

A Likelihood Method for Assessing Molecular Divergence Time Estimates and the Placement of Fossil Calibrations

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Abstract.—Estimating divergence times using molecular sequence data has become a common application of statistical phylogenetics. However, disparities between estimated ages for clades produced in different studies have become equally commonplace. Here, I propose a method for the objective assessment of the likelihood of inferred divergence times to evaluate the placement of fossil constraints using information from the broader fossil record. The inclusion of nodes from the Tree of Life for which credible age ranges are known, in addition to the fossil constraints used in the ingroup, will allow for the comparison of alternate fossil placements when the phylogenetic affinity of a fossil is ambiguous as well as provide a heuristic assessment of the global likelihood of estimated divergence times. The use of these “likelihood checkpoints” will allow for the comparison of inferred dates across data sets and across taxonomic groups to place divergence time estimates into a broader evolutionary timescale. The method is illustrated with an example using an expanded phylogenetic estimate of the Gnathostomata, inferred with relaxed-clock molecular dating methods. [Divergence time estimation; fossil calibrations; maximum likelihood; molecular phylogenetics; relaxed clocks.]

Divergence time estimation has emerged as one of the most useful extensions of phylogenetic inference (Renner 2005; Donoghue and Benton 2007). Although the assumption of a strict molecular clock rarely holds (Wu and Li 1985; Britten 1986), relaxed-clock methods can now provide robust chronograms, allowing researchers to answer numerous questions about the evolutionary history of organisms that require estimates of node ages or rates of molecular evolution (see Donoghue and Benton 2007). However, while individual studies each contain a particular ingroup and molecular data set, all represent components of a single unified Tree of Life (Benton and Ayala 2003). Therefore, divergence time estimates for particular groups do not exist in isolation, as inferred dates have broader implications about the timing of the evolution of all living organisms. While the fossil record may be scanty or nonexistent for some lineages and many crown groups, it provides information on the timing of the appearance of many major groups of organisms, particularly vertebrates (Benton 1990; Benton and Donoghue 2007). However, any study of divergence times can only reasonably incorporate a small fraction of this information, discarding the rest due to issues of computational intensity and the availability of molecular data.

This lack of complete calibration information can result in decreased precision for inferred dates when the temporal space in which divergence time estimates can vary is allowed to be excessively large, when in reality it should be constrained to a far narrower timescale. Absolute constraints on divergence time estimates (i.e., 4570 and 0 Ma) rarely enter into consideration. Thus, without broader calibration from the Tree of Life, the age of a clade is basically free to vary across any timescale defined by the fossil constraints. An extreme example

is the inferred age for the arthropod–nematode divergence given by Wang et al. (1999) at 1167 Ma, with a 95% confidence interval (CI) containing the formation of the universe in the big bang (Graur and Martin 2004).

The temporal interval in which the age of a clade may vary is defined not only by the fossil history of the clade itself, but also by the entire known fossil record and the geological history of the Earth. The use of fossils as minima for nodes with no or only broad maxima tends toward systematic overestimation of divergence times (Hugall et al. 2007), a phenomenon that has been addressed on an ad hoc basis by authors working with specific groups (e.g., Hugall and Lee 2004; Brown et al. 2007; Kitazoe et al. 2007; Marjanovic and Laurin 2007). Divergence times that are significantly older than that indicated by the fossil record, implying a huge temporal gap in fossilization, make one of two concrete statements. Either the root of the Tree of Life (or at least the higher level crown group) is far older than the fossil record and the majority of molecular divergence time studies have previously indicated, or the majority of divergences happened relatively early, placing extant taxa on very long branches. Such ancient dates would also posit that the majority of fossil taxa are members of crown groups, closely allied with modern organisms. Both these predictions are testable using available data.

Here, I propose an a posteriori method for evaluating the temporal likelihood of divergence time estimates for crown groups and an application for assessing alternative fossil calibration placements. The latter is an especially large contributor of variance to inferred divergence time estimates, and methods for evaluating fossil placement have been an active area of research (Near and Sanderson 2004; Near et al. 2005;

Rutschmann et al. 2007; Marshall 2008). Varying the placement of fossil calibrations can greatly affect inference of divergence times (Near and Sanderson 2004; van Tuinen and Hedges 2004; Rutschmann et al. 2007); thus, a method that allows one to objectively assess the likelihood of divergence time estimates and/or the placement of ambiguous constraints would be particularly useful. This is not merely a theoretical concern as authors often present several possible sets of divergence times based on alternate fossil placements (e.g., van Tuinen and Hedges 2004), occasionally going so far as to show multiple alternative chronograms and their attendant biogeographic implications based on different fossil calibration strategies (e.g., Zhang et al. 2008).

MODEL

Background

A comprehensive reading of the fossil record yields a basic framework of credible minimum and maximum dates for a number of major nodes in the Tree of Life (see <http://www.fossilrecord.net>; Benton and Donoghue 2007; Donoghue and Benton 2007; Table 1). If these nodes for which credible age ranges are known are present and unconstrained in a phylogeny, we can probabilistically assess divergence time estimates, and thus fossil calibration placement, based on the likelihoods of the inferred ages of those nodes. This will allow for the assessment of the likelihood of estimated divergence times and the placement of fossil calibrations, calculated using the probability densities of the estimated ages for the bounded nodes, regardless of the type of data or computational method used to generate the estimated dates.

Notation and Parameters

A dated chronogram typically consists of the observed number of substitutions along branches (the product of rate and time) and a set of fossil calibrations on the tree (F_t), from which the set of rates and times comprising the sum total of branch lengths are calculated. This yields an ultrametric tree (T), with branch lengths equal to time, yielding divergence time estimates for every node in the phylogeny. Various algorithms have been implemented for these calculations, allowing rates of evolution to be constant (i.e., a strict clock, Zuckerkandl and Pauling 1962), autocorrelated through time (Thorne et al. 1998; Sanderson 2002) or uncorrelated (Drummond et al. 2006). In addition to nodes on which fossil constraints have been placed, a set of additional nodes from the Tree of Life for which a temporal scale is known (sensu Benton and Donoghue 2007) will be present in the phylogeny or can be added through the inclusion of more taxa. These nodes, here dubbed "likelihood checkpoints," will be denoted N_t , consisting of V nodes, $N_{t(i)}$.

The actual location of these nodes (i.e., in the ingroup or as outgroups) is irrelevant, provided that their age bracketing can be robustly justified. If the ages of the nodes comprising N_t encompass a range of possible dates, they can be represented by probability distributions denoted $P(N_{t(i)})$. Thus, the likelihood of a chronogram T given the fossil constraints F_t can be assessed by calculating the joint probability densities of the inferred ages for the likelihood checkpoints, $\hat{N}_{t(i)}$, taken from T :

$$L(T|F_t) = \prod_{i=1}^V P(\hat{N}_{t(i)}). \quad (1)$$

TABLE 1. Putative oldest known fossils for selected extant clades of sarcopterygians

Clade	Fossil	Stratum	Age (Ma)	Reference
Coelacanthomorpha	<i>Eoactinistia foreyi</i>	Devonian (Praghatian/Emsian)	407	Johanson et al. (2006)
Dipnomorpha	<i>Styloichthys changae</i>	Devonian (Lochkovian/Praghatian)	411.2	Zhu and Yu (2002)
Tetrapodomorpha	<i>Kenichthys campbelli</i>	Devonian (Emsian)	397.5	Müller and Reisz (2005)
Lissamphibia	<i>Gerobatrachus hottoni</i>	Permian (Leonardian)	290	Anderson et al. (2008)
Salia	<i>Triadobatrachus massinoti</i>	Triassic (Scythian)	245	Rage and Røcek (1989)
Anura	<i>Eodiscoglossus oxoniensis</i>	Jurassic (Bathonian)	164.7	Evans et al. (1990)
Caudata	<i>Karaurus sharovi</i>	Jurassic (Tithonian)	150.8	Ivakhnenko (1978)
Gymnophiona	<i>Eocaecilia micropodia</i>	Jurassic (Sinemurian)	189.6	Jenkins and Walsh (1993)
Amniota	<i>Hylonomus lyelli</i>	Carboniferous (Vereiskian)	309.2	Dawson (1863)
Mammaliaformes	<i>Hadrocodium wui</i>	Jurassic (Sinemurian)	195	Luo et al. (2001)
Prototheria	<i>Steropodon galmani</i>	Cretaceous (Albian)	110	Archer et al. (1985)
Metatheria	<i>Sinodelphys szalayii</i>	Cretaceous (Barremian)	125	Luo et al. (2002)
Eutheria	<i>Eomaia scansoria</i>	Cretaceous (Barremian)	125	Ji et al. (2002)
"Reptilia"	<i>Eudibamus cursoris</i>	Permian (Asselian)	294.6	Berman et al. (2000)
Testudines	<i>Odontochelys semitestacea</i>	Triassic (Carnian)	220	Reisz and Head (2008)
Archosauriformes	<i>Euparkeria capensis</i>	Triassic (Anisian)	243	Müller and Reisz (2005)
Aves	<i>Archaeopteryx lithographica</i>	Jurassic (Kimmeridgian)	150.8	Von Meyer (1861)
Neognathae	<i>Neogaornis wetzeli</i>	Cretaceous (Maastrichtian)	70	Lambrecht (1929)
Paleognathae	<i>Diogenornis fragilis</i>	Paleocene (Thanetian)	55.8	Alvarenga (1983)
Crocodylia	<i>Effigia okeeffeae</i>	Triassic (Rhaetian)	210	Nesbitt (2007)
Rhynchocephalia	<i>Brachyrhinodon taylori</i>	Triassic (Carnian)	228	Huene, 1910; Benton (1990)
Lacertilia	<i>Tikiguania estesi</i>	Triassic (Carnian)	216.5	Datta and Ray (2006)
Iguania	<i>Bharatagama rebbenensis</i>	Jurassic (Lias)	175.6	Evans et al. (2002)
Serpentes	<i>Lapparentophis defrennei</i>	Cretaceous (Barremian)	130	Hoffstetter, 1959; Rage (1987)
Alethinophidia	<i>Haasiophis terrasanctus</i>	Cretaceous (Cenomanian)	95	Tchernov et al. (2000)

Note: This list is not intended to be comprehensive; groups were included that have fossils dating at least to the early Cenozoic. Ages given are either the age reported by the primary author or the lower bound of the stratum in which the fossil was found. No precision is intended by the listing of a single age without error. Fossils should be interpreted as stem group members of the indicated clade.

Thus, given n different sets of divergence time estimates/chronograms ($T_{(1)}, T_{(2)}, \dots, T_{(n)}$), which are based on n alternative fossil calibration strategies ($F_{t(1)}, F_{t(2)}, \dots, F_{t(n)}$), we can objectively choose between them based on the likelihoods of the estimated ages $\hat{N}_{t(i)}$ of the likelihood checkpoint nodes. In essence, we are choosing the fossil calibrations and resulting divergence time estimates that fit best into the global framework of the known fossil record, with the likelihood checkpoints forming a sort of “meta-prior” on the divergence time estimates. For this model, we will use a lognormal distribution with the min and max ages from the fossils as the bounds of the 95% CI to parameterize $P(N_{t(i)})$. This has several attractive features for divergence time estimation, as noted in Drummond et al. (2006). The use of the min and max ages as the 95% CI allows for enforcement of the fossil ages as soft rather than hard bounds (Yang and Rannala 2006), allowing for incorporation of error around the dating and stratigraphic assignment of the fossils themselves. The parameters of the lognormal distribution are given as:

$$\bar{N}_{t(i)} = \frac{\ln(F_{\min}) + \ln(F_{\max})}{2}, \quad \sigma_{t(i)} = \frac{\ln(F_{\max}) - \ln(F_{\min})}{3.92}, \quad (2a,b)$$

where F_{\min} and F_{\max} are the minimum and maximum fossil ages. Thus, given $\bar{N}_{t(i)}$ and $\sigma_{t(i)}$, we may define the lognormal $P(\hat{N}_{t(i)})$ as:

$$P(\hat{N}_{t(i)}) = \frac{e^{-(\ln \hat{N}_{t(i)} - \bar{N}_{t(i)})^2 / (2\sigma_{t(i)}^2)}}{\hat{N}_{t(i)} \sigma_{t(i)} \sqrt{2\pi}}. \quad (3)$$

For cases in which the fossil history is too fragmentary for justifiable bounds to be placed around nodes, the model can be parameterized to utilize a single minimum bound as a likelihood checkpoint. This may be helpful in cases where the most useful fossil information is the first appearance of the group in the fossil record (the oldest known fossil), used to calibrate the divergence between a clade and its sister group. In this case, $P(\hat{N}_{t(i)})$ may be defined using an exponential distribution with mean β , equal to the fossil age. This has the drawback of creating a very flat likelihood surface due to the long tail of the exponential distribution, where the inferred likelihoods of even drastically different dates may not be very different. However, the exponential distribution allows for the use of a single minimum bound without the error estimate that a lognormal distribution would require, which will be necessary when the fossil record for a group is sparse. $P(\hat{N}_{t(i)})$ is thus given by:

$$P(\hat{N}_{t(i)}) = \frac{1}{\beta} e^{-\hat{N}_{t(i)}/\beta}. \quad (4)$$

These likelihood checkpoints should ideally be distributed throughout the tree, associated with constraints at as many different temporal scales as possible. This is necessary to avoid potential bias arising as a tendency to select fossil constraints that are closer to individual

checkpoints, rather than ones that produce overall more likely date estimates. Evaluating the comparative likelihood of multiple dated chronograms, alternative fossil placements, and/or several potential calibration sets will then be relatively straightforward.

The Akaike information criterion (AIC; Akaike 1974) is often used to compare the relative goodness of fit of models of evolution (e.g., Felsenstein 1981); here, we will use the AIC to evaluate alternative divergence time estimates. The number of free parameters (k) for the alternative trees will be the number of fossils that differ in location between the sets of fossil calibrations. The variance contributed to \hat{N}_t by the shared fossil constraints is theoretically a consistent parameter across the multiple calibration sets, and thus, the identical constraints do not represent independent observations of an unknown variable. However, the unique fossils represent variable parameters for the estimation of $L(T|F_t)$. Given the potential nonequivalence of parameter estimates among different calibration sets, this use of the AIC can provide an initial metric by which to choose fossils or fossil placement but should be considered an approximation. Alternatively, one could employ the criterion of Edwards (1992), interpreting a difference of two likelihood units as a significantly better fit, or simply choose the calibration set or placement with the highest likelihood. These two approaches ignore the possibility of excess parameterization; however, this may not be a concern with smaller numbers of constraints.

Advantages, Assumptions, and Limitations

The primary assumption made is that the known fossil record of the ingroup or the immediate outgroups provides a sufficiently numerous and robust framework for the simultaneous use of fossils as likelihood checkpoints and testable calibrations. While this is not a limitation of the method itself, but rather of the quality of the known fossil record, it may pose challenges for the standardization of likelihood checkpoints for certain groups. This assumption may be valid for many groups with detailed fossil records (such as snakes and turtles; Rage 1987; Near and Sanderson 2004), whereas groups with more sparse fossil data may be forced to rely on deeper outgroup checkpoints to test ingroup calibrations, without a clear choice between checkpoints and calibrations. This also highlights the importance of increasing the availability and synthesis of the fossil record with evolutionary and phylogenetic studies of extant taxa through efforts such as <http://www.fossilrecord.net>. Other potential limitations include the broad likelihood surface introduced by the use of the exponential distribution, which may be unavoidable if a particular group lacks a detailed enough fossil record to place the upper and lower bounds on checkpoints as required by the lognormal distribution.

This method allows for the statistical evaluation of the placement of phylogenetically ambiguous fossils on different nodes. The use of some fossil information

as likelihood checkpoints rather than as constraints in the primary dating analysis may be desirable for two reasons. First, the addition of more fossil constraints may do nothing to highlight or combat improperly assigned calibrations because dates inferred using both “good” and “bad” fossils will necessarily be a compromise between the two. The effect of poorly fit fossils is not mitigated (at least not entirely) by the addition of more constraints (e.g., Near et al. 2005; Rutschmann et al. 2007). Rather than choosing the set of constraints that is most internally consistent (which is not necessarily indicative of accurate placement), the fossil calibration strategy that has the highest overall likelihood relative to the known fossil record can be chosen. Second, this method allows for the use of fossils as likelihood checkpoints that may not have a clear application as fossil constraints for dating. For instance, the origin of Lissamphibia in relation to the extinct Temnospondyli and Lepospondyli is contentious (Zhang et al. 2005; Marjanovic and Laurin 2007; Anderson et al. 2008), possibly rendering the use of fossils such as *Doleserpeton* and *Gerobatrachus* inappropriate for calibrating divergences between extant lissamphibian groups. However, the range from the oldest known hypothetical lissamphibian ancestors (Temnospondyli, ca. 355 Ma) to the putative stem batrachians *Gerobatrachus* (290 Ma; Anderson et al. 2008) provides a credible interval within which possible root times for Lissamphibia may be assessed without enforcing a particular phylogenetic hypothesis through the application of fossil constraints.

The flexibility of this model also allows for the incorporation of broader fossil calibration information into divergence time estimation without the need for full integration of that information into the primary analyses. In some cases, such as the example presented below, there may be enough well-supported fossil constraints and bounded nodes that may reasonably be withheld as checkpoints. If a set of calibrations yields accurate dates for checkpoint nodes in the absence of any actual constraints on those nodes, then the use of checkpoints will allow for the assessment of the likelihood of age estimates with little compromise in variance of inferred ages. In such a scenario, excluding some calibrations as checkpoints increases in our confidence in the robustness and validity of our calibration set and divergence time estimates, with little apparent decrease in accuracy or precision.

In many instances, the set of potential calibrations may not be large or varied enough to allow for the complete exclusion of some calibrations as checkpoints. If this is the case, the most robust application of this method, and the one potentially most likely to occur in practice, is to withhold the most confident calibrations as checkpoints to test the placement of other fossils and the overall likelihood of the divergence time estimates and then reemploy them as constraints in the final analysis. Such an approach may also avoid problems of circularity in consistently relying on certain fossils as checkpoints rather than as constraints and potential bias

arising from a preferential selection of fossil constraints closer to checkpoints. The most robust fossil calibrations are often constraints on the root of the tree; this is also where the application of a checkpoint is likely to be effective. Thus, researchers can easily test the placement of internal (i.e., ingroup) fossil calibrations using a root fossil as a checkpoint and then perform a final analysis employing all constraints, including the root fossil. Such an application of the method would provide an objective parametric assessment of molecular divergence time estimates, which should ultimately increase accuracy and precision.

EMPIRICAL APPLICATION

Divergence Time Estimates for Gnathostome Vertebrates

To illustrate this method on a large phylogeny with multiple potential fossil calibrations, I assembled a data set of the major gnathostomatan lineages, building on the RAG-1 data set of Hugall et al. (2007; hereafter HEA) with additional taxon sampling in Tetrapoda for more precise placement of fossil constraints. The data set comprises 129 taxa and 2619 bp. For deep-time likelihood assessment of alternate calibration topologies, two outgroups were added: a ray-finned fish (Actinopterygii: *Oncorhynchus mykiss*) as the sister group to Sarcopterygii and a cartilaginous fish (Chondrichthyes: *Negaprion brevirostris*) as the sister group to Osteichthyes (Supplementary Appendix 1, available from <http://www.sysbio.oxfordjournals.org/>). The goal was to compare two alternate sets of potential fossil calibrations.

Fossil Calibration and Likelihood Checkpoints

I considered two alternative sets of fossil calibrations, with a single constraint shared between the sets. The four well-constrained points proposed by Müller and Reisz (2005; hereafter MAR) were tested against the five constraints employed by HEA (Table 2). The point here was not specifically to reanalyze the results of HEA. The use of likelihood checkpoints supports fossil calibrations other than the ones employed by HEA; however, the results obtained with the more likely calibrations

TABLE 2. Two alternative sets of fossil constraints for Gnathostomata

Node	Calibration age (Ma)
Five Constraints (HEA)	
H1 Archosauromorpha	243–251
H2 Amniota	300.5–330.1
H3 Eutheria	71.2–113
H4 Trionychoidea	99.6–112
H5 <i>Heloderma–Elgaria</i>	93.5–112
Four Constraints (MAR)	
C1 <i>Alligator–Caiman</i>	66–71
C2 Archosauromorpha	243–251
C3 Reptilia	252–257
C4 Sarcopterygii (Tetrapodomorpha)	408–419

Note: Constraints are labeled on Figure 1.

are overall fairly consistent with those of HEA, with a few notable exceptions (see below), whereas those obtained using the constraints of HEA are considerably older. This is likely due to differences between relaxed-clock divergence dating in programs such as BEAST versus the penalized likelihood (PL) approach used by HEA, and their use of fixed node ages rather than probabilistic ranges. Although it is possible to directly compare divergence time estimates produced by any method using this approach I wish to illustrate the more direct application of choosing between different fossil constraints in a single analytical framework. Thus, for clarity, I have produced new analyses using the fossil constraints from HEA and MAR, avoiding direct comparison to the actual results of HEA.

A primary point of interest in this analysis is the comparison of ranged calibrations on nodes early in the tree, such as for Amniota. Although several authors have raised potential concerns about the use of this constraint at approximately 315 Ma (e.g., Graur and Martin 2004; Reisz and Müller 2004), it is still commonly employed in divergence time analyses of tetrapods (e.g., Zhang et al. 2005; Hugall et al. 2007). No direct tests of the accuracy of this constraint have been performed using alternative fossil calibrations in a quantitative framework. The rest of the constraints employed by HEA are fairly unambiguous and should have little effect on divergence time estimates. Eight fossil ranges were used as likelihood checkpoints on labeled nodes (lognormal distribution unless otherwise stated; Table 3; Fig. 1).

The program BEAST v1.4.6 (Drummond and Rambaut 2007) was used for divergence time estimation under the uncorrelated lognormal relaxed-clock model with a Yule process prior on speciation (Drummond et al. 2006). A General Time Reversible model with gamma-distributed rate heterogeneity and a proportion of invariant sites (GTR + Γ + I) partitioned by codon position was used, as per HEA. Fossil calibrations were enforced as lognormal priors on the time of the most recent common ancestor of the taxa subtended by the constraint. Analyses were run for 20 million generations, with the first 5 million discarded after calculating burn-in. Convergence and chain length were assessed by assuring that the estimated sample sizes for all parameters were greater than 200 (Drummond et al. 2006), which occurred prior to 5 million generations in all runs. Runs were replicated several times to ensure that global stationarity had been reached and that

individual analyses were not merely converging on local optima.

RESULTS

The topologies inferred from both analyses were identical to each other and to the topology recovered by HEA with respect to the relationships between the major clades. The dates inferred from the analysis employing the five constraints of HEA are on average significantly older than the those inferred using the four constraints from MAR (Table 4). This effect becomes more pronounced toward the root of the tree; the difference between the five constraint and four constraint node ages is significantly positively correlated with the age of the nodes ($r^2 = 0.72$, $P < 0.00001$). The results from the likelihood checkpoints (Table 5) indicate a significantly better fit of the four constraint (MAR) calibration set ($-\ln L = -72.64$, $AIC = 151.3$) to the broader fossil record than the five constraint (HEA) calibration set ($-\ln L = -328.99$, $AIC = 666.0$).

In addition to demonstrating a new method for evaluating divergence time estimates and fossil calibration placement, this study represents the first analysis of the timing of the divergence of the major Gnathostomatan lineages using relaxed-clock methods, which infer divergence times directly from the sequence alignment and generate error estimates automatically. The divergence between the Osteichthyes and Chondrichthyes lineages dates to 526 Ma, the Early/Middle Cambrian. Despite the robustness of the calibration set, any confidence placed in this date is tenuous at best. Early vertebrate fossils such as the putative agnathans *Haikouichthys* and *Myllokunmingia* are, however, known from older Cambrian strata (Shu et al. 1999). The divergence between Osteichthyes and Sarcopterygii dates to the Middle/Late Ordovician boundary; fossils of the putative stem gnathostomatan lineage Thelodonti are known from the same time period (Sansom et al. 1996). Regarding extant sarcopterygian clades of disputed age, support is found for an older Lissamphibia, approximately 338 Ma, consistent with the results of Zhang et al. (2005), but older than the dates suggested by Marjanovic and Laurin (2007). Within the Lepidosauria, the divergence between Rhynchocephalia and Squamata dates to 235 Ma, remarkably consistent with the oldest known Rhynchocephalians (ca. 228 Ma; Benton 1990). The radiation of Eutheria dates to 115 Ma, with stem eutherian

TABLE 3. Likelihood checkpoints for nodes based on credible age ranges from fossil bracketing

Node	Lower bound	Upper bound
Testudines	220 Ma (<i>Odontochelys</i> ; Reisz and Head 2008)	—
Gnathostomata	460.9 Ma (Thelodonti; Sansom et al., 1996)	530 Ma (Agnatha; Shu et al. 1999)
Lepidosauria	228 Ma (<i>Brachyrhinodon</i> ; Benton 1990)	—
Lissamphibia	290 Ma (<i>Gerobatrachus</i> ; Anderson et al. 2008)	355 Ma (Temnospondylia; Marjanovic and Laurin 2007)
Mammalia	162.5 Ma (Donoghue and Benton 2007)	191.1 Ma (Donoghue and Benton 2007)
Serpentes	130 Ma (<i>Lapparentophis</i> ; Rage 1987)	—
Teleostomi	421.57 Ma (Actinopterygii; Märss 2001)	460.9 Ma (Thelodonti; Sansom et al. 1996)
Tetrapoda	330.3 Ma (Donoghue and Benton 2007)	350.1 Ma (Donoghue and Benton 2007)

Note: Checkpoints without upper bounds used the exponential function (Equation 4).

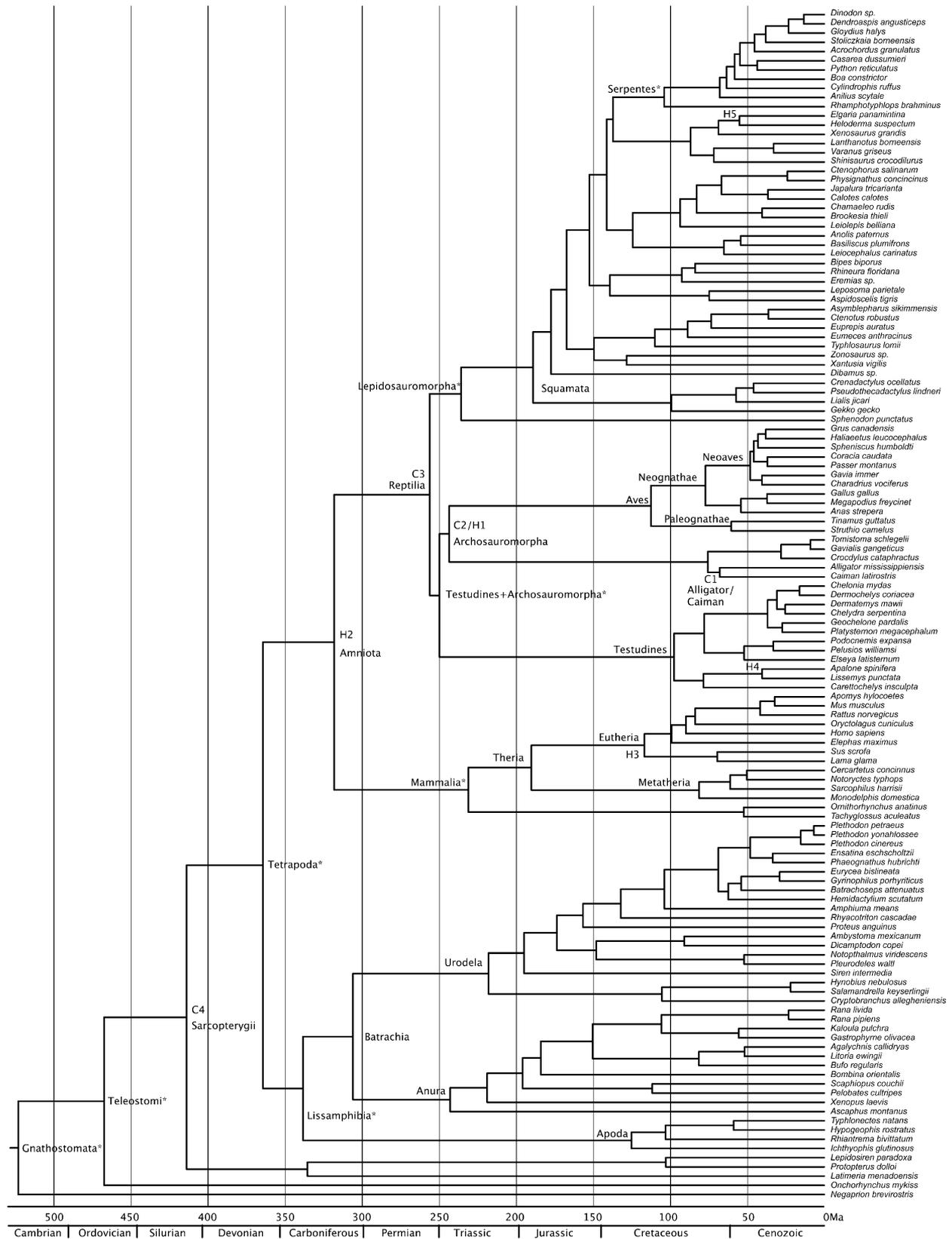


FIGURE 1. Chronogram of extant gnathostomatan vertebrates inferred using the four well-supported constraints from Müller and Reisz (2005), labeled as C1, and so forth. The five constraints from HEA are labeled H1, and so forth. Node labels refer to dates given in Table 4. The nodes used as likelihood checkpoints are indicated with an asterisk. The timescale is given in millions of years ago, with major stratigraphic eras indicated.

TABLE 4. Results and comparison of inferred ages (in Ma) for major gnathostomatan clades from the four and five constraint analyses, respectively

Node	Mean (4)	LCI	UCI	Mean (5)	LCI	UCI
Amniota	317.90	281.33	356.80	324.39	311.01	339.26
Anura	242.69	204.30	280.78	296.85	233.37	352.35
Apoda	125.10	65.12	192.55	118.18	76.15	167.34
Archosauromorpha	243.31	240.22	246.42	246.37	242.38	250.22
Aves	112.40	67.36	155.65	110.67	72.54	148.74
Batrachia	305.70	264.30	345.43	369.32	302.41	433.53
Eutheria	116.61	78.33	149.30	104.96	86.51	125.76
Gnathostomata	522.86	427.77	645.66	655.74	478.94	819.58
Lepidosauria	235.60	211.89	252.92	265.00	239.89	289.73
Lissamphibia	338.15	301.48	372.03	407.02	333.72	478.26
Mammalia	230.95	180.15	299.61	234.01	183.20	280.23
Metatheria	81.20	40.30	127.09	97.85	57.91	132.02
Neoaves	48.02	27.58	71.10	50.29	32.20	64.93
Neognathae	77.09	48.35	107.12	80.84	49.69	106.25
Teleostomi	467.17	415.39	540.24	598.71	457.90	727.41
Sarcopterygii	413.68	408.29	419.14	540.80	420.23	641.71
Squamata	188.84	162.88	213.31	207.73	178.65	234.12
Tetrapoda	364.10	329.96	391.18	434.38	357.62	504.29
Therians	189.96	141.70	252.89	193.24	134.75	239.80
Testudines	97.42	65.57	141.32	139.04	115.360	161.77
Testudines + Archosaurs	249.60	246.36	252.82	266.14	253.52	280.49
Urodela	217.80	171.10	259.74	239.00	176.35	295.06
Alligator/Caiman	67.84	65.20	70.27	11.58	4.29	20.03
Reptilia	256.00	253.68	258.30	288.98	272.26	306.67

Note: UCI and LCI represent the 95% highest posterior density of the posterior probability distribution of dates. LCI = lower credible interval; UCI = upper credible interval.

fossils known from approximately 125 Ma (Ji et al. 2002). The contentious date of the Neoaves radiation (Ericson et al. 2006; Brown et al. 2007; Ericson et al. 2007) is found to be well after the K–T boundary, approximately 49 Ma.

Based on the date for Testudines (97 Ma, 95% highest posterior density = 65.571 – 141.32), support is found for Joyce's (2007) reclassification of fossil turtles, suggesting a Late Jurassic or Cretaceous divergence of the Pleurodira and Cryptodira. A Cretaceous origin of the extant turtles is found here, indicating that fossil turtle fauna such as *Proganochelys* and *Condorchelys* likely represent stem lineages (Joyce 2007) rather than members of the crown groups Cryptodira or Pleurodira. This date is significantly younger than that recovered by HEA (207 Ma). In addition to Testudines, the date recovered for Crocodylia (76 Ma; Fig. 1) is the second major clade for which divergence times differ significantly between the four constraint data set (MAR) and the results of HEA, who recovered a date of 33 Ma for the extant crocodylians. The fossil record of putative stem crocodylians extends to the Triassic (Nesbitt 2007), with Cretaceous taxa such as *Albertochampsa* allied to

the modern alligatorids (Brochu 1999; Müller and Reisz 2005). Using the Cretaceous taxa as constraints for the extant alligatorids (Müller and Reisz 2005) suggests a Mesozoic rather than a Cenozoic origin of the extant crocodylians.

The inferred ages for the major clades within Sarcopterygii using the preferred four constraint topology (MAR) are broadly consistent with those found by HEA, with the notable exception of Testudines and Crocodylia, whereas the BEAST analyses using the five constraints of HEA are considerably older. As mentioned, this is likely due to differences in methodologies (i.e., the use of ranged calibrations in BEAST vs. fixed node ages using PL), illustrating the importance of considering fossil choice, calibration placement, and error estimation when inferring divergence times. The simultaneous inference of phylogeny and divergence times in BEAST allows for the incorporation of error in the former into the estimation of the latter, providing built-in CIs for inferred dates, although methods for error estimation for PL divergence dates are also available (Burbrink and Pyron 2008).

TABLE 5. Parameter and likelihood values for checkpoints used to assess fossil calibrations

Node	Type	Mean (SD)	Age (4)	–ln(4)	Age (5)	–ln(5)
Gnathostomata	Lognormal	6.203 (0.036)	522.86	–5.09	655.75	–35.55
Teleostomi	Lognormal	6.089 (0.023)	467.17	–6.54	598.71	–94.81
Tetrapoda	Lognormal	5.829 (0.015)	364.10	–13.25	434.73	–140.80
Lissamphibia	Lognormal	5.771 (0.052)	338.15	–4.30	407.02	–14.59
Lepidosauria	Exponential	228 Ma (—)	235.60	–6.46	264.98	–6.59
Testudines	Exponential	220 Ma (—)	245.60	–6.51	266.14	–6.60
Mammalia	Lognormal	5.172 (0.041)	230.95	–24.56	234.01	–26.71
Serpentes	Exponential	130 Ma (—)	137.12	–5.92	154.67	–6.06

Note: Mean and SD refer to the parameters of the checkpoint probability distribution, calculated from Table 3. Age (4) and (5) refer to the inferred ages of the labeled nodes using the 4 MAR and 5 HEA constraints, respectively. SD = standard deviation.

DISCUSSION

With this method, the likelihood of multiple alternative placements of multiple fossil constraints (e.g., stem vs. crown placements; Magallón and Sanderson 2001; Rutschmann et al. 2007) can be assessed using estimated dates for nodes in the Tree of Life (N_t) for which justifiable age bracketing is available from the fossil record. This provides a clear advantage over internal fossil cross-validation methods or ultrametric assessments (e.g., Near et al. 2005; Marshall 2008), in that the use of additional, putatively objective information from the fossil record allows for the comparison of divergence time estimates for researchers in any field. The success of such an approach hinges on the consistency and accuracy of the placement and dating of the checkpoints; such calibration references will require extensive validation via the use of existing methods of cross-validation, phylogenetic analysis of morphological data from the fossils themselves, and the integration of new information discovered in the fossil record.

Likelihood checkpoints may be thought of as an extension of the idea of stratigraphic consistency and congruence (e.g., Huelsenbeck 1994; Benton et al. 1999), in that we are using the fossil record in an attempt to objectively assess the quality of a phylogenetic estimate. However, in this case, we are concerned with inferred divergence times rather than branching order (e.g., Wills et al. 2008). No single criterion exists for determining what fossils to use as checkpoints (or constraints, for that matter), although this is a problem shared by any investigation into the paleontological record (see Paul and Donovan 1998). In general, the oldest known fossil of a group will provide at least a single exponential checkpoint, and a number of reliably bracketed nodes are given at <http://www.fossilrecord.net>. Taking mammals as an example, a study concerned with dating divergences within Rodentia may use a checkpoint on the root of the tree to assess divergence times in the ingroup. Such a checkpoint on the origin of the rodents would also be helpful for researchers studying other mammalian groups with rodents as an outgroup. For groups with a less detailed fossil record, the distinction may be somewhat muddled; in such cases, it may be desirable to report the likelihood of dates using some fossils as checkpoints as well as the dates estimated using all available fossils as constraints.

These results also indicate two important points regarding the general use of fossil constraints in divergence time estimation. First, the use of multiple ranged calibrations is preferable to the use of broad minimum ages with few or no fixed constraints, as the latter is often seen to be responsible for the inference of excessively old dates (Hugall et al. 2007). Second, the use of credible constraints near the base of the tree provides a more robust calibration of the entire tree than the use of calibrations only of nodes near the terminals. This is illustrated by the comparison between the results from the four versus five constraint analyses, where the discrepancy

between terminal nodes is minimal, but increases significantly toward the root (Table 4). The unconstrained root nodes in the five constraint analysis were inferred to be significantly older, likely due to the lack of calibration information from the distant terminal fossil constraints. This also indicates that the use of the common 315 Ma constraint of the Amniota is not supported by the broader fossil record, particularly when used as a primary constraint without additional calibration on the root of the tetrapods.

A fundamental question that must be asked in studies which estimate divergence times, and one which this study provides a preliminary framework for answering, is "are these node ages reasonable (i.e., not too old or too young)?" If one study suggests that the divergence between Chordata and Arthropoda occurred approximately 1200 Ma (Wray et al. 1996), and another study suggests an age of 627 Ma for that split (Lynch 1999), while neither may be 100% "correct," one must be closer to the truth than the other. The only objective information we have for assessing the validity of estimated divergence times is the fossil record. Although phylogenetic dating analyses are typically based on some paleontological information in the form of the fossil constraints, a greater synthesis of molecular and fossil data is needed for the accurate reconstruction of the evolutionary history of life. Additional future possibilities include the generation of phylogenies including fossil taxa using combined morphological and molecular data sets (e.g., Wiens 2009) and statistical models that can accommodate those taxa and date information for inferring node ages. This method provides one avenue for such a synthesis, allowing the assessment of how dates for one group fit in with those for other groups and the broader fossil record.

As has been illustrated, estimated divergence times imply something about the evolutionary timescale of all living things. The oldest known fossils of major sarcopterygian clades (Table 1) give a paleontological framework for the timing of the development of major vertebrate groups. This can be used as a rough metric for evaluating the temporal implications of inferred dates relative to their broader context in the Tree of Life. The placement of fossil constraints cannot be taken for granted; thorough studies of molecular divergence dating must take into account the variability in the calibrations as well as variability in the underlying molecular data. The use of calibrations on specific nodes, without consideration of the possible variability in the phylogenetic location of those fossils, may be a primary cause of inaccurate divergence time estimates. The use of likelihood checkpoints provides a method for broad unification of fossil and molecular data for inferring the timescale of the evolutionary history of life.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at <http://www.sysbio.oxfordjournals.org/>.

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