-RAG1-R (San Mauro *et al*., 2004), and solute carrier family 8 member 3 (*SLC8A3*) (Roelants *et al*., 2007) using primers SLC8A3_F/SLC8A3_R (Rovito, 2010). In addition to the set of 58 taxa sequenced for all of these loci, we sequenced the mitochondrial genes *16S* using primers MVZ117/MVZ98 (Palumbi *et al*., 1991) and *cytb* for five species of tropical bolitoglossines with tissue available but lacking published sequence data for inclusion in biogeographical analyses. PCR products were purified using 2 μL of 1:5 diluted ExoSAP-IT (USB Corporation, Cleveland, OH, USA), cycle sequenced, and run on an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, CA, USA). We edited sequences using either SEQUENCHER v. 4.2 (GeneCodes, Ann Arbor, MI, USA) or GENEIOUS v. 5.5 (BioMatters, Auckland, New Zealand).

Phylogenetic analyses

We aligned sequences for each fragment using MUSCLE v. 3.8 (Edgar, 2004) with default parameters. We deleted poorly aligned loop regions from the *12S* and *16S* genes and trimmed the ends of the sequences to the point where a majority of taxa had sequence data. Mitochondrial genes were concatenated for gene and species tree analyses. We inferred the gametic phase of the nuclear sequence data using the program PHASE 2.1 (Stephens, Smith & Donnelly, 2001) and tested for intralocus recombination using the single breakpoint analysis (Kosakovsky Pond *et al*., 2006) in the HyPhy package (Kosakovsky Pond, Frost & Muse, 2005) on the Datamonkey web server (Delport *et al*., 2010). We partitioned protein-coding mtDNA and nuclear genes by codon position and used these partitions, along with all tRNAs combined into a single partition, *12S*, and *16S* to find the optimal partitioning strategy using the program PartitionFinder v. 1.0.1 (Lanfear *et al*., 2012). This program selects the best-fit model of nucleotide substitution for each partition, uses a greedy algorithm to search partitioning schemes, and compares partitioning schemes using an information criterion. We used the Bayesian information criterion to compare partitioning schemes, and evaluated partitioning schemes

for mtDNA and nuclear genes separately. We only included the 24 substitution models used in MrBayes. For mtDNA, PartitionFinder selected the following partitioning scheme and substitution models: (1) codon position 1 of *ND1* and *cytb* and tRNAs: General Time Reversible (GTR) + proportion of invariable sites (I) + Gamma (G); (2) codon position 2 of *COI*, *COII*, *ND1*, and *cytb*: Hasegawa, Kishino, and Yano (HKY) + I + G; (3) codon position 3 of *COI*, *COII*, *ND1*, and *cytb3*: GTR + I + G; (4) codon position 1 of *COI* and *COII*: symmetrical (SYM) + I + G; (5) *12S* and (6) *16S*: GTR + I + G. For the nuclear genes, the selected partitioning scheme and substitution models were: (1) codon position 3 of *POMC*, *RAG1*, and *SLC8A3*: SYM + G; (2) codon position 1 of *POMC*, *RAG1*, and *SLC8A3* and codon position 2 of *POMC*: GTR + I + G; (3) codon position 2 of *RAG1* and *SLC8A3*: HKY + I + G. These partitioning schemes and substitution models were used in all phylogenetic analyses.

We used both maximum likelihood (ML) and Bayesian inference to estimate phylogenies using only mtDNA, only nuclear loci, and with a concatenated data set of mtDNA and the three nuclear loci. For ML analyses, we used RAxML v. 7.4.2 (Stamatakis, 2006) run on the CIPRES data portal (Miller, Pfeiffer & Schwartz, 2010) with 1000 bootstrap replicates and a GTR + G substitution model. Bayesian analyses were run in MrBayes v. 3.2 (Ronquist *et al*., 2012) for 20 000 000 generations (mtDNA) or 50 000 000 generations (concatenated data set), sampled every 1000 generations, with four chains per run and two runs per analysis. The first 5000 samples (mtDNA) or 10 000 samples (concatenated data set) were discarded as burn-in when estimating a consensus tree. *Hemidactylium scutatum* and *Eurycea bislineata* were used as outgroups for these analyses, and two species of *Batrachoseps* (the sister genus of the tropical bolitoglossines; Vieites *et al*., 2011) were also included.

Concatenation of all loci to produce a single phylogenetic hypothesis ignores the fact that coalescent stochasticity gives each independent locus a different evolutionary history, and concatenation can produce misleading results in some cases (Kubatko & Degnan, 2007). We used a multilocus coalescent species tree estimation method, *BEAST (Heled & Drummond, 2010) to estimate a species tree from mtDNA and nuclear loci while accounting for the independent history of each locus. The tropical bolitoglossines were constrained to be monophyletic. *Batrachoseps* was constrained to be the sister taxon of the tropical bolitoglossines, with *Hemidactylium* constrained to be the sister taxon to *Batrachoseps* + Bolitoglossini and *Eurycea* constrained to be the sister taxon of (*Hemidactylium* (*Batrachoseps*, Bolitoglossini)). The same partitioning strategy and substitution models were used as in the RAxML and MrBayes analyses. A relaxed lognormal molecular clock (Drummond *et al*., 2006) was used for each partition, and a Yule species tree prior was used. Two separate runs of 500 000 000 generations were conducted, sampled every 10 000 generations, and the two runs were combined using LogCombiner with the first 5000 samples of each run discarded as burn-in. TRACER v. 1.5 (Rambaut & Drummond, 2007) was used to check that effective sample size values were sufficiently high (> 200) and assess mixing. TreeAnnotator (Drummond & Rambaut, 2007) was used to construct a maximum clade credibility species tree from the posterior distribution of species trees. In addition to using mtDNA and nuclear loci, we estimated a species tree using only the three nuclear loci.

Finally, despite the fact that concatenation can be statistically inconsistent under some circumstances, we estimated a phylogeny by concatenating all loci, as well as all three nuclear loci. The same partitioning strategy and substitution models used for *BEAST analyses were used for the analyses of concatenated data sets. We used RAxML to estimate a phylogenetic tree with 1000 bootstrap replicates, and MrBayes with the same run parameters as in the mtDNA gene tree estimation.

Biogeographical analysis

For biogeographical analyses, we estimated a phylogeny including all described species of tropical bolitoglossines for which *16S* and/or *cytb* sequences were available either from GenBank or from additional species sequenced by us for this study (231 species total). We added the *16S* and *cytb* sequences to our existing alignment using MUSCLE. We estimated an ultrametric phylogenetic tree using BEAST v. 1.7.4 (Drummond *et al*., 2012) with the same partitioning strategy as in the *BEAST analysis, a lognormal relaxed clock for each partition, and a Yule tree prior. No fossils of tropical plethodontid salamanders are available for time calibration of our phylogeny. In order to gain a rough understanding of the timing of splits, we used a secondary calibration of 53 Mya for the split between *Hemidactylium* and *Batrachoseps* + Bolitoglossini (X. Shen, D. Liang, M. Chen, R. Mao, D. Wake & P. Zhang., unpubl. data). We used a normal prior distribution with a mean of 53 Mya and a SD of 3 Mya in order to place 95% of the prior distribution on 48–58 Mya, the 95% confidence interval reported by X. Shen *et al*. We ran the BEAST analysis for 50 000 000 generations, sampled every 10 000 generations, and discarded the first 1000 samples as burn-in when estimating a consensus tree. GenBank numbers and voucher information for sequence data are given in Table S1. We created an additional tree for biogeographical analyses following the same procedure, but constraining the topology of the RAxML tree to match that of the *BEAST species tree estimated from all loci. We used this RAxML tree as

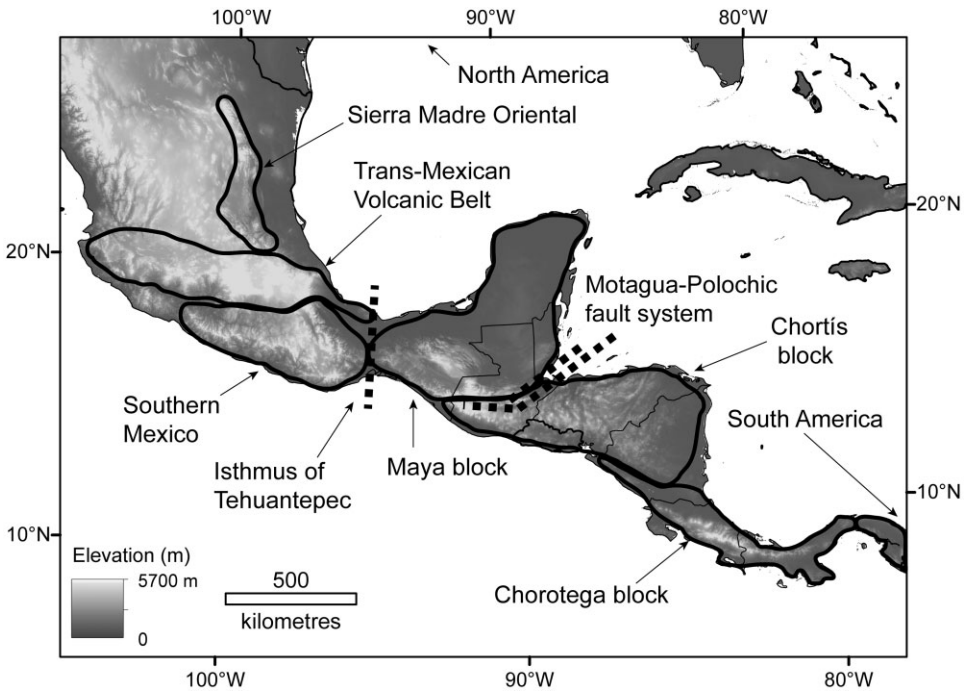

Figure 1. Areas used in biogeographical analyses of Mesoamerica. North and South American areas are not illustrated, except for a small portion of lowland Panama east of the Panama Canal contained within the South American area. Major geological faults corresponding to the Isthmus of Tehuantepec and the Motagua–Polochic fault system are indicated with dotted lines.

a starting tree for BEAST and disabled topological rearrangements to obtain an ultrametric tree for biogeographical analysis. Our phylogeny offers an unparalleled opportunity to test biogeographical hypotheses related to the radiation of Neotropical plethodontids. It includes a high proportion of all tropical bolitoglossines (231 of 291 species; AmphibiaWeb, 2015), many of which had no sequence data previously.

The presumed temperate North American origin of tropical bolitoglossines implies that the most likely dispersal route to the tropics would be through northern Mexico, with a subsequent dispersal into Central America. The high diversity of endemic salamanders, including several genera, in Nuclear Central America, however, implies a longer history in the region. We used the program LAGRANGE (Ree *et al*., 2005; Ree & Smith, 2008) to estimate distributions of ancestral nodes in the phylogeny. LAGRANGE implements the dispersal-extinction-cladogenesis model in a likelihood framework to estimate geographical range evolution along branches. During speciation events (nodes), one descendent species inherits a single area from the ancestral species' range, whereas the other species inherits either the remainder or the entirety of the ancestral range. LAGRANGE requires the delineation of geographical areas inhabited by species, and we divided Mesoamerica into six areas (Fig. 1): (1) Sierra Madre Oriental, Mexico; (2) Trans-Mexican Volcanic Belt (TMVB), (3) southern Mexico highlands, (4) Maya block, (5) Chortís block, (6) Chorotega block. We defined the boundary between the Maya and Chortís blocks as the Polochíc fault, and the Nicaraguan depression as the boundary between the Chortís and Chorotega areas. We did not include the Sierra Madre Occidental of Mexico; only a single, widespread species of plethodontid salamander [*Pseudoeurycea bellii* (Gray, 1850)] is known from there. Additionally, we included an area for northern South America, including all land east of the Panama Canal. Finally, we included temperate North America as a single area, as only the outgroups to the tropical bolitoglossines are found there.

We used the ultrametric tree generated using RAxML/BEAST including all species of tropical salamanders for which phylogenetic data were available for the LAGRANGE run. As we have no fossil calibrations to use in a dating analysis, we decided not to implement a time-stratified model, despite the changing geological configuration of Central America during the Cenozoic. We limited the number of areas an ancestral species could occupy to three, matching the highest number inhabited by any extant species; nearly all species are found in only one or two areas. We also constrained geographical ranges to be contiguous,

because no extant species have ranges that include disjunct areas as defined for our analysis, and allowed dispersal only through adjacent areas. There is geological evidence that the Chortís block may have been contiguous with southern Mexico in the Cretaceous and has been displaced approximately 1500 km east to its present position (Pindell & Dewey, 1982; Silva-Romo, 2009). We therefore allowed dispersal between southern Mexico and the Chortís block.

RESULTS

Phylogenetics

The results of the *BEAST species tree analysis of combined mitochondrial and nuclear loci show an initial divergence between genera endemic to Central America (*Bradytriton*, *Cryptotriton*, *Dendrotriton*, *Nototriton*, *Nyctanolis*, and *Oedipina*; posterior probability (PP) = 1.0; hereafter 'Central American clade') and those endemic to Mexico west of the Isthmus of Tehuantepec or present on both sides of the Isthmus (*Bolitoglossa*, *Chiropterotriton*, *Ixalotriton*, *Parvimolge*, *Pseudoeurycea*, and *Thorius*; PP = 1.0, hereafter 'Mexican/widespread clade') (Fig. 2). The lone exception to this pattern is *Ixalotriton*, which is within the second clade but occurs just east of the Isthmus of Tehuantepec. Within the Central American clade, all relationships are strongly supported (PP > 0.95) except the sister-taxon relationship between *Nototriton* and (*Oedipina*, *Bradytriton* + *Cryptotriton*; PP = 0.89). Within the Mexican/widespread clade, monophyly of all genera except for *Pseudoeurycea* is well supported (PP > 0.95). Within *Pseudoeurycea*, there are two well-supported clades: the first includes members of the *Pseudoeurycea bellii* and *Pseudoeurycea cephalica* species groups (Taylor, 1944; Parra-Olea, 2002), whereas the other includes all remaining members of *Pseudoeurycea* as well as the two species of *Ixalotriton*. Within this second clade, monophyly of the remaining species of *Pseudoeurycea* (to the exclusion of *Ixalotriton*) lacks strong support (PP = 0.84). The three species formerly assigned to *Lineatriton* [*Pseudoeurycea lineola* (Cope, 1865), *Pseudoeurycea orchileucos* (Brodie, Mendelson & Campbell, 2002), and *Pseudoeurycea orchimelas* (Brodie, Mendelson & Campbell, 2002)] are not monophyletic; *Ps. lineola* is placed as the sister taxon of *Pseudoeurycea leprosa* (Cope, 1869) + *Pseudoeurycea firscheini* Shannon & Werler, 1955 (PP = 0.95), whereas *Ps. orchileucos* and *Ps. orchimelas* are sister taxa and are most closely related to *Pseudoeurycea werleri* Darling & Smith, 1954, and *Pseudoeurycea obesa* Parra-Olea, García-París, Hanken & Wake, 2005 (PP = 0.93) (Fig. 2). The mtDNA gene tree matches the topology of the *BEAST tree exactly (Fig. 3).

The species tree estimated using only nuclear loci closely resembles that generated from all loci, in most respects (Fig. 4). There are again two main clades composed of the same genera (Central American endemics and Mexican endemics/widespread genera). Within the Central American clade, *Cryptotriton* is placed as the sister taxon of *Dendrotriton* (PP = 0.86), rather than of *Oedipina*. Within the Mexican/widespread clade, *Pseudoeurycea* is polyphyletic; the *Ps. bellii* and *Ps. cephalica* species groups are the sister taxon of *Bolitoglossa*, although with weak support. The polyphyly of species formerly assigned to *Lineatriton* is not strongly supported in this tree, although *Ps. lineola* is again placed as the sister taxon to *Ps. leprosa* + *Ps. firscheini*. There are no strongly supported conflicts within this major clade between the species tree estimated only from nuclear loci and the tree estimated using mtDNA and nuclear loci.

Analysis of the concatenated data set of all loci produced a phylogeny that resembles the *BEAST tree from the analysis of nuclear data in most respects (Fig. 5). *Cryptotriton* is the sister taxon to all genera in the Central American clade, except *Dendrotriton* (rather than the sister taxon of *Dendrotriton*, as in the nuclear-only *BEAST tree). *Parvimolge* is the sister taxon of the clade containing *Pseudoeurycea*, *Ixalotriton*, and *Bolitoglossa*, and unlike in the *BEAST tree estimated using all loci, the *Ps. bellii* and *Ps. cephalica* groups of *Pseudoeurycea* are the sister taxon of *Bolitoglossa*, rather than of the rest of *Pseudoeurycea* + *Ixalotriton*. Analysis of the concatenated nuclear loci only produced a tree that is identical in topology for all major clades to the *BEAST nuclear-only tree (Fig. 4).

Despite the lack of fossil calibration points for the Neotropical salamanders, our time-calibrated phylogeny (Fig. 6) provides some insight into the approximate and relative timing of divergence events within the bolitoglossines. We estimate that the divergence between *Batrachoseps* and the bolitoglossines occurred approximately 47 Mya (95% Highest Posterior Density: 41–53 Mya), and the initial split between the two major clades of bolitoglossines occurred roughly 42 Mya (36–47 Mya). The divergence of *Thorius*, one of the most morphologically distinct lineages of salamanders, from the rest of the Mexican/widespread clade occurred around 40 Mya (34–45 Mya), whereas the deepest divergence within extant *Thorius* dates to only 14 Mya (12–17 Mya). The divergence of *Bolitoglossa* from *Pseudoeurycea* (*sensu lato*) and *Ixalotriton* occurred relatively recently in the history of the bolitoglossines, around 28 Mya (24–31 Mya). The basal divergence within *Bolitoglossa* occurred around 22.5 Mya (19–26 Mya), and the most recent divergence between subgenera of *Bolitoglossa* was estimated to have occurred only 15 Mya (13–18 Mya). *Bolitoglossa* (*Eladinea*), which has the highest species diversity of any lineage of tropical salamanders, diverged around 21 Mya, and the clade containing all South American species diverged approximately 14 Mya (11–16 Mya).

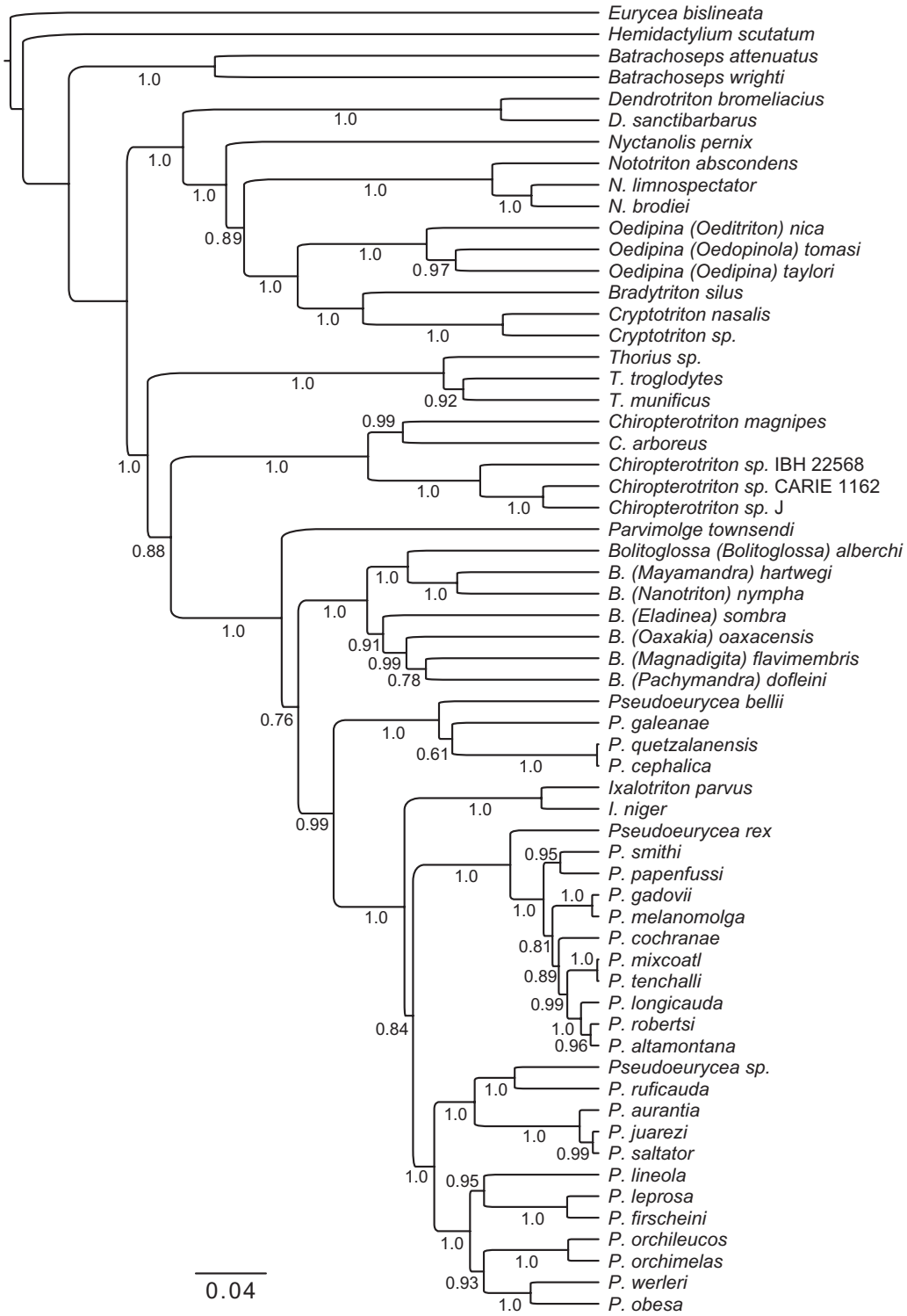

Figure 2. Species tree from *BEAST analysis of nuclear and mitochondrial loci. Numbers on branches indicate posterior probability of clades.

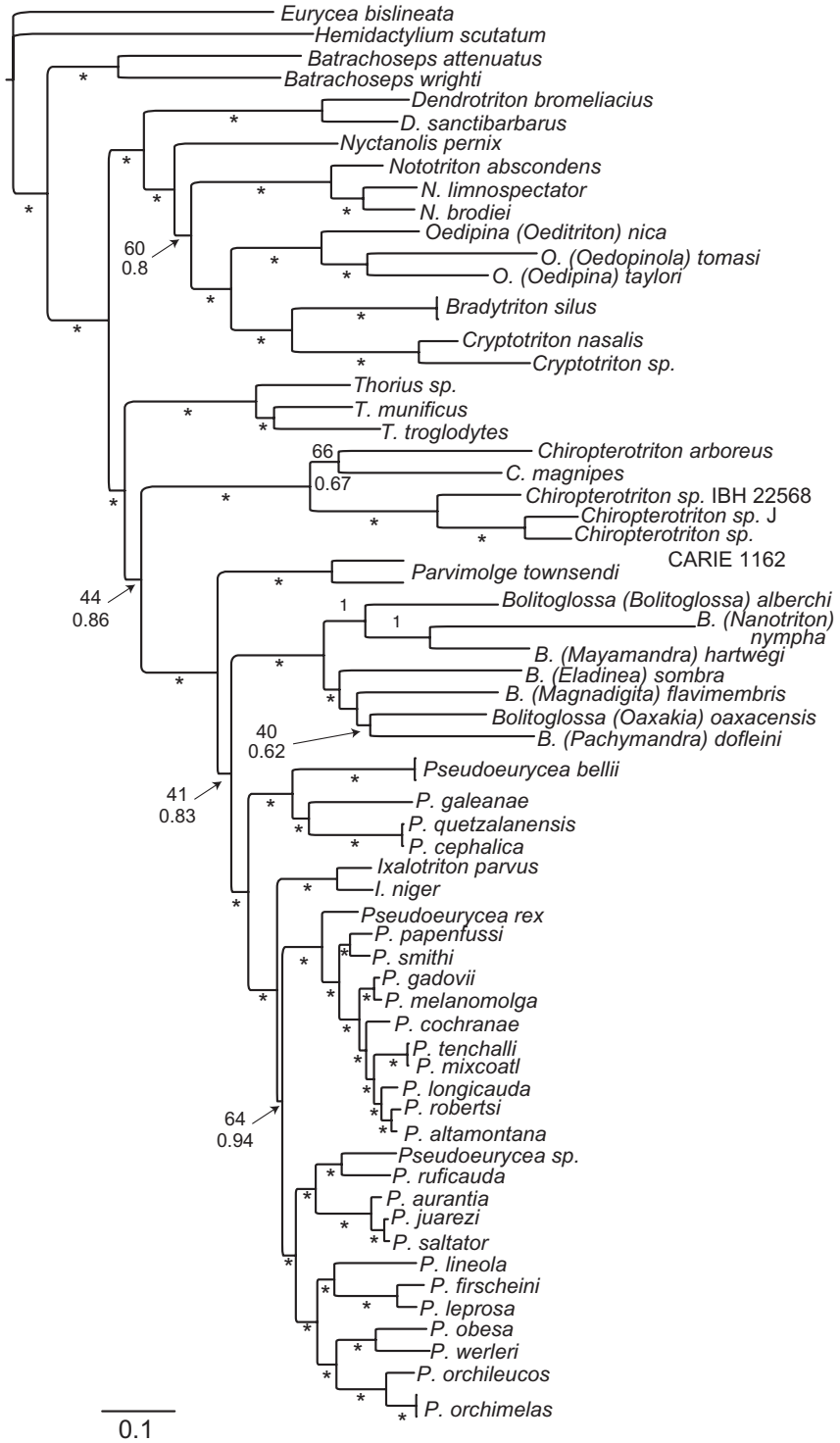

Figure 3. Mitochondrial gene tree from MrBayes analysis. Numbers above branches indicate bootstrap support (BS) from RAxML analysis, and numbers below branches are posterior probabilities (PP) from MrBayes analysis. Branches with an asterisk have BS > 70 and PP > 0.95.

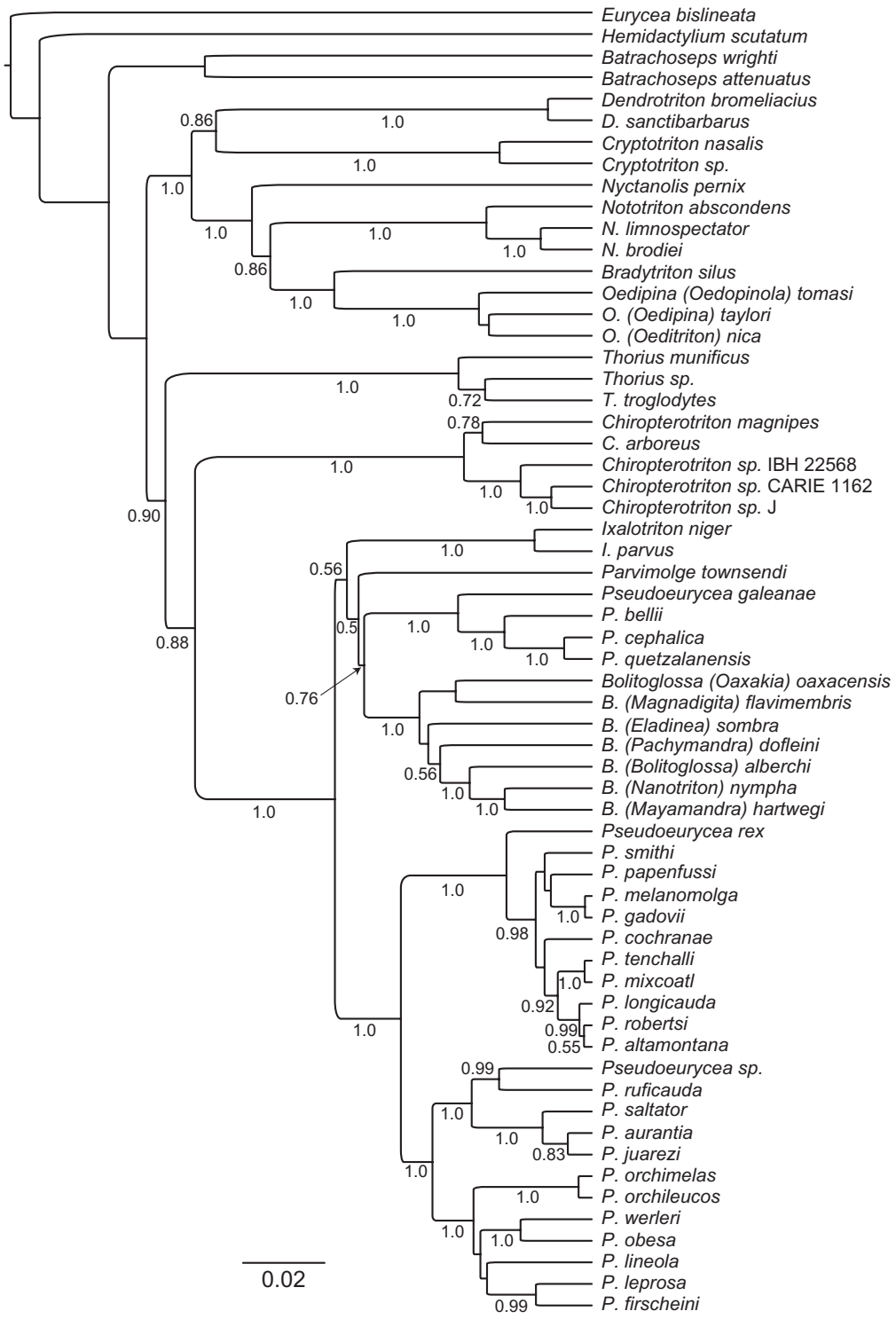

Figure 4. Species tree from *BEAST analysis of nuclear loci only. Numbers on branches indicate posterior probability of clades.

BIOGEOGRAPHY

Range estimates from LAGRANGE refer to all range-splitting scenarios contributing to 0.95 cumulative relative probability. Results from the LAGRANGE analysis using the phylogeny constrained to match the *BEAST topology were very similar to those obtained using the unconstrained phylogeny, and only the latter are discussed further. LAGRANGE estimated the split between

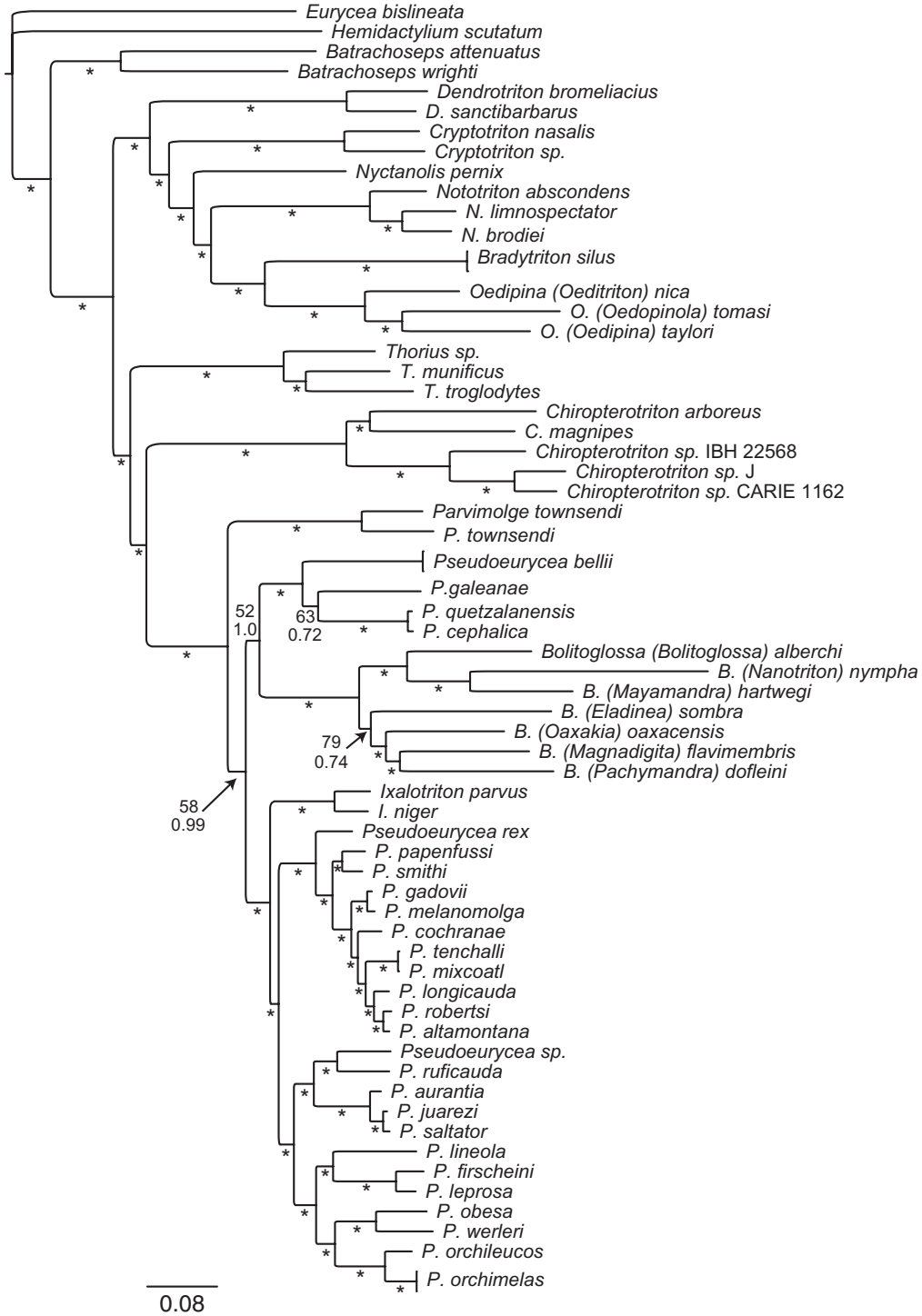

Figure 5. Phylogenetic tree from MrBayes analysis of concatenated mitochondrial and nuclear data. Upper numbers indicate bootstrap support from RAxML, and lower numbers show posterior probability from Bayesian analysis. Branches with an asterisk have BS > 70 and PP > 0.95.

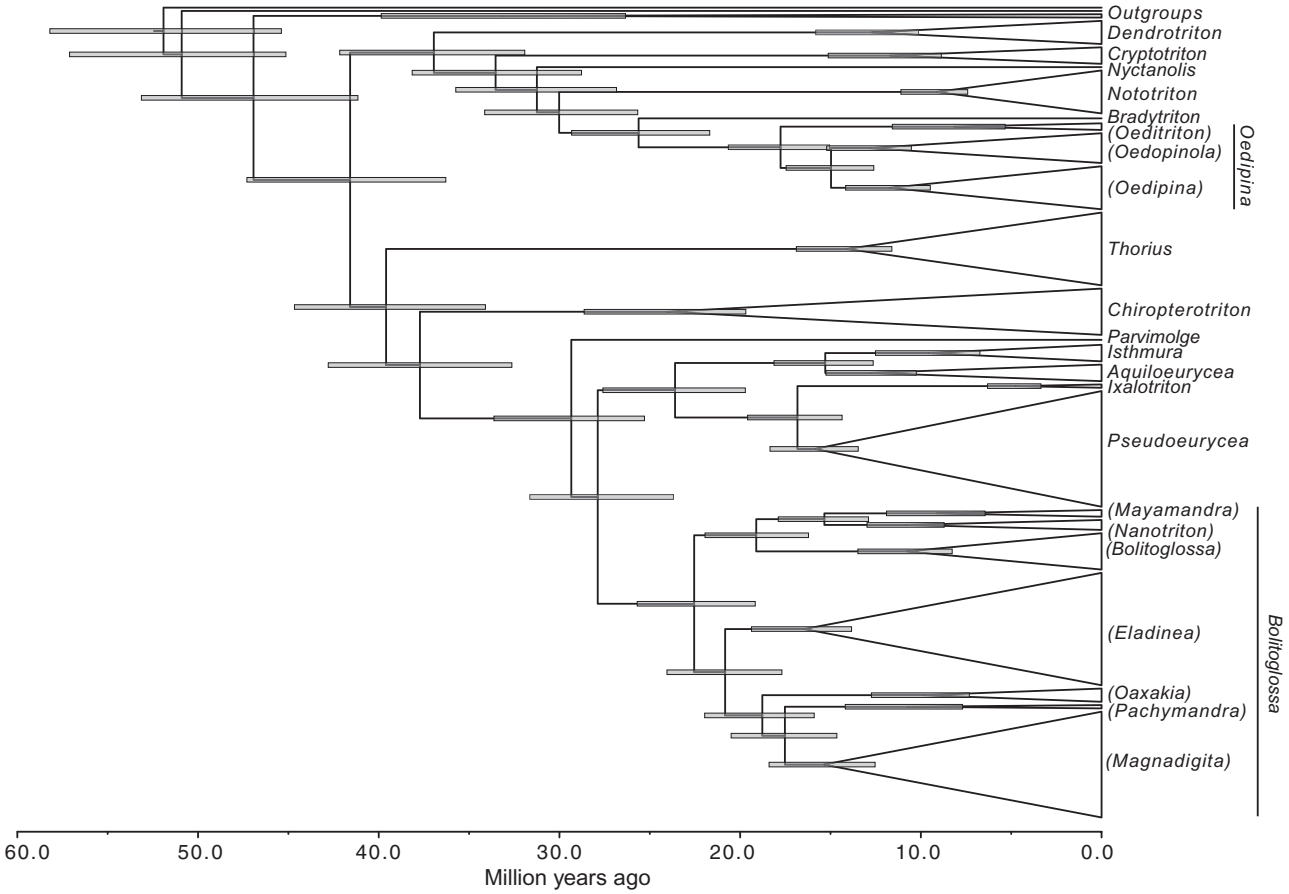

Figure 6. Time-calibrated phylogeny from BEAST analysis of concatenated data set including all species with sequence data available. Tips have been collapsed by lineage (genus or subgenus) for clarity. Bars on nodes indicate 95% highest posterior density intervals on node ages. *Aquiloeurycea* refers to the *Pseudoeurycea cephalica* species group, and *Isthmura* refers to the *P. bellii* species group.

Batrachoseps and the tropical bolitoglossines to have occurred between North America and either areas of Mesoamerica on both sides of the Isthmus of Tehuantepec [total relative probability (prob.) = 0.46], the Maya block and/or Chortís block (prob. = 0.23), the TMVB and/or southern Mexico (prob. = 0.14), or some other combination of these areas and North America. Range estimates for the ancestor at the time of the split between the two major clades of tropical bolitoglossines included five areas: the Maya block, Chortís block, TMVB, southern Mexico, and North America (Fig. 7). Scenarios involving a split between areas on opposite sides of the Isthmus of Tehuantepec accounted for 0.84 of the total relative probability, with the maximum likelihood estimate involving a split between the TMVB and the Maya block (prob. = 0.36). Scenarios including either the Maya or Chortís block accounted for a cumulative 0.93 relative probability. All range estimates for the ancestor of the Central American clade except two included the Maya and/or Chortís blocks, and only one (prob. = 0.0043) included an area of Mexico west of the Isthmus of Tehuantepec. Four of the less probable reconstructions also included North America (total prob. = 0.054).

At the split between *Thorius* and the remaining genera in the Mexico/widespread clade, all range estimates of the ancestor of the clade except two (which had total prob. = 0.070) included the TMVB, with some scenarios also involving southern Mexico, the Maya block, Chortís block, and North America (Fig. 7). The most likely scenario (prob. = 0.51) involved a split within the TMVB, and all scenarios except two involved the lineage leading to all genera except *Thorius* inheriting the TMVB as all or part of its range. The ancestral split within the widespread genus *Bolitoglossa*, which now is found in all the biogeographical areas except temperate North America, included the Maya and/or Chortís block in all but two scenarios (total prob. of ancestral range including Nuclear Central America = 0.89), with the clade including subgenus (sg) *Bolitoglossa*, *Mayamandra*, and *Nanotriton* inheriting a range containing the Maya block in most

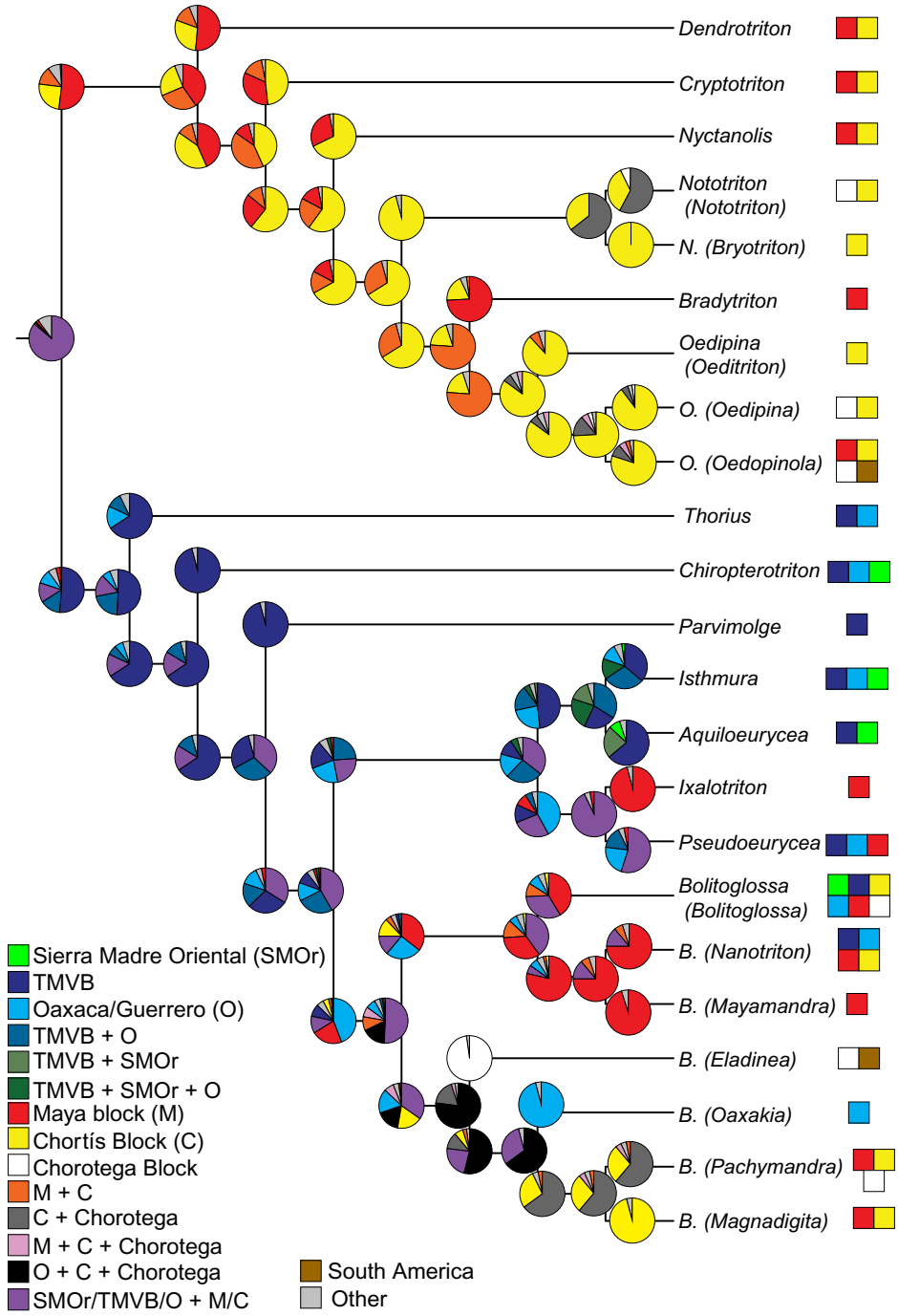

Figure 7. Summary at level of subgenera of results of LAGRANGE biogeographical analysis. Each node has three pie charts representing the range of the common ancestor and two daughter species involved in each speciation event. 'Other' range category includes ranges that include North America, as well as range reconstructions that did not contribute to 0.95 cumulative relative probability set. Boxes beside genus or subgenus names represent the areas in which each subgenus is found. TMVB, Trans-Mexican Volcanic Belt. *Aquiloeurycea* refers to the *Pseudoeurycea cephalica* species group, and *Isthmura* refers to the *P. bellii* species group.

scenarios (prob. = 0.53). The estimate of the range inherited by the latter clade included the TMVB, southern Mexico, Maya block, and/or Chortís blocks, whereas the remaining four subgenera inherited a range containing some combination of southern Mexico, Maya, Chortís, and Chorotega blocks.

DISCUSSION

PHYLOGENY

Our multilocus coalescent species tree estimates (Figs 2, 4) and our concatenated phylogeny (Fig. 5) suggest a novel set of relationships amongst tropical salamander genera. The phylogeny of Wiens *et al*. (2007), which included representatives of all genera but was based solely on mtDNA, recovered many of the relationships seen in our species trees but differed in several key aspects. First, all genera endemic to Central America (except *Ixalotriton*) were not in a single clade in their mtDNA gene tree, although the nodes in conflict between their topology and the species trees from our analyses were not well supported. *Cryptotriton* was found to be the sister taxon of all other tropical bolitoglossines (with weak support) in their analysis, whereas it is within the Central American clade in our species trees. *Dendrotriton* and *Nyctanolis* were strongly supported as sister taxa in their analysis, whereas *Dendrotriton* is either the sister taxon of all other genera in the Central American clade (based on all loci) or of *Cryptotriton* (nuclear genes only) in our species tree analyses. Wiens *et al*. (2007) recovered the other major clade in our species trees (Mexican/widespread genera). The placement of *Cryptotriton* as the sister taxon of *Bradytriton*, as in our species tree estimate from all loci, has not been found in any previous molecular or morphological analyses (Wiens *et al*., 2007; Pyron & Wiens, 2011), whereas the sister relationship between *Bradytriton* and *Oedipina* in our nuclear-only *BEAST tree and concatenated tree agrees with Wiens *et al*. (2007). The phylogeny of Wiens *et al*. (2007) differs from our species tree estimated from all loci in the placement of *Parvimolge* as sister to *Ixalotriton*, rather than as sister to all other genera in the clade (although with weak support), and from our nuclear gene results that place *Parvimolge* in a polytomy with *Bolitoglossa*, *Ixalotriton*, the *Pseudoeurycea bellii*/*cephalica* group, and all remaining species of *Pseudoeurycea*.

Although the phylogeny of Pyron & Wiens (2011) was based on nine nuclear and three mitochondrial genes, almost no nuclear data were included for tropical bolitoglossines. Their phylogeny showed few well-supported relationships between genera, and most of the deeper nodes within the tropical bolitoglossine clade were poorly supported. Their phylogeny included a clade comprised of *Bolitoglossa*, *Pseudoeurycea*, *Ixalotriton*, and *Parvimolge*, with *Bolitoglossa* as the sister taxon to the three remaining genera. Their phylogeny also included a clade comprising *Nototriton*, *Bradytriton*, and *Oedipina*, as in our nuclear-only species tree and concatenated species tree.

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The ancestral range estimates from LAGRANGE suggest a surprisingly important role for Nuclear Central America in the early diversification of tropical bolitoglossines (Fig. 7). Although the presence of multiple endemic genera (i.e. *Nyctanolis*, *Bradytriton*) and high species diversity has been cited as evidence of the importance of this region in the history of tropical salamanders (Wake & Lynch, 1976), the presumed North American ancestry of the clade implies a dispersal route through Mexico to reach Nuclear Central America. Surprisingly, the ancestral range of all tropical salamanders was estimated to most probably have included the TMVB and areas of Nuclear Central America, with most alternative range estimates including some or all of Nuclear Central America. Although the Sierra Madre Oriental provides the most direct dispersal route between North America and central Mexico, it was not included in any of the ancestral range estimates for the basal node of tropical salamanders, or for the ancestor of the Mexico/widespread clade (Fig. 7). This result suggests the possibility that plethodontid salamanders followed an alternate dispersal route to Central Mexico. Given that the sister genus of tropical bolitoglossines occurs in California, Baja California, and Nevado de Colima, Mexico, a dispersal southward along the Pacific coast directly to the area now occupied by the TMVB could also be considered as a possibility. In fact, if the most recent common ancestor between *Batrachoseps* and the bolitoglossines dates back to the Late Cretaceous (Vieites *et al*., 2007), or even to the early Palaeogene (X. Shen, D. Liang, M. Chen, R. Mao, D. Wake & P. Zhang., unpubl. data), a Pacific route is the most likely given palaeogeographical reconstructions. Until the Late Cretaceous, the Western Interior Seaway divided North America into two independent landmasses (Kauffman, 1984; Hay *et al*., 1999): eastern Appalachia, completely isolated from any then-emerged land in Mexico or Central America, and western Laramidia. The latter extended continuously at least into areas of southern Mexico, allowing dispersal for the ancestor of bolitoglossines into the Neotropical region. Our dating analysis, however, suggests an Eocene date for the divergence of the bolitoglossines from *Batrachoseps*. By this time, the interior seaway had disappeared but it had been replaced by the Western Inland Coastal Plain (Bonett *et al*., 2013), which would have been inhospitable for these salamanders or their ancestors. However,

we must continue to consider dispersal from either eastern or western North America as a possibility.

According to our time estimates and biogeographical analysis, ancestors of the bolitoglossines had already reached Central America during the early to middle Eocene. The geological history of this region is complex and there are still discrepancies about its formation (Pindell *et al*., 2006; Rogers & Mann, 2007; Silva-Romo, 2009). The hypotheses concerning the Chortís block are particularly controversial, with three different scenarios considered. Some authors have proposed that this block drifted from an isolated position far into the Pacific Ocean, reaching its current position no earlier than the Middle to Late Palaeogene (Keppie & Morán-Zenteno, 2005), dates within the highest posterior density of our estimates. Alternatively, other authors have argued that the Chortís block had contact with southern Mexico at least since Cretaceous times, either in a similar position as today (James, 2005) or attached to the Pacific coast area at the southern part of the North American plate (Rogers & Mann, 2007). In any case and according to most palaeogeographical reconstructions, the potential establishment of Bolitoglossini ancestors in the Maya and/or Chortís blocks seems possible at least since the Palaeogene, supporting our biogeographical results and age estimates for the main clades within the group. A similar timing of diversification was proposed for *Craugastor* (Crawford & Smith, 2005). The ancestor of this anuran genus dispersed northward from South America, but it also reached the Chortís block early, probably during the early Palaeogene. Subsequent diversification has resulted in a highly diverse group, with 113 recognized species mostly present in the same areas as bolitoglossines.

Within the Mexican/widespread clade, the Maya and Chortís blocks of Central America appear to have played an important role in the diversification of *Bolitoglossa*, given that they were included in nearly all range reconstructions of the ancestor of all *Bolitoglossa*. Taken together with the unambiguous reconstruction of the ancestor of the Central American endemic clade within Nuclear Central America, this region was probably much more important in the initial divergences of major groups of tropical salamanders than has been previously appreciated. The importance of Nuclear Central America in the diversification of the tropical bolitoglossines is also evident from examining the number of lineages present in each biogeographical region. Counting each genus or subgenus (for *Bolitoglossa* and *Oedipina*) as a lineage and including two lineages raised to generic status below, a strong pattern of increasing lineage diversity is evident from either extreme of the tropical bolitoglossine distribution towards the centre (Table 1). Only four lineages (containing a total of eight species) are present in the Sierra Madre Oriental of Mexico, increasing to eight lineages each in the TMVB and Oaxaca highlands (when undescribed species of *Chiropterotriton* from Oaxaca are included). Both the Maya and Chortís blocks of Nuclear Central America have 11 different lineages, dropping to six in the Chorotega block. South America contains only two lineages of tropical salamanders, and one of these lineages (*Oedipina* sg *Oedopinola*) has only two species in the region. Wake & Lynch (1976) noted that the eastern TMVB and northern Oaxaca have the highest generic diversity, but that Nuclear Central America had slightly higher species richness. New discoveries and taxonomic changes since their analysis have increased species diversity in nearly all regions, but the number of new lineages (including *Bradytriton*, *Nyctanolis*, and *Oeditriton*) in Nuclear Central America has made this region not only richer in species but also in lineages. Furthermore, when the Maya and Chortís blocks are considered together as Nuclear Central America, this region is high in endemic lineages. Of the 15 lineages occurring in Nuclear Central America, eight [*Bolitoglossa* (*Magnadigita*), *Bolitoglossa* (*Mayamandra*), *Bradytriton*, *Cryptotriton*, *Dendrotriton*, *Ixalotriton*, *Nyctanolis*, and *Oeditriton*] are endemic. The other regions of high species and lineage diversity do not attain this level of lineage endemism; even when considered together, the TMVB and southern Mexico contain nine lineages, only two of which [*Bolitoglossa* (*Oaxakia*) and *Thorius*] are endemic (Table 1).

The timing of divergence events in our time-calibrated tree agrees with an ancient history of the bolitoglossines in Nuclear Central America. Although the Central American genera (besides *Ixalotriton*) have been present in Nuclear Central America for tens of millions of years, they have attained only modest species diversity; *Cryptotriton* and *Dendrotriton* have only six and eight species, respectively, and *Bradytriton* and *Nyctanolis* are both monotypic. Despite having diverged more recently, *Bolitoglossa* and *Pseudoeurycea* together account for most of the diversity of tropical salamanders, and all these species appear to have evolved within the last 30 Myr. Our dating estimate for the arrival of *Bolitoglossa* in South America, sometime between 16 Mya (upper estimate of divergence of South American clade from Costa Rica/Panama *Eladinea*) and 8.4 Mya (lower estimate of divergence of *Bolitoglossa taylori* Wake, Brame & Mayers, 1970 from other South American *Eladinea*) places this divergence within the Miocene. This is in agreement with previous studies (Hanken & Wake, 1982; Parra-Olea, García-París & Wake, 2004; Wiens *et al*., 2007; Elmer *et al*., 2013) showing that salamanders entered South America long before the final closure of the Isthmus of Panama in the Pliocene around 3 Mya (Coates & Obando, 1996). Our estimate agrees more closely with

Table 1. Number of described species of each lineage (genera and subgenera) present in regions used in biogeographical analyses. Subgenus listed in parentheses after genus where applicable. *Aquiloeurycea* refers to the *Pseudoeurycea cephalica* species group, and *Isthmura* refers to the *P. bellii* species group.

Genus (subgenus)	Sierra Madre Oriental	TMVB	Southern Mexico	Maya block	Chortís block	Chorotega block	South America
Thorius	0	9	15	0	0	0	0
Chiropterotriton	4	8	0	0	0	0	0
Parvimolge	0	1	0	0	0	0	0
Aquiloeurycea	2	4	0	0	0	0	0
Isthmura	1	3	3	0	0	0	0
Ixalotriton	0	0	0	2	0	0	0
Pseudoeurycea	0	13	23	3	4	0	0
Bolitoglossa (*Bolitoglossa*)	1	2	1	7	4	4	0
Bolitoglossa (*Eladinea*)	0	0	0	0	0	27	40
Bolitoglossa (*Magnadigita*)	0	0	0	11	27	0	0
Bolitoglossa (*Mayamandra*)	0	0	0	4	0	0	0
Bolitoglossa (*Nanotriton*)	0	1	1	3	2	0	0
Bolitoglossa (*Oaxakia*)	0	0	5	0	0	0	0
Bolitoglossa (*Pachymandra*)	0	0	0	1	1	1	0
Dendrotriton	0	0	0	6	2	0	0
Cryptotriton	0	0	0	1	5	0	0
Nyctanolis	0	0	0	1	1	0	0
Nototriton	0	0	0	0	9	8	0
Bradytriton	0	0	0	1	0	0	0
Oedipina (*Oedipina*)	0	0	0	0	10	12	0
Oedipina (*Oeditriton*)	0	0	0	0	3	0	0
Oedipina (*Oedopinola*)	0	0	0	1	4	7	2

TVMB, Trans-Mexican Volcanic Belt.

recent, but controversial, geological evidence showing that the Isthmus may have been emergent much earlier, with only a narrow strait separating Central America from South America by the early Miocene, facilitating intercontinental exchanges of terrestrial organisms by the Miocene (Montes *et al*., 2012). This hypothesis is also supported by molecular and fossil data from an array of taxa including arachnids (Zeh, Zeh & Bonilla, 2003), freshwater fishes (Bermingham & Martin, 1998; Perdices, Doadrio & Bermingham, 2005), frogs (Wang, Crawford & Bermingham, 2008), and mammals (Woodburne, 2010).

Similar patterns of diversification to the bolitoglossines, but at more recent timescales, are seen within different groups of mammals. Like bolitoglossine salamanders, shrews have a northern origin; the two main genera found in the Neotropics, *Sorex* and *Cryptotis*, are distributed from North America south to Guatemala and the northern Andes of South America, respectively. They show much lower levels of diversity than the bolitoglossines (16 Mexican and Guatemalan species of *Sorex*, Carraway, 2007; 30 species of *Cryptotis*, Moreno Cárdenas & Albuja, 2014), however, and appear to be more recent arrivals in Mesoamerica; *Sorex* probably arrived in southern Mexico and Guatemala in the late Miocene (Esteva *et al*., 2010). On an even more recent timescale, the rodent *Ototylomys phyllotis* arrived in Nuclear Central America around 3.4 Mya, prior to the Great American Biotic Interchange, from which it later spread (3.2–2.8 Mya). The species showed an initial northward dispersal to the Chiapas and Guatemala highlands (2.3 Mya), with later dispersals (1.8 Mya) toward both the south (Nicaragua, Costa Rica) and the north-east (Belize) (Gutiérrez-García & Vázquez-Domínguez, 2012). There are several examples of dispersal from northern Central America and subsequent diversification in Mexico in different kinds of organisms, including plants, invertebrates, and vertebrates. These examples show that Central America played an important role in the evolution of these groups, but many of them occurred at different evolutionary scales (see Gutiérrez-García & Vázquez-Domínguez, 2013 and references therein).

Morphology

Extensive homoplasy with regards to morphology and ecology within the bolitoglossines was highlighted by Wake (1966) and then extended further by Wake &

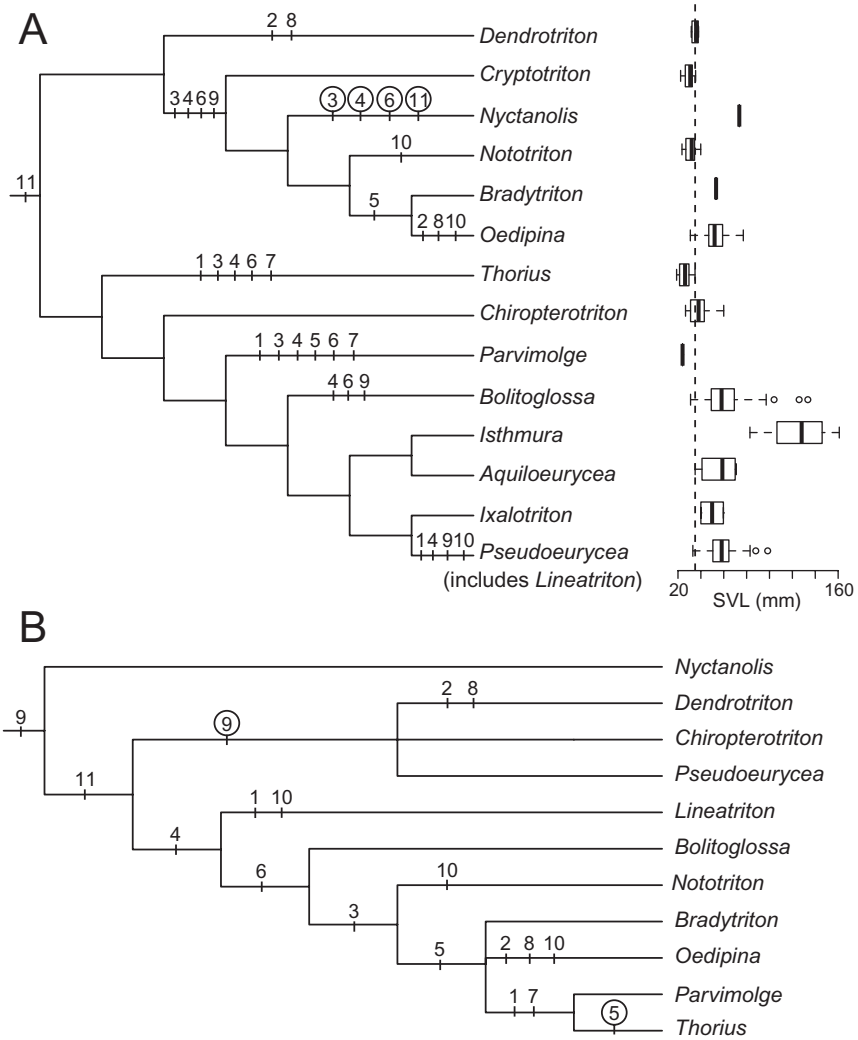

Figure 8. A, morphological characters from Wake & Elias (1983) mapped on a cladogram (summarized at genus level) from our analysis of concatenated sequence data, with box plots of snout–vent length (SVL) for each genus shown at right. A dotted line marks 35 mm SVL, a size limit often used to define miniaturized species. Only species formerly assigned to *Lineatriton* show derived states for characters 1, 4, 9, and 10 within *Pseudoeurycea*. B, most parsimonious tree for these morphological characters (redrawn from Fig. 8 of Wake & Elias, 1983). *Nototriton* includes *Cryptotriton*, which had not yet been described at the time of publication of Wake & Elias (1983), and *Pseudoeurycea* includes *Aquiloeurycea* and *Isthmura* (elevated to genus status below). The outgroup to the tropical bolitoglossines, *Batrachoseps*, was used to determine the ancestral state for each character. Circled characters indicate reversals to the ancestral state.

Elias (1983), Wake (1987), Wake (1991), and Parra-Olea & Wake (2001). The new phylogeny requires considerably more homoplasy than found in the last formal treatment, by Wake & Elias (1983). For example, miniaturization probably has evolved many times: either once within the Central American clade (with multiple reversals to larger size; Fig. 8) or at least four times (in *Cryptotriton*, *Dendrotriton*, *Nototriton*, and *Oedipina*), once more at the base of *Thorius*, again within *Chiropterotriton*, once again in *Parvimolge*, and perhaps at the base of *Bolitoglossa*, or if not there (see Jaekel & Wake, 2007), then independently within its subgenera *Nanotriton* and *Eladinea*.

Wake & Elias (1983) identified 18 characters in their analysis of nonmolecular traits for phylogenetic inference. Briefly, derived states for their characters 1–10 were: (1) mineralized mesopodials, (2) tibial spur absent/reduced, (3) ulnare and intermedium fused, (4) distal tarsals 4 and 5 fused, (5) mental gland externally obscure, (6) stylus of operculum reduced or absent, (7) preorbital processes of vomers absent, (8) prefrontal bones absent, (9) septomaxillary bones absent, and (10)

frontal processes of premaxillary fused at point of origin. They recorded extensive homoplasy in their two alternative trees. However, *Cryptotriton* and *Ixalotriton* had not been recognized as distinct genera at that time. Furthermore, both of their alternative trees, a maximum parsimony tree and one based on zoogeographical and phylogenetic arguments of Wake & Lynch (1976), differ substantially from the species trees that we recovered (Fig. 8). For clarity, we compared their results to those obtained using our concatenated tree. Their first tree differs from the concatenated tree of the present analysis in several important respects. They found *Nyctanolis* to be sister to the remaining genera, which were organized in two major clades, one including an unresolved tritomy of *Chiropterotriton*, *Dendrotriton*, and *Pseudoeurycea* and the other including *Lineatriton*, *Bolitoglossa*, *Nototriton* (including *Cryptotriton*), *Bradytriton*, *Oedipina*, *Parvimolge*, and *Thorius* (Fig. 8). Synapomorphies were identified on the tree for six stems, but two of these were reversed higher in the tree (Fig. 8). Five additional homoplasies were identified. The alternative tree again found *Nyctanolis* to be the sister taxon of everything else, and above it was an unresolved polytomy of *Pseudoeurycea*, *Chiropterotriton*, (*Lineatriton* – sister to *Parvimolge* + *Thorius*), *Dendrotriton*, and (*Bolitoglossa* – sister to the remaining taxa, *Bradytriton* – sister to the remaining taxa, *Nototriton* + *Oedipina*). This tree differed from the first in having three rather than six unique synapomorphies, and more homoplasies (ten vs. five, but with no reversals). Both trees differ dramatically from our concatenated tree; the only clade recovered in the older and newer phylogenetic analyses contains *Bradytriton*, *Nototriton*, and *Oedipina*. All of the polytomies have been resolved, and *Pseudoeurycea* has turned out to be diphyletic.

Of the 18 characters used by Wake & Elias in their phylogenetic analysis, only ten were potential synapomorphies for multigeneric clades. Their character 11, fused premaxillary bones, as a derived state, was a synapomorphy for all genera except *Nyctanolis*. In our analysis, the presence of two premaxillary bones in *Nyctanolis* is a reversal, and hence a homoplastic state with respect to outgroup taxa. Their characters 12–18 each have a derived state as an autapomorphy for only one genus. We think that all of these are correct except that character 12, absence of a sublingual fold in *Bolitoglossa*, is a reversal, and again a homoplastic state with respect to outgroup taxa. We will not discuss the remaining autapomorphic characters.

Although a detailed analysis of homoplasy should be carried out at the level of species rather than genera, we can explore our phylogenetic hypothesis for possible synapomorphies amongst their first ten characters, which were elusive in the study of Wake & Elias (1983). No clade in the most parsimonious tree analysed by Wake & Elias was supported by more than a single synapomorphy. In their alternative tree, however, three clades were supported by three synapomorphies each: *Lineatriton* + (*Parvimolge* + *Thorius*), *Parvimolge* + *Thorius*, and *Bolitoglossa* + (*Bradytriton* + [*Nototriton* + *Oedipina*]) (Fig. 8). We recovered none of these clades, an indication of extensive homoplastic evolution.

In our concatenated phylogeny one clade is supported by four synapomorphies: *Cryptotriton* + (*Nyctanolis* + [*Nototriton* + {*Bradytriton* + *Oedipina*}]) (Fig. 8). However, *Nyctanolis* shows reversal for all but one of these characters (character 9, it lacks septomaxillary bones, a derived trait). Characters 3, 4, 6, and 9 can all reasonably be associated with miniaturization, as evidenced by the fact that 3, 4, and 6 are found in the minute salamanders, *Thorius*, 3 and 4 are found in the miniaturized *Parvimolge*, and 4, 6, and 9 are found in *Bolitoglossa*, which is suspected to have had a miniaturized ancestor (Jaekel & Wake, 2007). All of these are homoplasies, and 4 and 9 are also found within *Pseudoeurycea* (in those species formerly assigned to *Lineatriton*). Characters 4 and 6 could be mapped equally parsimoniously as a synapomorphy for the Bolitoglossini with five and four reversals, respectively, but we feel that the mapping shown in Figure 8 is more likely to be the result of their association with miniaturization. Only one other multitaxon clade (*Bradytriton* + *Oedipina*) has even a single synapomorphy (character 5). Just with this rather superficial (in the sense of not having been able to examine many species, although all genera are included) analysis of homoplasy, we found all ten characters to be subject to homoplasy and recorded three instances of homoplastic reversal (all in *Nyctanolis*, a large, broad-headed member of a clade otherwise characterized by miniaturized or generally small species with narrow heads). Homoplasy would be even more extensive if all species were considered, because characters such as septomaxillary bones and prefrontals are variable within genera.

The extensive morphological homoplasy revealed by our phylogeny may be a result of alteration of a common ancestral developmental programme, in part owing to miniaturization of multiple lineages of salamanders across the phylogeny. Alberch & Alberch (1981) showed that the loss of prefrontal bones (character 8) in *Bolitoglossa occidentalis*, a small species, was the result of truncation of the ancestral developmental programme before these bones had formed. The loss of phalangeal elements in some fully webbed species was explained by a similar phenomenon. Hanken (1984) hypothesized that a reduction in size or presence of skull bones, as well as increased variability in osteological characters both within and between species, could be the result of truncation of skull

development. Both of these studies showed that tropical bolitoglossines follow a shared generalized ontogenetic trajectory (Alberch *et al*., 1979), and that skeletal elements that ossify later in the trajectory (such as the prefrontals or septomaxillae) are more likely to be lost in miniaturized species. *Nyctanolis*, a relatively large species in the Central American clade, which is comprised mostly of small species, continues further along the shared ontogenetic trajectory and has features not seen in the miniaturized species, as listed above. Alterations in the developmental sequence (Alberch *et al*., 1979) may lead to the presence of septomaxillae (usually the last bone to form) even when prefrontals are absent (as in *Dendrotriton*). Truncation of development in the presence of precocious ossification, however, as in *Thorius*, can lead to the development of nearly all of the skull bones, if only at very small size and dubious functional utility (Hanken, 1984), and *Parvimolge* has both prefrontals and septomaxillae as well as a robust skull, despite being one of the smallest salamanders. This combination of truncation and shuffling of the developmental trajectory, perhaps largely in response to organismal-level selection on features such as body size (Hanken, 1984; Hanken & Wake, 1993), may be responsible for much of the homoplasy evident from our phylogeny. Detailed studies of developmental series (currently lacking for most genera) and functional morphology will be necessary to disentangle the roles of selection on particular characters vs. organismal-level selection affecting multiple traits, but our results reiterate the importance of viewing morphological evolution in both a phylogenetic and developmental context.

TAXONOMY

The new phylogeny finds *Pseudoeurycea* to be diphyletic. Furthermore, species previously assigned to *Lineatriton* are nested within one of the two clades of *Pseudoeurycea.* In order to rectify this situation and produce monophyletic taxa while recognizing the morphological diversity present within the currently recognized genus, we propose a new taxonomy for *Pseudoeurycea*, as most recently treated comprehensively by Parra-Olea (2002). We endorse the reduction of *Lineatriton* to synonymy with *Pseudoeurycea*. Furthermore, we raise the recently named *Pseudoeurycea* (*Isthmura*) to full generic level, and we subdivide *Isthmura*, naming a new genus. The formal taxonomy is as follows:

PSEUDOEURYCEA TAYLOR, 1944

Type species: Spelerpes leprosus Cope, 1869.

Synonymy: Lineatriton Tanner, 1950.

Diagnosis: Moderate to small salamanders, with moderate to short limbs, moderately robust to slender to very slender bodies and moderately to very long tails, with limbs of moderate to short length and short fifth toes.

Assigned taxa: Pseudoeurycea ahuitzotl Adler, 1996; *P. altamontana* (Tayor, 1939); *P. amuzga* Perez-Ramos & Saldaña de la Riva, 2003; *anitae* Bogert, 1967; *P. aquatica* Wake and Campbell, 2001; *P. brunnata* Bumzahem & Smith, 1955; *P. cochranae* (Taylor, 1943); *conanti* Bogert, 1967; *P. exspectata* Stuart, 1954; *P. firscheini* Shannon & Werler, 1955; *P. gadovii* (Dunn, 1926); *P. goebeli* (Schmidt, 1936); *P. juarezi* Regal, 1966; *P. kuautli* Campbell, Brodie, Blancas-Hernández & Smith, 2014; *P. leprosa* (Cope, 1869); *P. lineola* (Cope, 1865); *P. longicauda* Lynch, Wake & Yang, 1983; *P. lynchi* Parra-Olea, Papenfuss & Wake, 2001; *P. melanomolga* (Taylor, 1941); *P. mixcoatl* Adler, 1996; *P. mixteca* Canseco-Márquez & Gutiérrez-Mayén, 2005; *P. mystax* Bogert, 1967; *P. nigromaculata* (Taylor, 1939); *P. obesa* Parra-Olea, García-París, Hanken & Wake, 2005; *P. orchileucos* (Brodie, Mendelson & Campbell, 2002); *P. orchimelas* (Brodie, Mendelson & Campbell, 2002); *P. papenfussi* Parra-Olea, García-París, Hanken & Wake, 2005; *P. rex* (Dunn, 1926); *P. robertsi* (Taylor, 1939); *P. ruficauda* Parra-Olea, García-París, Hanken & Wake, 2004; *P. saltator* Lynch & Wake, 1989; *P. tenchalli* Adler, 1996; *P. teotepec* Adler, 1996; *P. tlahcuiloh* Adler, 1996; *P. tlilicxitl* Lara-Góngora, 2003; *P. unguidentis* (Taylor, 1941); *P. werleri* Darling & Smith, 1954.

Comments: Pseudoeurycea has long been a problematic taxon because of the absence of morphological synapomorphies for the included taxa. The genus with the revised content is robustly supported as a clade by the molecular data presented herein, but several species are assigned tentatively because of their rarity and the absence of tissue samples (*P. amuzga*, *P. aquatica*, *P. kuautli*, *P. teotepec*, *P. tlilicxitl*). We found robustly supported clades within *Pseudoeurycea*, one corresponding to the *leprosa* group of Parra-Olea (2002) and the other to her *gadovii* group. We have not named these groups because we know of no diagnostic characters. Each of these contains additional subclades, and we especially point out the division of the *leprosa* group into a *leprosa* subclade (including *P. leprosa*, *P. firscheini*, *P. lynchi*, *P. mystax*, *P. nigromaculata*, *P. obesa*, and *P. werleri*, as well as the species formerly assigned to *Lineatriton*: *P. lineola*, *P. orchileucos*, and *P. orchimelas*) and a *juarezi* subclade (including *P. juarezi*, *P. aurantia*, *P. ruficauda*, *P. saltator*, and *P. unguidentis*).

ISTHMURA RAFFAËLLI & DUBOIS, 2012

Type species: Spelerpes bellii Gray, 1850.

Diagnosis: Large to very large black salamanders, usually boldly marked with segmentally arranged segments of red or red-orange to pinkish coloration; slight interdigital webbing with well-developed fifth toe.

Assigned taxa: Isthmura bellii (Gray, 1850), new comb.; *Isthmura boneti* (Alvarez & Martín, 1967), new comb.; *Isthmura gigantea* (Taylor, 1939), new comb.; *Isthmura maxima* (Parra-Olea, García-París, Papenfuss & Wake, 2005), new comb.; *Isthmura naucampatepetl* (Parra-Olea, Papenfuss & Wake, 2001), new comb.; *Isthmura sierraoccidentalis* (Lowe, Jones & Wright, 1968), new comb. The last taxon is raised to full species status on the basis of its different ecology and distribution, and extreme reduction of reddish coloration.

AQUILOEURYCEA GEN. NOV.

Type species: Spelerpes cephalicus Cope, 1869.

Diagnosis: Salamanders of moderate to small size lacking any organized colour pattern; digits, especially fifth toe, relatively short and with moderate amounts of interdigital webbing.

Assigned taxa: We assign the following taxa to *Aquiloeurycea*: *Aquiloeurycea cephalica* (Cope, 1865), new comb.; *Aquiloeurycea cafetalera* (Parra-Olea, Rovito, Márquez-Valdelamar, Cruz, Murrieta-Galindo & Wake, 2010), new comb.; *Aquiloeurycea galeanae* (Taylor, 1941), new comb.; *Aquiloeurycea quetzalanensis* (Parra-Olea, Canseco-Márquez & García-París, 2004), new comb.; *Aquiloeurycea scandens* (Walker, 1955), new comb. The enigmatic *Aquiloeurycea praecellens* (Rabb, 1955), new comb. known only from the unique holotype, is provisionally assigned to this genus.

Etymology: From *aquilo*, L., referring to the northerly distribution of the genus relative to most other genera of bolitoglossines, and *eurycea,* possibly a reference to Greek mythology used by Rafinesque (1822) to refer to brook salamanders of the USA, and subsequently to the genus *Pseudoeurycea*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Voucher information and GenBank numbers for sequences used in phylogenetic analyses. Collection abbreviations: CARIE, Colección de Referencia de Anfibios y Reptiles del Instituto de Ecología, Asociación Civil; IBH, Colleción Nacional de Anfibios y Reptiles, Instituto de Biología, UNAM; MVZ, Museum of Vertebrate Zoology, University of California, Berkeley; MZFC, Museo de Zoología 'Alfonso L. Herrera', Facultad de Ciencias, UNAM.