

Precision of molecular time estimates

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Molecular clocks are used to estimate the time of divergence of genes and species and have helped illuminate the evolutionary history of life. In a recent article, Graur and Martin raised concerns regarding confidence intervals of molecular time estimates and fossil calibrations. Although our past work was singled out, their concerns apply generally to the field, including their own previous studies. We believe that those concerns are unfounded. Graur and Martin use *ad hoc* methods for estimating confidence intervals and for the interpretation of fossil evidence, which led them to calculate overly wide confidence intervals and to make erroneous conclusions. In a separate article, Reisz and Müller propose that the bird–mammal fossil calibration used by us, and others, should be abandoned in favor of a bird–lizard calibration. However, other paleontologists disagree, and Reisz and Müller have failed to provide robust fossil evidence or to consider sequence availability. Molecular clocks have great potential but must be calibrated carefully.

In a recent *Trends in Genetics* article, Graur and Martin [1] raised some concerns about time estimations using molecular clocks. Although their article includes a large amount of hyperbole, biblical references and other non-scientific discourse, their primary point is that molecular time estimates (MTEs) have larger errors than that reported in the literature. In an accompanying article, in this issue of *Trends in Genetics*, Reisz and Müller [2] recommend abandoning the widely used bird–mammal calibration in favor of a bird–lizard calibration. Here, we discuss these issues and point out that Graur and Martin [1] are incorrect because the research community has interpreted clock calibrations as minimum times, not as central values or ranges. Similarly, Reisz and Müller's [2] proposal is unwarranted because the bird–mammal calibration is one of the best constrained in the vertebrate fossil record and the use of their alternative calibration would result in smaller datasets and reduced precision of time estimates.

The origin of the 310 MYA calibration

Graur and Martin [1] first discuss the source of the fossil calibration time [310 million years ago (MYA)] that we have used in our previous studies [3]. Their claim that it was 'bibliographically misattributed' is incorrect. We referred to a fossil reference source [4] that identified

the earliest pertinent fossil (*Hylonomus*) and indicated the geological formation (Vereiskian stage of Moscovian epoch) in which it was found. Because the temporal boundaries for the Vereiskian stage are narrowly defined as 309–311 MYA in the widely used geological timescale [5] cited in the introduction to that fossil reference source, we used the midpoint (310 MYA) following standard practice [6]. Most paleontologists [2,4,6–10] have agreed that these fossils from the Vereiskian stage are the oldest from either the bird or the mammal lineages. Although Graur and Martin question the phylogenetic position of *Hylonomus*, Reisz and Müller [2] correctly point out that the time of that split does not rely on *Hylonomus* because other relevant fossils exist from the same time period (discussed in the following section). Many genes are available for use with the bird–mammal calibration and, thus, it is one of the best calibration points in the tree of life.

Temporal constraints on the bird–mammal divergence

When we first used the 310 MYA bird–mammal calibration in the mid-1990s [3,11], we indicated that it was a minimum time and we explicitly discussed the maximum limit, which pertained to earlier fossils. For example, we stated that: 'The earliest ancestors of mammals (synapsids) and birds (diapsids) are lizard-like and first appear in the Carboniferous period, at ~310 Myr [million years] ago. The fact that the fossil record documents a morphological transition from lobe-finned fishes to stem tetrapods at 370–360 Myr ago, and records the appearance of stem amphibians at 338 Myr ago, indicates that the time of the diapsid–synapsid split (within amniotes) is unlikely to be a considerable underestimate.' [11] Therefore, the mammal–bird calibration range was discussed in our articles, which both Graur and Martin [1] and Reisz and Müller [2] failed to acknowledge. Instead, they pointed to stem amniote fossils (~340 MYA) as if they were being noted for the first time. Two recent studies [10,12] that have scrutinized these calibration points have reached conclusions similar to our own.

The statistical distribution of uncertainty in the fossil calibration is different from and more complex than envisaged by either Graur and Martin [1] or Reisz and Müller [2]. The error is directional in part and not easily represented by parametric statistics. First, the minimum time is defined by the formation containing the fossil and the time of divergence can not be younger than the age of a well-identified, well-dated fossil. The maximum age of the fossil calibration is qualitatively different because we are unlikely to ever find a true 'stem' fossil, one that was

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literally on the ancestral lineage leading to the divergence in question. If there is a reason to believe that the fossils defining the minimum age are close in time to the splitting event, the probability distribution might be better represented as a triangular, negative exponential or lognormal, with a peak closest to the minimum age. As we stated in one of our earlier articles [13], we proposed this pattern for the bird–mammal calibration (Figure 1) because the earliest representatives of the bird and mammal lineages were similar in morphology and no earlier representatives have been discovered since the mid-1800s. At the maximum end (370 MYA), it is logically a much lower probability that the synapsid and diapsid lineages split immediately after the transition from fish to tetrapods. The use of a normal or uniform distribution when there is evidence of non-normality or non-uniformity will introduce bias in the time estimate. Although some probability-based methods have appeared recently that facilitate the incorporation of fossil-based error [14,15], none is able to accommodate these complexities.

Graur and Martin [1] use a value of 288 MYA for the minimum date, citing publications by Reisz [16] and Lee [17]. However, Reisz and Müller [2] disagree and indicate a minimum date of 313–316 MYA for the bird–mammal

divergence. Apparently, Graur and Martin [1] had misread the earlier article by Reisz [16], which indicated fossil evidence older than 295 MYA. Graur and Martin also use an outdated geological timescale for the age of that boundary [5]. Later timescales show the Carboniferous–Permian boundary as 292–295 MYA [16,18], which is only 5% younger than our date of 310 MYA. Although one recent study [12] questioned the classification of *Hylonomus* (earliest bird ancestor) and *Protoclepsyrops* (earliest mammal ancestor), thereby disagreeing with Reisz and Müller's [2] interpretation of the earliest fossils, the revised minimum date in that study for the bird–mammal divergence (306 MYA) is within 1% of 310 MYA. Radiometric error is often small (~1%) [18,19] and ignored in time estimation but should be considered if it is large [12]. Despite minor differences of opinion regarding some fossils, which is inevitable, most authors still consider the minimum bird–mammal divergence to be within ~1% of 310 MYA; therefore, we see no reason to revise that widely used date for the calibration.

Inferences based on mammal–bird calibration point

A primary concern raised by Graur and Martin [1] is our use of the bird–mammal minimum time (310 MYA) as a point estimate rather than a range (or confidence interval) for fossil calibration. Before discussing why this criticism is baseless, we note that the use of minimum-point estimates for calibrations has been the standard approach in the field for more than two decades (see articles by Aranason *et al.* [20], Martin *et al.* [21], Fitch [22], Doolittle *et al.* [23], Wray *et al.* [24], Springer *et al.* [25], Cooper and Penny [26], Bromham *et al.* [27], Ayala *et al.* [28] and Nei *et al.* [29], among others). Therefore, it is unclear why Graur and Martin [1] omitted this fact and singled out our research for comment. Nonetheless, neither we nor others have stated that fossil calibrations are errorless, as contended by Graur and Martin ('310 ± 0 MYA') [1]. Instead, minimum time estimates are used to obtain statistically conservative estimates of molecular divergence.

Furthermore, these time estimates are regarded as minimum time estimates, and this has been taken into account by us (and others) in drawing our previous conclusions. For example, our use of a minimum calibration was least likely to overturn the major hypotheses (Type I error) tested in our studies [11,13], such as the Cretaceous–Tertiary ordinal diversification of placental mammals and Cambrian origin of animal phyla. The use of an older calibration (>310 MYA) for the bird–mammal divergence would only have resulted in older MTEs and a higher probability of rejection of the hypotheses that were already rejected by our results.

The number of calibration points

Time estimation with molecular clocks would be mostly unnecessary if the fossil record provided an accurate and complete representation of evolutionary history. Unfortunately, the fossil record is largely incomplete and biased [8,30]. For example, a third of living animal phyla have no fossil record, despite the presumed existence of each lineage for at least 500 million years [31]. Also, the earliest representatives of any two lineages are expected to be

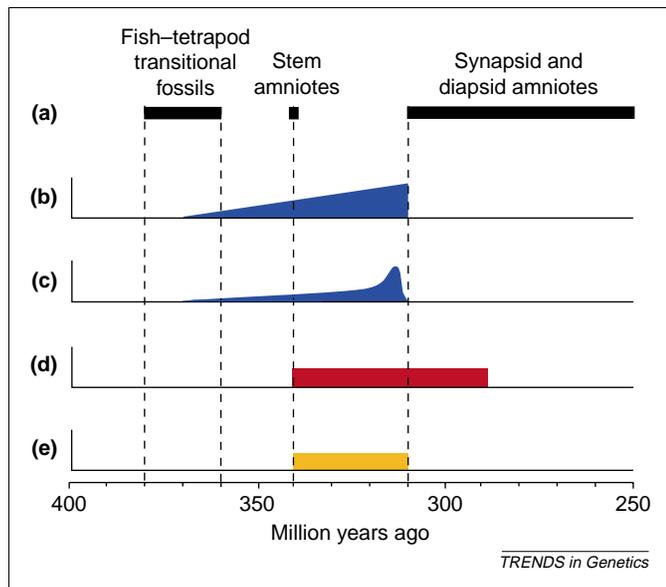


Figure 1. Temporal constraints on the bird–mammal fossil calibration. (a) Fossils that are relevant to constraining the calibration point include the sarcopterygian fish in the Devonian period and early tetrapod transitional fossils (380–360 MYA), early Carboniferous stem amniotes (~340 MYA) and the earliest representatives of the bird (diapsid) and mammal (synapsid) amniote lineages in the late Carboniferous (~310 MYA), as detailed in Hedges *et al.* [3] and Kumar and Hedges [11]. (b) The probability distribution (triangular) roughly corresponding to textual description of error limits in studies by Hedges *et al.* [3], Kumar and Hedges [11] and Wang *et al.* [13]. Broad error limits (370–310 MYA) were recognized, with a peak of probability near the minimum limit based on the nature and history of the earliest fossils (see main text). (c) A lognormal distribution shows similar characteristics to the triangular distribution but permits a depressed tail. (d) The probability distribution (uniform) corresponding to the description of error limits in Graur and Martin [1]. A narrower range (338–288 MYA) is recognized, with a mean of 313 MYA. However, the minimum age limit (288 MYA) is based on an outdated timescale and ignores earlier fossils, and the fixed maximum limit (~340 MYA) is not based on evidence for a fixed event with sharp probability boundary. (e) The probability distribution (uniform) corresponding to the description of error limits in Reisz and Müller [2]. In this case, the minimum limit is correctly set to the age of the earliest ancestors of birds and mammals but the maximum age (~340 MYA) is not based on evidence for a fixed event (see main text). Abbreviation: MYA, million years ago.

virtually indistinguishable. Therefore, the fossil record always underestimates when two lineages diverged and often it is a considerable underestimate. For this reason, the choice of fossils used in calibrations is crucial to obtaining reliable MTEs. At least one calibration point is always required and, although more are desirable, they must be well constrained. Greater accuracy in the MTE is obtained with one or more tightly constrained fossil calibrations close to the speciation event, rather than with many calibrations that are poorly constrained (Figure 2).

Another major requirement for calibrating molecular clocks is sequence availability. Large numbers of genes are needed to improve the precision and reduce any bias of the time estimated [3,11,13]. To meet both requirements, we have frequently – but not exclusively – used the bird–mammal calibration [3] because the minimum date is well constrained in the fossil record and it permitted time estimates from an abundance of living species that are represented in the public databases. We never advocated the use of only a single calibration point or only the bird–mammal calibration, as

implied by Graur and Martin [1]. We have used other robust fossil and geological calibrations in our studies as appropriate [32–36].

Reisz and Müller [2] presented three alternative calibrations to the bird–mammal calibration. However, they overlooked a more comprehensive discussion of tetrapod fossil calibrations that included those same calibrations, published by another paleontologist [6]. Furthermore, they failed to provide evidence for a temporal series of transitional fossils on maximum limit, to justify such a narrow interval (252–257 MYA) for the bird–lizard split. Thus, there is no supporting evidence that *Youngina* diverged from the stem immediately before the first fossils of that taxon at 257 MYA. Hence, the maximum limit could be earlier. Finally, the use of such calibrations, if found to be robust, is currently hampered by the fact that crocodylians, lizards and lungfish are among the most poorly represented vertebrates in the public sequence databases (Figure 2). We have previously shown that many fossil-based minimum times of divergence among vertebrates correspond relatively closely to molecular dates based on bird–mammal calibrations

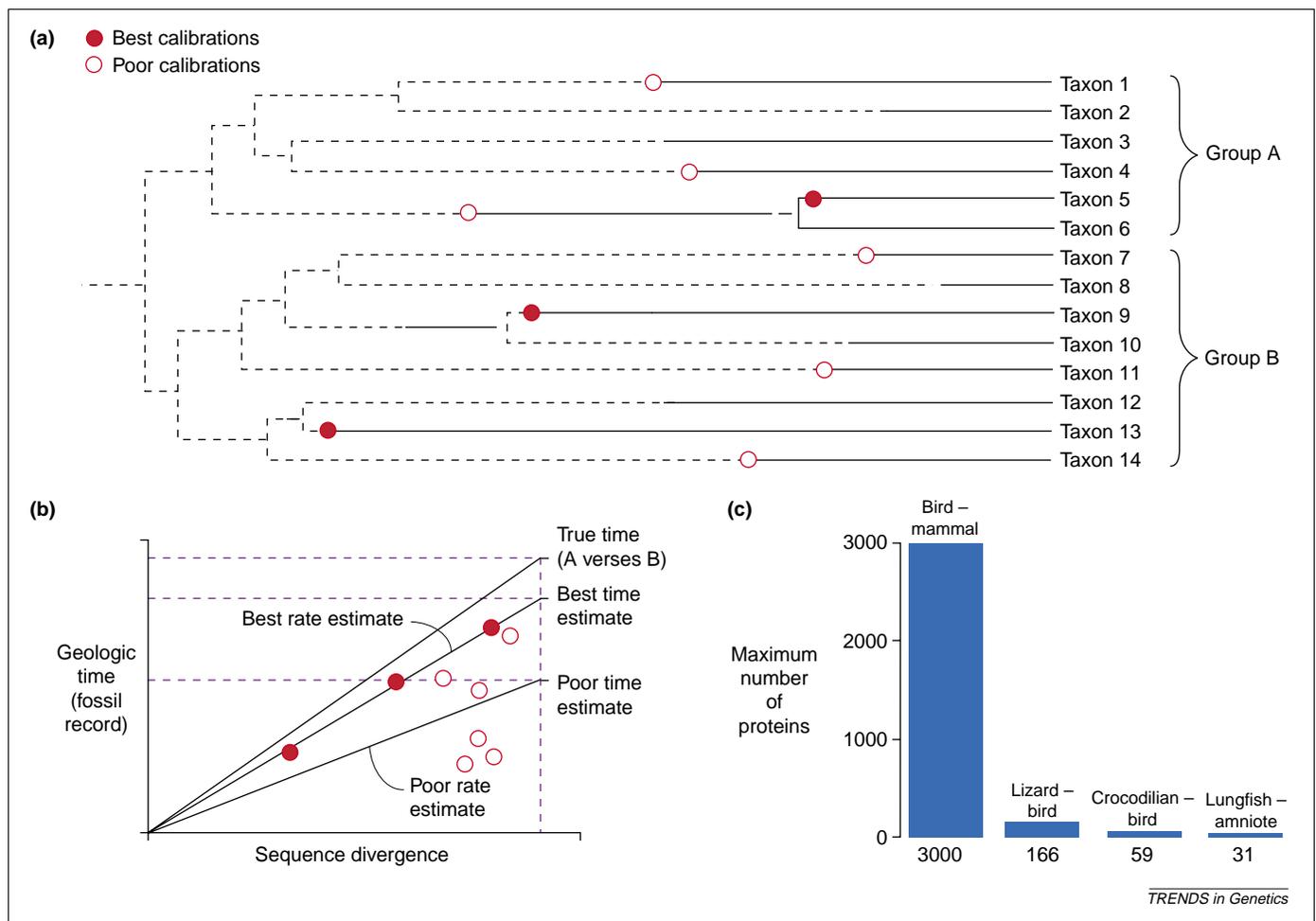


Figure 2. The considerations for selecting calibrations in molecular clock analyses are robustness of the fossil record and sequence availability. **(a)** In a hypothetical phylogeny of ten species, nine calibration points are available for use in estimating the time of divergence of groups A and B (the solid line signifies the fossil record; the broken line signifies the true phylogeny). **(b)** The use of all nine calibrations results in poor rate and time estimates because six of the fossil divergences represent large underestimates. Instead, exclusive use of the three best calibrations results in the best rate and time estimates. Sequence availability must also be considered. **(c)** Three fossil calibrations suggested by Reisz and Müller [2], the lizard–bird, crocodylian–bird and lungfish–amniote calibrations, are all limited in sequence availability compared with the bird–mammal calibration. The histogram shows the number of unique proteins, with > 100 amino acids, in GenBank. The actual number of proteins available for calculating the time of any particular divergence will be lower because additional taxa must be considered.

[11,37]. Therefore, we are optimistic that, as sequence databases grow, additional robust fossil calibrations will become available for use with molecular clocks.

Use of secondary calibrations

After divergences in a phylogeny are calibrated to geological time, with one or more calibrations, any node (or MTE) in the tree can be used as a secondary calibration point in a separate analysis, if there are no known biases. Secondary calibrations might be used to minimize the difference between the calibration point and the divergence to be timed, thereby increasing the number of applicable genes and the overall precision of time estimates [36]. This is because genes evolve at different rates: sequences of distantly related organisms can not be aligned for fast-evolving genes, whereas sequences of closely related organisms might have no genetic differences for slowly evolving genes. In addition, large extrapolations can amplify any existing biases in divergence times and the comparison of sequences from the same region of a phylogeny helps to minimize errors in the determination of sequence orthology. Furthermore, if fossil calibration points are available for a particular region of a phylogeny (e.g. Precambrian era) but are not robust, the resulting MTEs would have less accuracy than if a secondary calibration, derived from a robust primary calibration, were used. For all of these reasons, we have used secondary calibrations to improve the precision and accuracy of our time estimates [11,13,36].

Graur and colleagues [1,38] claim that secondary calibration leads to inconsistent results, in the sense that the original calibration time can not be recovered from the secondary calibration time by reversing the analysis (non-reciprocity). This claim is based largely on Shaul and Graur's [38] flawed reanalysis of a 75-gene dataset that we used originally to estimate the divergence times of animal phyla and more-ancient divergences [13]. Shaul and Graur used a subset of our data containing only 21 genes. Using the rodent-primate divergence of 110 MYA (secondary calibration), they attempted to recover the original 310 MYA bird-mammal divergence. They reported a mean bird-mammal divergence time of 393 MYA with a 95% confidence interval of 315–471 MYA, which excluded the original calibration time of 310 MYA. However, this result is based on an incorrect assumption, namely that the multigene times have a gaussian distribution. Instead, the distribution of bird-mammal MTEs is highly skewed because of a small sample size and large extrapolation. The mode [39] (302 MYA) and median (326 MYA) are more appropriate measures of a central value for such highly skewed distributions [11,39,40]. These estimates are close to the target date of 310 MYA and indicate that their finding of inconsistency is based on invalid premises. Hedges and colleagues [36] also found 98% consistency in the 310 MYA calibration when using a much larger dataset of 120 proteins to address the arthropod-deuterostome divergence. Therefore, this and other unrealistically large confidence intervals stated in the article by Graur and Martin [1] have resulted from their *ad hoc* approaches and are without merit.

Confidence intervals of time estimates

Graur and Martin are in error when they suggest that we, and by implication others in the research community, have inferred overly narrow confidence intervals of MTEs. Instead, they have calculated confidence intervals incorrectly by assuming that the calibration range and time-estimation error are additive. For example, they claim that by 'considering the uncertainties in the primary calibration (i.e. 338–288 MYA), the diversification of avian orders can be said to have occurred with 95% confidence within the time interval of 132–67 MYA.' [1] However, their calculation extends the original interval by 18 million years, which is incorrect. The correct calculation [41] adds only 3.2 million years. Graur and Martin's [1] method produces exaggerated and erroneous confidence intervals, including some extending > 14 billion years. In addition, their suggestion that the use of confidence intervals is more appropriate than presenting standard errors of the mean is a matter of choice and both are commonly used [3,11,34,42]. Graur and Martin [1] claim that our use of standard error was misleading in a previous study [3] but they overlooked that we stated (in a figure legend) that the mean \pm one standard error was plotted.

Disputed divergence times among mammals

In our *Trends in Genetics* review [40], we noted (in a figure legend) that we could not place divergence times involving rodents on the timescale figure because of large differences in the published MTEs [43–45]. However, Graur and Martin [1] altered a quotation from our paper by adding the word 'discarded' and thus changing the meaning of the sentence. They inferred that we discarded rodent MTEs, which is incorrect. Graur and Martin [1] also draw attention to our summary time for the divergence of primates and cattle, 90–98 MYA (see Figure 6 in Ref. [40]), noting that it is 'a statistical novelty'. On the contrary, our figure legend lists the references for the nine different studies used in the construction of the figure, including one study [3] that lists the central value for this divergence as low as 90 MYA and another [45] as high as 98 MYA (hence, 90–98 MYA). We also did not state that they are confidence intervals. Because most numbers in the figure are not ranges (they are central values), they could not be interpreted logically as confidence intervals, revealing that the criticisms by Graur and Martin [1] are exaggerations and are neither scientifically valid nor objective.

Similarly, Graur and Martin [1] suggested that the 95% confidence intervals of 119–74 MYA (primate and rodent), 117–67 MYA (primates and artiodactyls) and 145–85 MYA (artiodactyls and rodents) falsify the continental breakup hypothesis of Hedges *et al.* [3]. Even if these intervals were correct, this conclusion is unwarranted because all of those confidence intervals are in the Mesozoic era (251–65 MYA) when continents were breaking up, not during the Cenozoic era (65–0 MYA) when the areas of land were establishing connections. In addition, subsequent studies since 1996 have supported Mesozoic era divergences in birds and mammals [11,26,29,41,44,45]. Also, the prediction of this hypothesis (that super orders of these animals were once restricted to ancient landmasses)

has been supported by the discovery of Afrotheria, a super order of mammals [25,46].

Concluding remarks

All recent reviews of molecular clocks have concluded that they have broad utility in evolutionary biology, in addition to genetics, biomedicine and astrobiology [40,47–52]. By contrast, Shaul and Graur [38] recently concluded ‘as an extreme measure, we would suggest not to derive divergence dates from molecular data at all.’ We disagree with this pessimistic view, extended by Graur and Martin in Ref. [1] because they have not provided valid evidence that supports their opinion. Instead, they use histrionics, melodrama, hyperbole and an abundance of biblical references in their critique, while taking *ad hoc* approaches to expanding confidence intervals. This has only served to confuse and mislead those unfamiliar with the literature in this field. Obviously, we welcome reevaluation of MTEs that we (and others) have produced and expect that some past results will be revised with new evidence. Finally, we hope that our explanations will help dispel any confusion resulting from the article by Graur and Martin [1] and convey that there is a bright future for the use of molecular clocks in establishing the temporal component of the tree of life as methods are improved and genomes are sequenced.

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