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Divergence Time Estimation Using Fossils as Terminal Taxa and the Origins of Lissamphibia

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Abstract.—Were molecular data available for extinct taxa, questions regarding the origins of many groups could be settled in short order. As this is not the case, various strategies have been proposed to combine paleontological and neontological data sets. The use of fossil dates as node age calibrations for divergence time estimation from molecular phylogenies is commonplace. In addition, simulations suggest that the addition of morphological data from extinct taxa may improve phylogenetic estimation when combined with molecular data for extant species, and some studies have merged morphological and molecular data to estimate combined evidence phylogenies containing both extinct and extant taxa. However, few, if any, studies have attempted to estimate divergence times using phylogenies containing both fossil and living taxa sampled for both molecular and morphological data. Here, I infer both the phylogeny and the time of origin for Lissamphibia and a number of stem tetrapods using Bayesian methods based on a data set containing morphological data for extinct taxa, molecular data for extant taxa, and molecular and morphological data for a subset of extant taxa. The results suggest that Lissamphibia is monophyletic, nested within Lepospondyli, and originated in the late Carboniferous at the earliest. This research illustrates potential pitfalls for the use of fossils as post hoc age constraints on internal nodes and highlights the importance of explicit phylogenetic analysis of extinct taxa. These results suggest that the application of fossils as minima or maxima on molecular phylogenies should be supplemented or supplanted by combined evidence analyses whenever possible. [Divergence time estimation; evolutionary rates; fossil constraints; Lissamphibia; molecular clock; Tetrapoda.]

As many as 50 billion species, up to 99.9% of all organisms that have ever existed, have gone extinct (Raup 1993) and cannot be included in phylogenetic analyses based on molecular data. However, these taxa can potentially provide a rich source of information regarding the origins of extant groups based on their age and phylogenetic position. A major goal of systematics is to produce an accurate "time tree" of life, describing the relationships between organisms (both extant and extinct), and their dates of origin (e.g., Kumar and Hedges 1998; Benton and Ayala 2003; Donoghue and Benton 2007; Hedges and Kumar 2009). However, the occasionally incomplete nature of the synthesis of paleontological and neontological data in molecular divergence time estimation has been noted by many authors, as fossils are usually applied only as broad minima or maxima on internal nodes (e.g., Benton and Ayala 2003; Müller and Reisz 2005; Donoghue and Benton 2007; Parham and Irmis 2008). New approaches are needed for more accurate divergence time estimation to extract not only temporal but also phylogenetic information from paleontological data sets, as previous studies have typically done only one or the other (e.g., Kumar and Hedges 1998; Gatesy et al. 2003).

Fossil data are typically employed subsequent to molecular phylogenetic analysis as minimum or maximum ages on the divergence times of internal nodes (e.g., Donoghue and Benton 2007; Ho and Phillips 2009). Methods for assessing the quality of these calibrations and the validity of their placement at different nodes have become fairly common (e.g., Near et al. 2005; Rutschmann et al. 2007; Marshall 2008; Lee et al. 2009; Pyron 2010), which is crucial given the errors that can arise due to calibration uncertainty and fossil misspecification (e.g., Near and Sanderson 2004; van Tuinen and Hedges 2004; Hug and Roger 2007; Burbrink and Pyron 2008; Ho and Phillips 2009; Ruane et al. 2011). Additionally, calibration points are often duplicated across different analyses by different researchers. Thus, although results may differ between studies, many times calibration strategies are not independent (Graur and Martin 2004).

Importantly, most fossils do not represent specific nodes on a phylogeny, but distinct taxa that diverged at a unique point in time, with their own branch length resulting from varying rates of molecular and morphological evolution. Thus, the use of fossils as minima or maxima for divergences between extant taxa is a conservative, but potentially incomplete solution to a complex problem. Many authors have discussed issues involving the utilization and utility of fossil data in phylogenetic inference (e.g., Gauthier et al. 1988; Donoghue et al. 1989; Huelsenbeck 1991), and numerous attempts have been made to build phylogenies including both extant and extinct taxa using molecular and morphological data (e.g., Eernisse and Kluge 1993; Shaffer et al. 1997; Gatesy et al. 2003; Rothwell and Nixon 2006; O'Leary and Gatesy 2008; see Lee et al. 2009). However, few studies have tried to explicitly infer divergence times based on the ages of the fossils contained in such trees (e.g., Manos et al. 2007; Marjanović and Laurin 2007; Sauquet et al. 2009; Magallón 2010).

Fossil dates are often used without explicit consideration of the phylogenetic placement of the extinct taxon itself, such as through estimation of a phylogeny containing the fossil of interest. This may lead to significant errors if fossils are misspecified (see van Tuinen and Hedges 2004; Near et al. 2005; Lee et al. 2009; Ruane et al. 2011). However, empirical and theoretical results suggest that phylogenetic estimation may be improved by the addition of fossil taxa (e.g., Donoghue et al. 1989; Cobbett et al. 2007; Wiens 2009). Thus, in cases where sufficient morphological data are available for both the extant and the extinct taxa, it should be possible to infer divergence times from phylogenies containing both. Thus, the molecular clock for the extant species is calibrated by the placement of the extinct taxa of known age, and the morphological data are directly incorporated into the phylogenetic analyses.

Using this approach, fossil placement is determined analytically based on the morphological data rather than by post hoc potentially nonphylogenetic hypotheses. Both the molecular and the morphological branch length estimates can be parameterized based on extinct and extant taxa, with the proportionality of morphological and molecular evolution (e.g., Omland 1997) providing calibration for the overall clock rates. Such analyses might be preferable to those including only extant taxa for estimating the ages of origin and diversification for many groups. However, although methods for divergence time estimation using molecular data are common (e.g., Thorne et al. 1998; Sanderson 2002; Drummond et al. 2006), there have been few explicit techniques developed for inferring node ages for trees containing fossils (e.g., Magallón 2010).

In this paper, I examine techniques for estimating divergence times for phylogenies containing both extant and extinct taxa, inferred using molecular and morphological data. I combine methods for phylogenetic inference using morphological data (Lewis 2001) with relaxed-clock methods for inferring molecular divergence times (Drummond et al. 2006) and methods for dating trees with noncontemporaneous terminal taxa (Rambaut 2000). I estimate divergence times by combining relaxed-clock models for molecular data and models for morphological data with methods that allow for noncontemporaneous taxa to analyze a data set containing fossil and extant taxa sampled for morphological and molecular data. I use these methods to estimate the age and phylogenetic placement of Lissamphibia (frogs, salamanders, and caecilians), using a combined molecular and morphological data set containing several amphibian groups and a number of tetrapod outgroups, both living and extinct.

The origin of the extant lissamphibians is the subject of some debate (e.g., San Mauro et al. 2005; Zhang et al. 2005; Marjanović and Laurin 2009; Roelants et al. 2007; Zhang and Wake 2009b). Competing hypotheses suggest that the group is nested within Temnospondyli, within Lepospondyli, or polyphyletic, with caecilians (Gymnophiona) related to lepospondyls, and frogs (Anura) and salamanders (Caudata), collectively known as Batrachia, related to temnospondyls (reviews in Lee and Anderson 2006 and Ruta and Coates 2007). This uncertainty also impacts estimates of the age of the group (e.g., Marjanović and Laurin 2007; Anderson et al. 2008). Using molecular divergence time methods with internal fossil calibrations, age estimates for the group have ranged from 267 to 380 Ma (San Mauro et al. 2005; Zhang et al. 2005; Hugall et al. 2007; Marjanović and Laurin 2007; Roelants et al. 2007; Inoue et al. 2010). These dates have been used to support both the lissamphibian temnospondyl (Zhang et al. 2005) and lissamphibian polyphyly hypotheses (Lee and Anderson 2006). Purely stratigraphic approaches expand this range to 255–380 Ma (Marjanović and Laurin 2008), though fossil-based analyses suggest a Permian origin of the group, <300 Ma (Marjanović and Laurin 2007).

Given the considerable topological and temporal variation that apparently results from the consideration of extinct amphibian species, it would clearly be desirable to integrate the temporal and phylogenetic information of both extinct and extant taxa, using morphological and molecular data, to estimate phylogeny and divergence times for Lissamphibia. Promisingly, several molecular and morphological data sets have been produced for the group (e.g., Vallin and Laurin 2004; San Mauro et al. 2005; Hugall et al. 2007; Wiens 2007). I combine these to simultaneously estimate phylogeny and divergence times for both the extant lissamphibians and the fossil relatives to test not only the ancestry (i.e., polyphyly, temnospondyl relation, or lepospondyl relation) but also the timing (i.e., older [∼380 Ma] or recent [∼260 Ma]) of lissamphibian origins. I further discuss the importance of phylogenetic analysis of extinct taxa. Analytical issues affecting traditional divergence time estimates such as branch length estimation (Phillips 2009), missing data (Wiens 2003; Lemmon et al. 2009), and rate heterogeneity among characters and partitions (Mueller 2006; Clarke and Middleton 2008) may be amplified in such an analysis and their impacts are documented. The use of fossils as constraints on divergences between extant taxa while excluding them from phylogenetic inference should be supplanted by the integration of both extant and extinct taxa in phylogenetic inference and divergence time estimation whenever possible.

Materials and Methods

Molecular Data

I combined portions of the RAG-1 data sets from Wiens (2007) and Hugall et al. (2007) and added a few additional samples to generate a new matrix containing single representatives from almost all lissamphibian families, with Synapsida as an amniote outgroup for which molecular and morphological data were also available (Appendix Table A1; Vallin and Laurin 2004). Although multilocus data sets are generally preferable to single-locus estimates, the use of the RAG-1 data is attractive for four reasons. First, the behavior of the

locus as a deep time marker for tetrapod phylogenetics has been thoroughly characterized (Hugall et al. 2007). Second, it decreases the potential for the interaction between among-locus molecular rate variation and missing data affecting branch length estimation (Lemmon et al. 2009). Although the RAG-1 matrix is not 100% complete, missing data within single loci have typically been shown not to negatively affect phylogenetic inference when the overall number of characters is large (e.g., Wiens 1998, 2003), whereas asymmetrical absences in multilocus molecular data sets have been (e.g., Lemmon et al. 2009). Third, it decreases the parameterization necessary to account for rate variation across branches and among loci, which can negatively affect Markov chain Monte Carlo performance (Rannala 2002). Fourth, the RAG-1 data have been used in at least five previous studies to estimate ages for Lissamphibia and Tetrapoda (San Mauro et al. 2005; Hugall et al. 2007; Wiens 2007; Inoue et al. 2010; Pyron 2010). This will allow for a direct comparison between the results of this study and previous divergence dating analyses using traditional fossil calibration methods with the same data set.

The matrix was produced using the alignment of Hugall et al. (2007) as a guide and comprises 2652 bp for 34 species. In terms of coverage, 24% of taxa (8 of 34) have complete or nearly complete sequence data, whereas 71% (24 of 34) have a length of ∼1500 bp. One taxon (Rhinophrynidae) had a length of 1144 bp, whereas the representative from Leiopelmatidae had 895 bp. Alignments were performed using the MUSCLE algorithm (Edgar 2004) with the default settings, trimmed for maximum coverage, and checked by assuring that all sequences were in reading frame and contained no stop codons. I also performed several additional analyses to assess the impact of missing data on the analyses (see below).

Morphological Data

The morphological data are taken from the matrix presented by Vallin and Laurin (2004), who scored 161 characters measuring various aspects of cranial, axial, and appendicular skeletal morphology for 49 taxa. The data set is 78.32% complete, accounting for both missing data and ambiguities. Characters in this matrix were intentionally chosen to maximize homology and scoring potential across tetrapods (Laurin 1998; Laurin and Reisz 1997). The data set includes a mixture of ordered and unordered characters (see Vallin and Laurin 2004). The matrix includes eight extant groups (seven lissamphibian families and one Synapsida) and 41 fossil taxa. As per Vallin and Laurin (2004), the outgroup taxon in all analyses including the morphological data is Osteolepiformes. Though this taxon is known to be paraphyletic (Cloutier and Ahlberg 1995), the coding was derived primarily from *Eusthenopteron* and thus still represents an appropriate outgroup (Laurin and Reisz 1997).

Phylogenetic Analysis

To assess the impact of the addition of extinct taxa to molecular phylogenetic analyses, I performed three separate analyses using the morphological and DNA sequence data in MrBayes v3.1.2 (Ronquist and Huelsenbeck 2003). First, I analyzed the morphological data alone, using the Markov variable (Mkv) model specified by Lewis (2001), with gamma-distributed rate heterogeneity (Γ), and a subset of the characters defined as ordered (see Clarke and Middleton 2008). Second, I analyzed a data set containing the 49 taxa from the morphological data set, combined with the DNA sequence data for the eight taxa that were also represented in the molecular analysis, with relative rates estimated for the molecular and morphological partitions. In this analysis, all 49 taxa had morphological data, and 8 of 49 (16%) had both morphological and molecular data. Third, I performed a combined analysis of the morphological and DNA data, using a matrix containing all 75 taxa from both data sets, of which 26 (35%) had only DNA sequence data, 41 (55%) had only morphological data, and 8 (11%) had both molecular and morphological data. The nucleotide data were assigned a GTR + Γ + *I* model partitioned by codon position (Hugall et al. 2007). Again, the molecular and morphological partitions were assigned separate relative rates, which were estimated empirically. All analyses were performed using two runs of four chains each, continued for 50 million generations. Convergence was assessed using Gelman and Rubin's *r* (Gelman et al. 2003), and stationarity was assumed when values of *r* approached 1. This occurred prior to 5 million generations, which were discarded as burn-in in all analyses.

Divergence Time Estimation

To infer divergence times using both fossil and extant taxa, I used a modification of the relaxed phylogenetics approach described by Drummond et al. (2006), implemented in the program BEAST v1.5.4 (Drummond and Rambaut 2007). The BEAST suite now implements several models that can analyze multistate character data, including the multistate stochastic Dollo (MSSD) model of Alekseyenko et al. (2008) and the Mkv model of Lewis (2001), which is a subtype of the stochastic Dollo-like models. I used the Mkv model, as it has been most thoroughly evaluated with respect to phylogenetic analysis of morphological data (Müller and Reisz 2005; Wiens 2009).

The morphological characters were separated into ordered and unordered partitions and given Mk model elements with the appropriate number of states for each character (e.g., a partition for unordered three-state characters, ordered five-state characters), whereas the models for the DNA data remained as specified in MrBayes. Relative rate multipliers were assigned to the combined morphological partition and each of the three codon positions of the molecular data to allow for rate heterogeneity among and within partitions.

All parameters were unlinked across partitions, with a single uncorrelated lognormal relaxed-clock model (1-clock) for overall branch rates shared by both data types. This is an approximation of the phylogenetic analysis performed in MrBayes while estimating divergence times using both the molecular and morphological data. I also performed a second analysis to assess the potential impact of rate heterogeneity among data types and the correlation between molecular and morphological rates. In this analysis, unlinked clock distributions were assigned to the molecular and morphological data (2-clock). Clock rates were thus drawn from a lognormal distribution unique to each partition (see Drummond et al. 2006).

To extract the temporal information from the 41 extinct taxa, the age of each fossil taxon (online Appendix A, available from http://www.sysbio.oxfordjournals.org) was entered as a noncontemporaneous tip date representing millions of years before the present (see Rambaut 2000; Drummond et al. 2006). I obtained the geological stage of each fossil from Benton (1993) and Laurin (2004; Gradstein et al. 2004; online Appendix A). The lower (most recent) temporal bound of the age range of that stage was used as the tip date. For higher taxa that may span a substantial stratigraphic range, I used the age of the basal node of the taxon. As a conservative prior on the age of the root of the tree (see Pyron 2010), I used a lognormal distribution with the 95% highest prior density (HPrD) bounding the earliest known fossils associated with the outgroup taxon (374.5 Ma; see Laurin 2004) to the earliest evidence of putative stem tetrapods from the Devonian (395 Ma; Niedźwiedzki et al. 2010), with a median age of 384.3 Ma. Thus, the median prior age is concordant with previous stratigraphic assessments (~380–386 Ma; Laurin 2004), and the 95% HPrD encompasses the dates spanned by the known fossil record of the stem tetrapods (e.g., Benton 1993; Niedźwiedzki et al. 2010).

The combined molecular and morphological data were analyzed under a partitioned mixed model analysis using an uncorrelated lognormal distribution of evolutionary rates. Thus, the molecular clock estimation of age was parameterized using both the root prior and the relative divergence between the fossil and extant taxa based on the estimated morphological and molecular divergence between those taxa. Two replicates of each single-chain analysis (1-clock and 2-clock) were run for 50 million generations, sampled every thousandth. Analyses were also run with empty data matrices (all characters changed to "?") to sample from the prior distribution for comparison with the posterior. Convergence was assessed by examining concordance between estimated ages from the replicated analyses and by measuring the effective sample size (ESS) of the inferred parameters. Convergence was assumed when ESS values reached 200 for most or all parameters (e.g., Drummond et al. 2006), which occurred prior to 5 million generations for both analyses, and these first 5 million were discarded as burn-in. All input files and results are available at the Dryad data repository (doi:10.5061/dryad.8120).

Missing Data, Branch Lengths, and Evolutionary Rates

As fossil taxa will necessarily introduce a large proportion of missing data in any combined data set (Wiens 2009), the impact of these absent characters on branch length estimation and support may be substantial. In particular, the interaction between among partition rate variation and missing data may negatively affect phylogenetic inference (Lemmon et al. 2009). Additionally, heterogeneity in evolutionary rates between the morphological characters may also affect estimated branch lengths (Clarke and Middleton 2008). To evaluate the impact of these potential sources of confounding variance, I performed several additional analyses on the estimated phylogenies.

I assessed the impact of missing data on support and branch length estimation on the three primary MrBayes analyses, the morphology-only tree, the morphology supplemented with DNA tree, and the combined molecular and morphological tree. I tested the impact of missing data three ways. First, I tested for a correlation between the support at each node and the mean proportion of data present for all taxa subtended by that node. Second, I used the strategy of Wiens et al. (2005), testing for a correlation between the support for the placement of terminal taxa and the proportion of data for each taxon. Finally, I tested for a correlation between the support for terminal taxa and the minimum data proportion for sister species pairs or single terminals, sensu Pyron et al. (2011). The first two analyses may be underpowered due to a partial nonindependence of data points, whereas the latter is extremely sensitive to highly incomplete taxa. However, together these analyses should be able to detect any significant trends between the support and missing data proportion.

To evaluate the impact of missing data on branch length estimation, I tested for a correlation between the proportion of data present for each taxon and the length of the terminal subtending branch. This analysis was also performed on the trees from the three primary MrBayes analyses. These tests were not performed on the time-calibrated chronograms from BEAST, as the extant taxa necessarily have longer branches than the extinct taxa. The former extend to time zero, whereas the latter terminate at fixed points, primarily in the distant past. All tests were performed using all compatible clades rather than the majority rule consensus trees to avoid overlooking poorly supported nodes collapsed into polytomies, which may have otherwise contributed to a significant pattern. All correlations were tested using the nonparametric Spearman's rank coefficient.

Finally, I assessed rate heterogeneity within the morphological partition and correlation with the molecular rates in a number of ways. First, estimates of

the α parameter for the gamma distribution of rate heterogeneity for the morphological data in both the MrBayes and the BEAST analyses give an estimate of the amount of rate variation in the morphological data. Values of α closer to zero indicate higher heterogeneity, whereas larger values suggest lower overall variance in substitution rate. Accounting for Γ distributed rates has been shown to significantly improve models for morphological evolution (Clarke and Middleton 2008). Second, as mentioned above, I ran two divergence time analyses: one with a single clock shared between the morphological and molecular partitions and one in which each data type was given a separate clock. The partition-specific relative rate multipliers from the first analysis give an estimate of the degree of relative rate heterogeneity among the partitions. The concordance or lack thereof between the rate and date estimated from the two analyses will give an indication of the degree of correlation between the evolutionary rates of the two data types and the degree of absolute rate heterogeneity across the data set.

Results

Morphological Analyses

The Bayesian analysis of the morphological data yields results highly similar to the parsimony analysis presented by Vallin and Laurin (2004), with a few exceptions (Fig. 1). The lissamphibians are strongly supported (Pp = 1.0) as a monophyletic group, including the extinct taxa Eocaecilia (sister to Gymnophiona, i.e., stem group Apoda), Karaurus (nested within Caudata), and Triadobatrachus (sister to Anura, i.e., stem group Salientia), though relationships within the group are not well resolved. The placement of the extant amphibians within the lepospondyls is strongly supported (Pp = 0.95), as suggested by Vallin and Laurin (2004) and Marjanović and Laurin (2007), contrary to the suggestions of Zhang et al. (2005) and Lee and Anderson (2006), and the results of Anderson et al. (2008). The reptiliomorphs are sister to the lepospondyl–lissamphibian group (Pp = 1.0; Fig. 1). The temnospondyls and seymouriamorphs, respectively, are sister to the extant tetrapods (Pp = 0.92 and 0.63), forming the crown group Tetrapoda (Laurin 2004). A number of stem tetrapods are weakly placed near the root, and the stegocephalians are strongly supported as the basal tetrapods (Fig. 1).

Morphological Data Supplemented with DNA

The addition of molecular sequence data for the eight extant taxa in the morphological matrix does not alter the deeper structure of the tree but strongly influences the resolution of the lepospondyls and the lissamphibians (Fig. 2). The lissamphibians are still nested within the lepospondyls (Pp = 0.93), though with the exception of Aïstopoda, the other lepospondyl taxa form a weakly supported (Pp = 0.68) clade exclusive of the lissamphibians. The primitive lepospondyl group Aïstopoda is inferred as the sister group to the lissamphibians, with moderate support (Pp = 0.91; Fig. 2). The shift in resolution and topology among the lepospondyls illustrates the impact that combining extinct and extant species can have on phylogenetic inference (e.g., Gauthier et al. 1988; Cobbett et al. 2007; Wiens et al. 2010). The relationships among the lissamphibians are fully resolved and well supported, with Caudata + Anura sister to Gymnophiona (Fig. 2). The relationships among the amniotes, temnospondyls, and stem tetrapods are unaffected by the addition of molecular data (Fig. 2).

Combined Morphological and Molecular Data

The addition of a number of additional lissamphibian taxa does not result in an appreciably different tree from the phylogenetic estimate inferred using only the fossil species and the extant taxa that had both molecular and morphological data (Fig. 3). The placement of the lissamphibians in the lepospondyls, the position of the temnospondyls, and the relationships among the stem tetrapods remain unchanged. The inferred relationships among the extant amphibian families are very similar to those estimated by Wiens (2007), Hugall et al. (2007), Zhang and Wake (2009a, 2009b), and San Mauro (2010). The support for the sister relationship between Caudata and Anura is slightly weaker in the combined analysis (Pp = 0.86) than in the supplemented analysis (Pp = 0.97), though the monophyly of Lissamphibia remains strongly supported (Pp = 1.0; Figs. 2 and 3).

The addition of multiple extant lissamphibian families offers a more detailed picture of the placement of the fossil amphibian species (Fig. 3). The Jurassic apodan Eocaecilia is sister to the extant gymnophionans (see Zhang and Wake 2009a), part of the stem group Apoda. The Jurassic salamander Karaurus, putatively representing the salamander stem group Urodela, is nested within the crown group Caudata, sister to Hynobiidae (Pp = 0.84). Perhaps most notably, the Triassic taxon Triadobatrachus, typically thought to be a stem group frog (Salientia), is nested within the crown group Anura, forming a polytomy with the neobatrachian frogs exclusive of Ascaphidae and Leiopelmatidae, though the monophyly of this group is weakly supported (Pp = 0.63). However, the monophyly of Anura + Triadobatrachus is strongly supported (Pp = 1.00; Fig. 3). These results may be somewhat suspect, as both Karaurus and Triadobatrachus exhibit a number of putatively primitive features lost in their respective crown groups (e.g., Cannatella and Hillis 1993; Roček and Rage 2000; Ruta and Coates 2007).

A number of conclusions regarding divergence times can be drawn from this uncalibrated phylogeny (Fig. 3), though the placement of some of the extinct taxa necessitates some caution in interpretation (Manos et al. 2007). The stem group age of Lissamphibia dates at a minimum to the early Carboniferous, ~318–359 Ma.

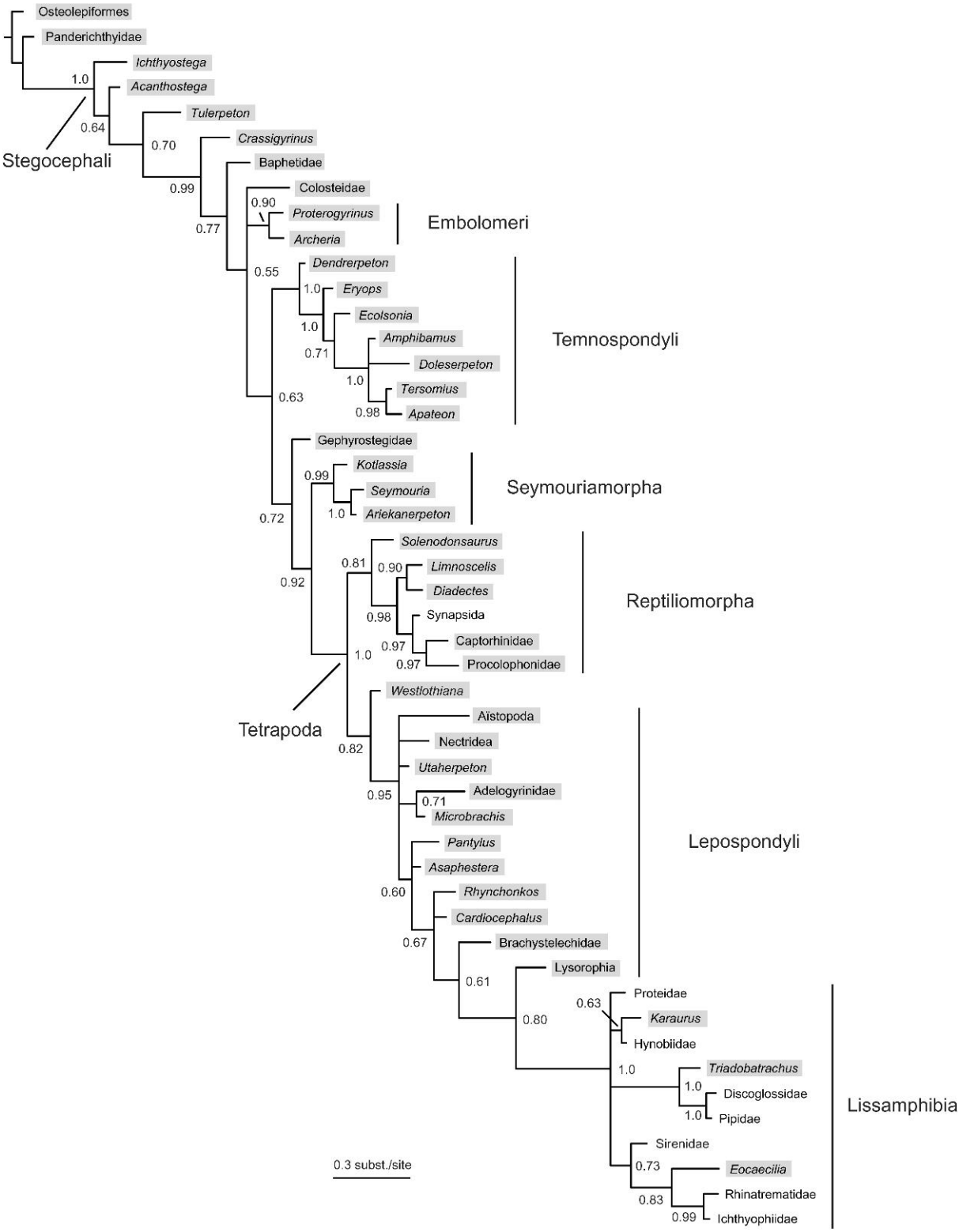

FIGURE 1. Phylogeny of lissamphibians and related tetrapods based on Bayesian inference analysis of 161 morphological characters in MrBayes. Numbers above nodes represent Pp values based on 45 million post-burn-in generations. Taxa covered by gray boxes are fossils.

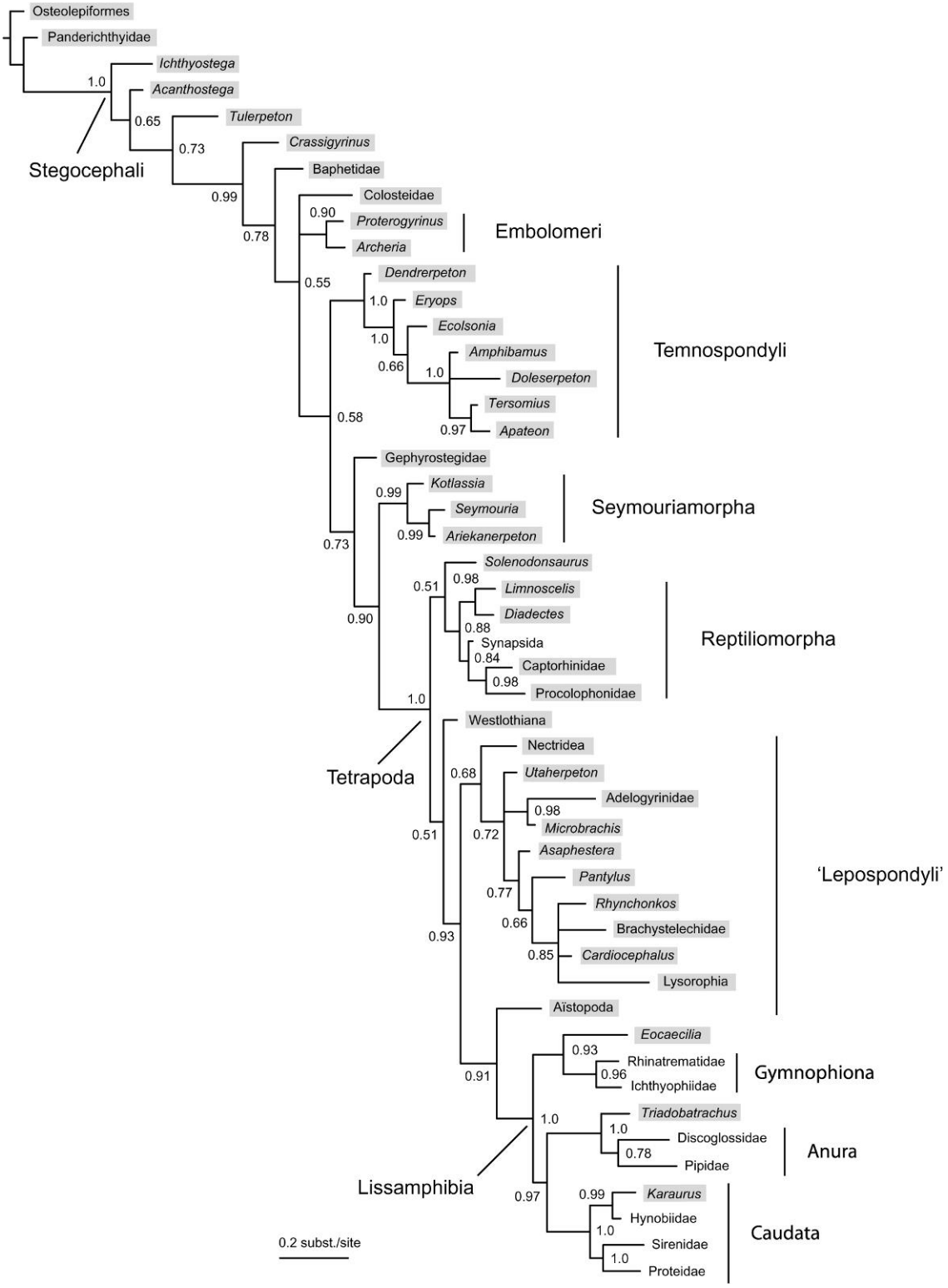

FIGURE 2. Bayesian phylogeny of the lissamphibians and related tetrapods based on 161 morphological characters, supplemented with molecular data (RAG-1, 2652 bp) for the eight extant taxa. Numbers above nodes represent Pp values based on 45 million post-burn-in generations from MrBayes. Taxa covered by gray boxes are fossils.

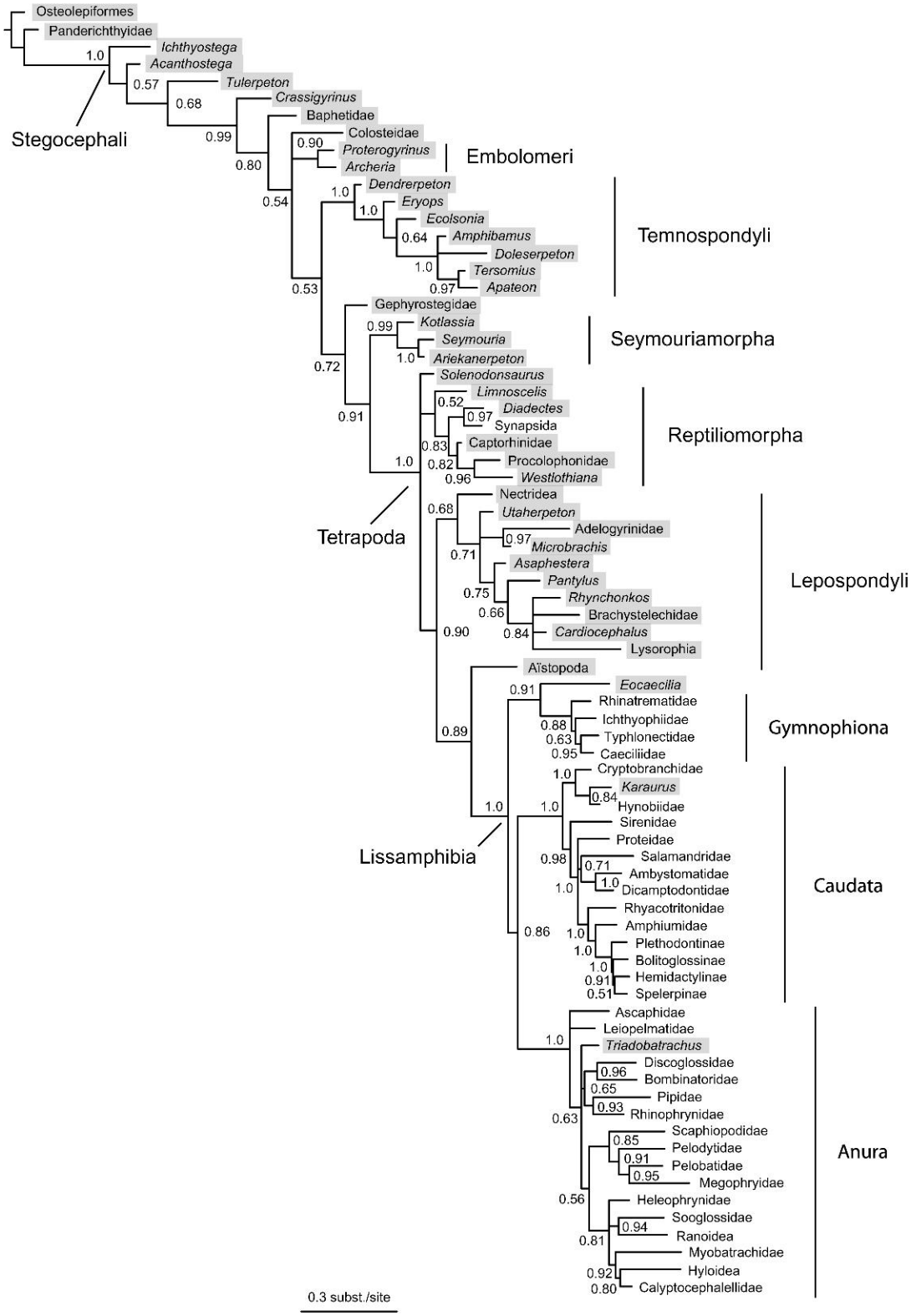

FIGURE 3. Bayesian phylogeny of the lissamphibians and related tetrapods based on 161 morphological characters for 41 fossil taxa, combined with molecular data (RAG-1, 2652 bp) for 34 extant taxa, eight of which also had morphological data. Numbers above nodes represent Pp values based on 45 million post-burn-in generations from MrBayes. Taxa covered by gray boxes are fossils.

The divergence between Karaurus and Hynobiidae occurred during or prior to the late Jurassic (145–161 Ma), though this date can likely only be considered a credible minimum for the stem group age of Urodela (see Cannatella and Hillis 1993; Ruta and Coates 2007). The divergence between the extant gymnophionans and Eocaecilia (i.e., the most recent common ancestor [MRCA] of Apoda) occurred prior to the early Jurassic, 175–199 Ma. The divergence between the neobatrachian and "mesobatrachian" frogs would be given a tentative minimum of 243–251 Ma based on the placement of Triadobatrachus, though this date provides a more credible minimum date for the age of the anuran stem group Salientia, based on the previously hypothesized affinities of the fossil (see Roček and Rage 2000).

Divergence Time Estimation

The combination of morphological and molecular data to parameterize relaxed-clock divergence time estimation yields stable age estimates for both the ingroup and the fossil outgroups. For both the 1-clock and 2-clock analyses, the two runs were combined, with the first 10 million of the 100 million generations discarded as burn-in. This yielded large ESS values (>500) for the majority of parameters, including the prior, posterior, and most date estimates. The two analyses produced somewhat concordant results (see below), and I primarily report the results from the 1-clock runs. The 1-clock analysis was highly congruent with the MrBayes analysis with respect to the monophyly of Lissamphibia, the lepospondyl affinity of the amphibians, and placement of the reptiliomorphs and temnospondyls. However, Triadobatrachus was inferred as the sister taxon to Anura. This is far more consistent with morphological evidence and existing phylogenetic hypotheses (e.g., Cannatella and Hillis 1993; Roček and Rage 2000) and likely to yield dates more consistent with stratigraphic evidence (e.g., Marjanović and Laurin 2007, 2008). Additionally, Karaurus was weakly supported as the sister taxon to Cryptobranchidae + Hynobiidae (Cryptobranchoidea; Fig. 4), though still within Caudata.

The results sampling from the prior yield a root age of 401.9 Ma (95% highest posterior density [HPD] = 392.5–411.6 Ma). Given the topological constraint of the ingroup to exclude Osteolepiformes, the estimated ages of all labeled nodes (e.g., Lissamphibia, Tetrapoda, Anura) were slightly younger (∼400 Ma) for all nodes of interest. Thus, all ingroup node age estimates represent strong departures from the prior distribution. The root of the tree dates to the early Middle Devonian boundary, 402.3 Ma (95% HPD = 393.0–412.2 Ma). This is somewhat older than recent stratigraphic consensus (e.g., Laurin 2004) but broadly consistent with the earliest putative Osteolepiform fossils (e.g., Benton 1993) and recently discovered trackways which putatively represent early tetrapods (e.g., Niedźwiedzki et al. 2010). The crown group age of the extant Tetrapoda (the amniote–amphibian divergence) dates to the late Devonian, 367.5 Ma (95% HPD = 354.9–380.6 Ma). The stem leading to the lissamphibians is estimated at 328.6 Ma (95% HPD = 314.5–344.1 Ma) in the early Carboniferous. The crown group age of Lissamphibia dates to the late Carboniferous, 305.5 Ma (95% HPD = 278.0–332.0 Ma), consistent with Marjanović and Laurin (2007, 2008), Zhang and Wake (2009a), and San Mauro (2010), but much younger than San Mauro et al. (2005), Zhang et al. (2005), Hugall et al. (2007), Roelants et al. (2007), Pyron (2010), and Inoue et al. (2010). Comparison of dates for the lissamphibians with previous molecular estimates are given in Table 1. Note that Wiens (2007) is not included, as crown group ages were fixed in that study.

The age of Gymnophiona + Eocaecilia (i.e., Apoda), 239.9 Ma (95% HPD = 184.3–297.7 Ma), is similar to inferred crown group ages from other studies (e.g., Roelants et al. 2007; Zhang and Wake 2009a, 2009b). The age of the extant crown group is much younger at 97.9 Ma (concordant with Marjanović and Laurin 2007), albeit with a very wide credible interval (95% HPD = 18.8–206.5 Ma). The age of the crown group Caudata, including Karaurus, is younger than estimates from most other researchers (e.g., San Mauro et al. 2005; Roelants et al. 2007) at 229.3 Ma (95% HPD = 176.5–290.6 Ma). The age of the frog stem group Salientia (Anura + Triadobatrachus) at 264.3 Ma (95% HPD = 245.0–289.9 Ma) is similar to estimates for the crown group Anura from previous studies (e.g., San Mauro et al. 2005; Roelants et al. 2007). However, the crown age of Anura, 225.5 Ma (95% HPD = 159.2–276.4 Ma), is more consistent with other previous molecular estimates (e.g., Roelants et al. 2007; Zhang and Wake 2009b; Pyron 2010) and stratigraphic evidence (e.g., Marjanović and Laurin 2007, 2008) given the basal placement of Triadobatrachus in the BEAST analyses (see Cannatella and Hillis 1993).

Although the 2-clock analysis also yielded good apparent convergence, the topology and dates for the ingroup nodes were unstable compared with the MrBayes trees and the 1-clock analysis. The placement of the extinct outgroups and stem tetrapods did not differ between the two analyses, and the lepospondyl affinities and monophyly of Lissamphibia were strongly supported (Pp > 0.95). However, weak support (Pp = 0.59) was estimated for a gymnophionan–caudatan relationship (i.e., Procera; Feller and Hedges 1998; Ruta and Coates 2007), which was also found by Vallin and Laurin (2004). The extinct species Triadobatrachus, Eocaecilia, and Karaurus were all placed sister to their respective crown groups. For Karaurus, support was moderate (Pp = 0.70), though this relationship is more consistent with the primitive character states of the taxon (Ruta and Coates 2007).

Though the root age is similar to the 1-clock analysis (399.7 Ma, 95% HPD = 390.1–409.5 Ma), internal node ages were uniformly younger (Table 1). Whether this represents a weak correlation between the molecular and morphological rates, or merely a lower amount of

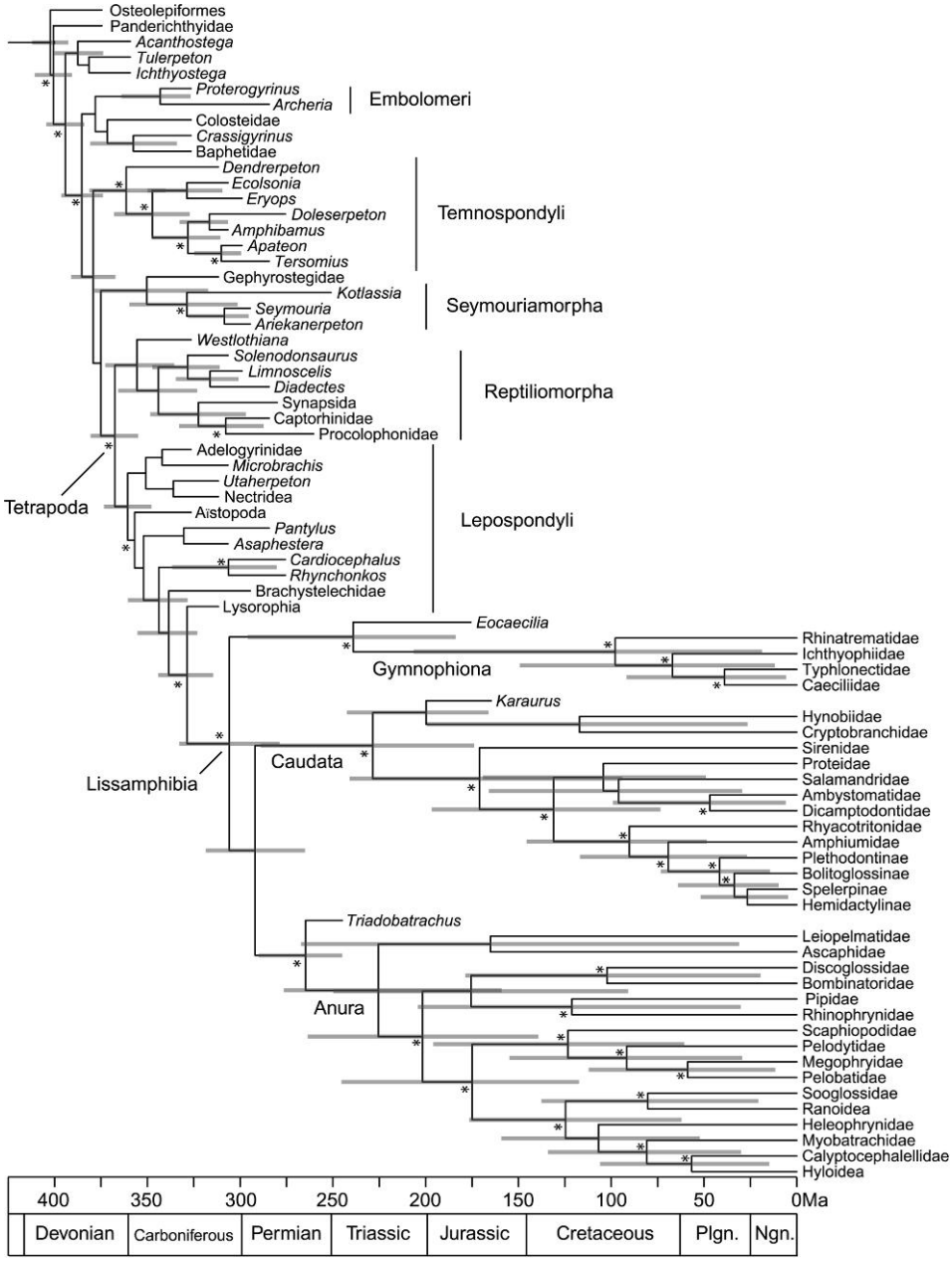

FIGURE 4. Dated chronogram with branch lengths equal to time in millions of years, produced using the combined morphological and molecular data set (Fig. 3) under the 1-clock model in BEAST. Bars at nodes represent the 95% HPD for the mean age based on 90 million post-burn-in generations. Nodes without bars are supported at Pp < 0.50. Nodes supported at Pp > 0.95 are indicated with an asterisk. Taxa present at time 0 are extant, whereas noncontemporaneous tips are extinct species. Tip dates taken from Benton (1993) and Laurin (2004) and are given in online Appendix A. The abbreviations 'Plgn.' and 'Ngn.' represent the Paleogene and Neogene, respectively.

phylogenetic signal in the morphological partition, is unclear. It does, however, illustrate the effect that the variable placement of fossils can exert on ingroup nodes while still constraining root divergences. This is particularly apparent for groups such as Caudata, where it should be noted that the posterior age distribution for this group was weakly bimodal in both analyses (1-clock and 2-clock), reflecting the potential alternative placements for Karaurus (see Lee et al. 2009; Ruane et al. 2011).

Missing Data, Branch Length Estimates, and Evolutionary Rates

None of the three missing data analyses revealed a significant relationship between taxon completeness

TABLE 1. Age estimates (Ma) for major lissamphibian crown groups

Reference	Lissamphibia	Batrachia	Gymnophiona	Anura	Caudata
1-Clock model	306 (279–333)	292 (265–319)	98 (19–206)	226 (159–276)	229 (174–289)
2-Clock model	290 (262–316)	270 (239–302)[a]	75 (40–110)	198 (157–238)	148 (108–190)
RAG-1					
San Mauro et al. (2005)	367 (328–417)	357 (317–405)	214 (177–256)	262 (223–305)	273 (238–312)
Hugall et al. (2007)	323 (304–342)	274 (253–295)	115 (99–131)	—	—
Pyron (2010)	338 (302–372)	306 (264–345)	125 (65–193)	243 (204–281)	218 (171–260)
Inoue et al. (2010)	380 (334–443)	367 (324–427)	213 (176–257)	267 (225–315)	276 (239–322)
Other data					
Zhang et al. (2005)	337 (321–353)	308 (289–328)	—	—	197 (176–219)
Marjanović and Laurin (2007)	267 (250–291)	246 (227–263)	—	—	180 (170–200)
Roelants et al. (2007)	369 (344–396)	358 (333–385)	226 (197–254)	243 (217–264)	249 (220–282)
Zhang and Wake (2009a, 2009b)	294 (271–319)[a]	264 (255–276)[a]	228 (195–160)[b]	—	183 (167–201)[a]
San Mauro (2010)	316 (292–343)	292 (263–321)	213 (178–246)	206 (172–239)	191 (157–225)

[a]Dates for the weakly supported Procera grouping for the 2-clock model for Batrachia.

and node support for the morphology-only data set ($P > 0.05$), the morphology supplemented with DNA ($P > 0.05$), or the combined data set ($P > 0.05$). Concordantly, terminal branch length was not significantly related to data proportion in terminal taxa for the morphology-only data set ($P > 0.05$). Terminal branch length was significantly negatively related to data proportion for the morphology + DNA ($r_s = -0.40$, $P = 0.005$), as both the mean and variance in branch length estimates were higher for the 41 extinct taxa than the eight extant taxa. However, the relationship was not significant for the combined data set ($P > 0.05$). This suggests that the significant relationship for the supplemented data set is an artifact of the imbalance in sample size between the extinct and extant taxa in that data set, as the mean branch length estimates were similar across all three data sets (<0.25 substitutions per site). In general, the combined data set greatly exceeds the dimensions of anomalous zones identified for inaccurate divergence time estimates based on branch length misestimation, with branches <1.0 substitutions per site, and total alignment length >1 kb (Schwartz and Mueller 2010).

Recent studies have suggested that missing data may mislead model-based phylogenetic analyses through the estimation of strongly supported but erroneous relationships and misestimation of branch lengths (Lemmon et al. 2009). However, it is important to note here that the topological estimates and support values are highly similar to nonmodel–based analyses of the morphological data (Vallin and Laurin 2004) and numerous independent molecular estimates of lissamphibian relationships based on data sets with almost no missing data (e.g., Zhang et al. 2005; Roelants et al. 2007; Zhang and Wake 2009a, 2009b). Finally, branch length estimates do not appear to be strongly influenced by missing data for extinct or extant taxa, and divergence time estimates for the extant ingroup nodes are broadly consistent with many of these studies (e.g., Zhang and Wake 2009b; San Mauro 2010). There do not appear to be any obvious pathological biases in the analyses presented here stemming from missing data in the extinct taxa.

Posterior estimates of α were strongly unimodal and greater than 1 for all MrBayes analyses (1.2–1.8), which is concordant with the estimates from the BEAST analyses of 1.92 (1-clock; 95% HPD = 1.37–2.53) and 1.74 (2-clock; 95% HPD = 1.27–2.27). In contrast, estimates of α from the prior distributions were evenly distributed on the uniform interval from 1 to 100, indicating strong signal for the posterior estimates. These values suggest that within-partition substitution rate heterogeneity is relatively low for the morphological data. Posterior values for mean relative rate multipliers in the MrBayes analyses ranged from 1.04 to 1.06, whereas the 1-clock BEAST estimate (this parameter was not included in the 2-clock analysis) was slightly higher, at 1.19 (95% HPD = 0.97–1.44). The mean values from the BEAST prior distribution was 2.76 (95% HPD = 0–9.51), again indicating strong signal from the data in the posterior distribution.

For the 2-clock analysis, the mean rates for the morphological and molecular data over all branches (total substitutions per site divided by total time) were of a similar magnitude, at 0.0053 (95% HPD = 0.0022–0.001) and 0.0012 (95% HPD = 0.001–0.0014), respectively, compared with 0.0016 (95% HPD = 0.0013–0.0018) for the 1-clock analysis. The mean per branch rates (the sum of the branch rates divided by the number of branches) were 0.012 (95% HPD = 0.0046–0.024) and 0.0013 (95%HPD = 0.0011–0.0016) for the morphological and molecular data, respectively, compared with 0.0035 (95% HPD = 0.0025–0.0048) for the 1-clock runs. Thus, although the morphological rates appear to be somewhat faster, they are not highly divergent from the molecular rates. This suggests that among-partition rate heterogeneity between the DNA and morphological data is not particularly high and that substitution rate dynamics are relatively proportional for both partitions across branches. However, among-branch heterogeneity in the rates of morphological evolution appears to be higher than for the molecular data, as evidenced by the difference in the mean and per branch rates.

Discussion

Phylogeny and Divergence Time Estimation Using Extinct Taxa

These results suggest that simultaneous inference of phylogeny and divergence times using extinct taxa can improve resolution and support not only for the placement of those taxa (e.g., Huelsenbeck 1991; Eernisse and Kluge 1993; Cobbett et al. 2007; Santini and Tyler 2004; Manos et al. 2007; Sauquet et al. 2009; Wiens 2009; Wiens et al. 2010) to test hypotheses regarding the historical origins of living group (e.g., Lee and Anderson 2006) but also for the estimation of internal node ages (e.g., Magallón 2010; Manos et al. 2007; Marjanović and Laurin 2007). Despite the molecular characters outnumbering morphological characters by a factor of 16, and 93.9% missing data for 55% of the taxa, the analyses presented here yield well-resolved, well-supported estimates of both phylogeny and divergence times for the lissamphibians and related stem tetrapods. The departure of the posterior densities from the prior distribution indicates that a strong signal is present in the ingroup for estimating both phylogeny and divergence times, whereas the estimated ages exhibit a much stronger fit with stratigraphic evidence than most previous molecular studies (see below).

I find concordance between the model-based Bayesian analyses of the combined data with previous parsimony analyses of the molecular data alone with respect to the placement of the extinct taxa (Vallin and Laurin 2004). This suggests that both the topological location of the extinct taxa and the branch length estimates are not significantly affected by the presence or addition of missing data cells. Additionally, I do not find a significant relationship between missing data and either node support or branch length. Although missing data may cause problems for model-based analyses, particularly when rates vary among partitions (e.g., Lemmon et al. 2009), simulations indicate that phylogenies based on both molecular and morphological data can be inferred with confidence even with a large proportion of missing cells (Wiens 2003, 2009).

This strategy does have some potential drawbacks. First, it is unclear how strong of a correlation between rates of morphological divergence and rates of molecular evolution exists (e.g., Smith et al. 1992; Omland 1997), and whether this can be used to parameterize a molecular clock for extant taxa. This method assumes a certain degree of proportionality between the morphological and molecular clocks. Hopefully, moderate differences in rate across data types can be dealt with by allowing relative rate variation or unlinking clocks across partitions. Here, relative rate multipliers and unlinked clocks for the molecular and morphological data suggest that overall rates do not differ drastically between partitions in this particular data set, though among-branch rate heterogeneity is somewhat higher for the morphological data.

However, the results from the 2-clock analysis appear to be unstable for the lissamphibian ingroup. Whether this is due to differences in among-branch differences in substitution patterns or simply due to a lack of phylogenetic signal in the morphological partition for robust independent rate estimates is unclear. This illustrates the need for increasing both taxonomic and character sampling in morphological data sets as well as careful consideration of model parameterization for phylogenetic and temporal inference. Ultimately some degree of correlation between morphological and molecular divergence will be necessary to yield accurate parameterization of node ages. However, even under a 1-clock model if the correlation is weak, the fossils included in the analyses still act as traditional minima on node ages, as the MRCA of taxa subtended by the node representing the fossil is thereby given a minimum age to calibrate the molecular clock for the nucleotide data. Thus, when phylogenetic signal is present in the morphological data, it should generally be possible to utilize such data for estimating divergence times.

Second, phylogenetic uncertainty in the placement of fossils can still affect age estimates (e.g., Lee et al. 2009) when these taxa are included in the primary analyses. For example, the variable placement of the extinct taxa *Triadobatrachus* and *Karaurus* in the MrBayes and BEAST analyses can have a strong effect on our interpretation of the crown group ages of the caudates and anurans (Table 1). If those fossils were highly nested within the crown groups, their ages would necessarily be substantially older than estimated here and elsewhere based on both stratigraphic and molecular evidence (see Marjanović and Laurin 2007, 2008). In particular, the placement of *Karaurus* sister to Cryptobranchoidea appears to be based on a single synapomorphy (a posteromedial vomerine tooth row; Laurin and Reisz 1997; Vallin and Laurin 2004). However, this source of variability is not unique to this dating strategy and also affects all previous fossil calibration methods (e.g., van Tuinen and Hedges 2004). Rigorous assessment of fossil placement continues to be a crucial concern in the estimation of divergence times (e.g., Lee et al. 2009; Sauquet et al. 2009; Ruane et al. 2011).

Finally, many ingroup nodes have fairly broad credible intervals (Fig. 4), many of which are not associated with lowered support or phylogenetic uncertainty (Lee et al. 2009). This likely results from the relatively broad "prior" on divergence times for the ingroup, stemming from the relative paucity of extinct ingroup taxa compared with the outgroups (Fig. 4). This suggests that increasing the number and interdigitation of crown group fossils will improve results (see Wiens 2009). Nodes that subtend both extinct and extant taxa, such as Lissamphibia, have much more precise credible intervals (Figs. 3 and 4). There are numerous other well-preserved amphibian species that can potentially be added to analyses such as this in the future (Estes 1981; Sanchiz 1998; Holman 2003, 2006; Marjanović and Laurin 2007; Evans et al. 2008), offering opportunities for expanding not only taxonomic sampling but character sampling as well.

Other projects such as MorphoBank (http://www.morphobank.org/) provide an increasingly detailed resource for gathering morphological data for phylogenetic analyses. The development of new methods for analyzing multistate data, such as the MSSD model of Alekseyenko et al. (2008), open further avenues for incorporating additional data into molecular divergence time analyses. Data such as nuclear introns (e.g., Creer et al. 2006) or chromosome number (e.g., Mayrose et al. 2010) may further increase the amount of information that can be used to parameterize molecular clocks and infer divergence time estimates. As the integration and expansion of paleontological and molecular data sets continues, it will be imperative that character sampling increases along with taxonomic sampling in order to yield the highest phylogenetic accuracy.

Origin and Diversification of Lissamphibia

The results of this study corroborate the previous results of Vallin and Laurin (2004) and Marjanović and Laurin (2007) in strongly supporting a lepospondyl–lissamphibian relationship. This is in contrast to previous studies finding support for either a temnospondyl–lissamphibian grouping (e.g., Zhang et al. 2005; Ruta and Coates 2007) or a polyphyletic origin of the group (Lee and Anderson 2006). Previous phylogenetic analyses have inferred a temnospondyl–lissamphibian grouping from data sets including extinct taxa (e.g., Ruta and Coates 2007) but utilized only morphological characters and did not use model-based phylogenetic analyses. Although the use of alternative data sets may often produce different results (see Schoch and Milner 2004), the sampling of extant taxa in studies such as Anderson et al. (2008) is not conducive to the analysis presented here. In the future, it will be important for morphological analyses to include not only large numbers of characters but also as many extant and extinct taxa as possible to allow for combined evidence phylogenetic inference (Wiens 2009; Wiens et al. 2010; Ruane et al. 2011).

A primary point of interest in this study is the comparison of the estimated dates with previous molecular and stratigraphic studies (Table 1). Unless indicated, I discuss ages from the 1-clock analysis. The 2-clock analyses, which putatively have the most accurate placement of the extinct lissamphibian ingroup taxa, yield dates that are highly concordant with other stratigraphic estimates (e.g., Marjanović and Laurin 2007, 2008). However, the ages for more recent clades may be untenably young based on what is known from other studies of these groups based on both molecular phylogenetic analysis and historical biogeography (e.g., Bossuyt et al. 2006; Wiens 2007).

For the 1-clock analysis, the inferred crown group age for the lissamphibians (1-clock: 305.5 Ma and 2-clock: 290.4 Ma; Fig. 4) is similar to the molecular results of Zhang and Wake (2009b) and San Mauro (2010) and the combined molecular and paleontological data presented by Marjanović and Laurin (2007, 2008). This estimate is much younger than most previous molecular studies utilizing fossils as post hoc constraints (e.g., San Mauro et al. 2005; Zhang et al. 2005; Roelants et al. 2007; Inoue et al. 2010). However, a Carboniferous–Permian origin of the lissamphibians (1-clock 95% HPD = 278–332 Ma and 2-clock 95% HPD = 262–316 Ma) is far more consistent with the known stratigraphic record of the lissamphibians and associated stem groups (e.g., Lepospondyli, Temnospondyli; Marjanović and Laurin 2007, 2008) than older estimates ranging up to 360–380 Ma (e.g., San Mauro et al. 2005; Roelants et al. 2007; Inoue et al. 2010). Earlier Carboniferous or Devonian dates for the extant lissamphibians would predate all known crown group amphibian fossils (Marjanović and Laurin 2007) and would likely only be well supported by the fossil record under a scenario of lissamphibian polyphyly (Lee and Anderson 2006), which is strongly rejected by most molecular studies (e.g., Hugall et al. 2007; Zhang and Wake 2009b), including this one.

The divergence of Batrachia, the split between Anura and Caudata (292 Ma), is concordant with several previous studies (e.g., Zhang et al. 2005; Hugall et al. 2007) and much younger than many prior molecular estimates (e.g., San Mauro et al. 2005; Roelants et al. 2007; Inoue et al. 2010). This is also somewhat older than the stratigraphic ranges suggested by Marjanović and Laurin (2007) and the molecular estimates of Zhang and Wake (2009b) based on those ranges (∼265 Ma). This discrepancy may be due to the differential placement of Karaurus, which is placed inside the crown group Caudata in these analyses, sister to Cryptobranchoidea. Nevertheless, estimates for the crown group age of salamanders (Caudata; 227 Ma) are younger than many previous studies (e.g., San Mauro et al. 2005; Roelants et al. 2007; Inoue et al. 2010) but slightly older than others (e.g., Zhang et al. 2005), including both stratigraphic and molecular dates (∼155–180 Ma; Marjanović and Laurin 2007; Zhang and Wake 2009b). Finally, the age for the origin of extant caecilians (Gymnophiona; 98 Ma) is significantly younger than most other recent estimates (e.g., San Mauro et al. 2005; Roelants et al. 2007; Zhang and Wake 2009a), though remarkably consistent with the oldest putative crown group gymnophionan, which dates from the late Cretaceous (Marjanović and Laurin 2007). Interestingly, the stem group age for this group (Apoda; Cannatella and Hillis 1993) at 239 Ma is consistent with the crown group age estimates from Roelants et al. (2007) and Zhang and Wake (2009b).

The estimates presented here are uniformly younger than the majority of previous molecular studies, most of which utilized internal node age constraints based on the post hoc application of fossil dates. In contrast, these ages are generally highly concordant with both stratigraphic consensus estimates (Marjanović and Laurin 2007, 2008), and some molecular studies (e.g., Zhang and Wake 2009b; San Mauro 2010), despite lacking any similarity in calibration strategy. These estimates are based solely on information gained by the inclusion of

fossil species as terminal taxa, extracted from calibration of internal node ages subtending the extinct taxa. This stratigraphic concordance is particularly important, as the known fossil record provides the only objective metric for assessing the likelihood of molecular divergence time estimates (Pyron 2010; Ruane et al. 2011). Although the results of Zhang and Wake (2009b) and San Mauro (2010) suggest that a high degree of integration between molecular and paleontological data can result in robust age estimates from a molecular analysis, the inclusion of extinct species in phylogenetic inference potentially allows for the removal of a final layer of potential idiosyncracy in the application of fossil dates for divergence time estimation.

Conclusions

The prevailing use of fossils as minimum or maximum ages for internal nodes has previously been shown to have a strong potential for error when not based on an explicit phylogenetic analysis containing both extinct and extant taxa. When sufficient morphological data are available for both fossil and extant taxa, combining these data using a strategy such as the one described here will likely be preferable to previous analytical frameworks using fossils as age constraints. It employs a larger amount of data and more realistically accounts for the phylogenetic placement of extinct taxa while yielding phylogenetic and temporal results exhibiting higher stratigraphic concordance than many previous studies. This method is dependent on the assumption that the morphological phylogenetic analysis is accurate. As morphological data sets become larger and more complete, concerns about accuracy should be alleviated to some extent based on the apparent power with which extinct taxa can be placed and the apparent positive effect they can have on combined evidence phylogenetic inference. Inferring phylogenies and divergence times using both extinct and extant taxa in a single analysis represents another step toward a unified time-calibrated tree of life.

Supplementary Material

Supplementary material, including data files and/or online-only appendices, can be found at http://www.sysbio.oxfordjournals.org/ and in the Dryad data repository (doi:10.5061/dryad.8120).

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APPENDIX

TABLE A1. GenBank accession numbers for exemplar taxa used in molecular analyses

Clade	Species	Accession number
Ambystomatidae	*Ambystoma mexicanum*	EF551561
Amphiumidae	*Amphiuma pholeter*	AY650128
Ascaphidae	*Ascaphus truei*	AY323754
Bolitoglossinae	*Pseudoeurycea rex*	AY650125
Bombinatoridae	*Bombina orientalis*	AY323756
Caeciliidae	*Dermophis mexicanus*	AY650148
Calyptocephalellidae	*Calyptocephalella gayi*	AY583337
Cryptobranchidae	*Cryptobranchus alleganiensis*	AY650141
Dicamptodontidae	*Dicamptodon copei*	AY691695
Discoglossidae[a]	*Discoglossus galganoi*	AY583338
Heleophrynidae	*Heleophryne regis*	AY323764
Hemidactylinae	*Hemidactylium scutatum*	AY691711
Hyloidea	*Litoria ewingii*	EF551562
Hynobiidae[a]	*Hynobius nebulosus*	AY650144
Ichthyophiidae[a]	*Ichthyophis glutinosus*	EF551563
Leiopelmatidae	*Leiopelma hochstetteri*	AY583342
Megophryidae	*Megophrys sp.*	AY323760
Myobatrachidae	*Lechriodus melanopyga*	AY583341
Pelobatidae	*Pelobates cultripes*	AY323758
Pelodytidae	*Pelodytes punctatus*	AY583343
Pipidae[a]	*Xenopus laevis*	L19324
Plethodontinae	*Kersinia koreana*	AY887135
Proteidae[a]	*Proteus anguinus*	AY650138
Ranoidea	*Rana sylvatica*	DQ019511
Rhinatrematidae[a]	*Rhinatrema bivittatum*	EF551564
Rhinophrynidae	*Rhinophrynus dorsalis*	AY874302
Rhyacotritonidae	*Rhyacotriton kezeri*	AY650129
Salamandridae	*Pleurodeles waltl*	AJ010258
Scaphiopodidae	*Scaphiopus couchii*	AY323759
Sirenidae[a]	*Siren intermedia*	AY650140
Sooglossidae	*Nesomantis thomasseti*	AY323778
Spelerpinae	*Stereochilus marginatus*	AY691713
Synapsida[a]	*Homo sapiens*	M29474
Typhlonectidae	*Typhlonectes natans*	EF551566

[a]The presence of that taxon in the morphological matrix.