Calibration and Error in Placental Molecular Clocks: A Conservative Approach Using the Cetartiodactyl Fossil Record

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Abstract

The nature of the molecular and fossil record and their limitations must be ascertained in order to gain the most precise and accurate evolutionary timescale using genetic information. Yet the majority of such timescales are based on point estimates using fossils or the molecular clock. Here we document from the primary literature minimum and maximum fossil age estimates of the divergence of whales from artiodactyls, a commonly used anchor point for calibrating both mitogenomic and nucleogenomic placental timescales. We applied these reestimates to the most recently established placental timescale based on mitochondrial rRNA and several nuclear loci, and present an attempt to account for both genetic and fossil uncertainty. Our results indicate that disregard for fossil calibration error may inflate the power of the molecular clock when testing the time of ordinal diversification in context with the K-T boundary. However, the early history of placentals, including their superordinal diversification, remained in the Cretaceous despite a conservative approach. Our conclusions need corroboration across other frequently used fossil anchor points, but also with more genetic partitions on the linear relationship between molecular substitutions and geologic time.

Multiple studies of molecular evolutionary timescales agree that the initial diversification of extant placental mammals occurred in the Cretaceous. This result is consistent despite sometimes large variation in molecular divergence time estimation with different calibration points, methods, or datasets (Bromham et al. 1999; Smith and Peterson 2002). However, it is often overlooked that substantial error may also exist within a single dataset with a fixed calibration and model of evolution. This error derives from uncertainty in the fossil age of calibration points, branch-length estimation, and tree topology (Nei and Kumar 2000). In the last 30 years, while phylogenetic and branch-length uncertainties were sometimes acknowledged in error estimation, most molecular clocks have been calibrated with a fixed fossil age. Fossil records often have been prioritized for estimating molecular rates, specifically those that are documented in the paleontologic literature as diagnostic (e.g., the divergence of human from chimp, or horse from rhinoceros) or continuous due to exceptional preservation (e.g., regarding the origin of whales or amniotes). The tacit assumption of error-free fossil ages from these records and the statistical implications of this assumption have received little attention in the molecular clock literature. Hence it is unclear what role the specific error associated with empirical data will play in resolution of the placental timescale.

Understanding variation in divergence time estimation within and between studies is of particular importance in testing whether placental biodiversity has been marked by major perturbation events across the Cretaceous-Tertiary boundary. Of particular interest is the recent indication that diversification within extant primates, Rodentia, Eulipotyphla, and Xenarthra started in the Late Cretaceous (Springer et al. 2003), suggesting that these lineages may have been affected less by K-T events than other orders. This conclusion is supported by analysis of the largest available (>16 kb) concatenated dataset for placentals, sophisticated modeling of evolutionary rates across the placental tree, multiple fossil constraints, and broad taxon sampling. Nonetheless, because point estimates for the initial diversification within those four orders predate the K-T boundary by only 5–12 million years (MY), the effect of molecular error may be especially critical.

We investigate the role of genetic and fossil error in resolution of the early placental timescale. We focus on the most frequently used internal calibration point in multiorder mammalian molecular clock studies: the divergence of modern whales from artiodactyls (Artiodactyla-Cetacea or A/C-60) (Arnason and Gullberg 1996). The return of mammals to the aquatic realm is considered to be one of the largest macroevolutionary events in amniote history (Carroll 1997) and is linked with a myriad of distinctive morphological features. Among mammals, modern whales and dolphins comprise the largest aquatic adaptive radiation combined with the greatest morphological departure from terrestrial ancestors. Because of their distinctive and diagnostic body form, the whale fossil record includes several archaic whales intermediate to modern lineages in time, morphology, and ecology (Thewissen 1998). Over the last 20 years, important transitional fossils have aided our understanding of how, why, where, and when Cetacea and Artiodactyla diverged (Gingerich and Uhen 1998; Thewissen 1998), but without significantly influencing the time of their initial divergence. Relative stasis in the timing of cetacean origin has been essential to its utility as a calibration in placental molecular clock studies.

Methods

To investigate the use of fossils in calibrating the larger placental clock from the literature, we performed a keyword search in Scisearch using "molecular clock" spanning 1963–2002. In addition, we reviewed molecular clock studies in five journals with frequent focus on mammalian evolution: *Molecular Biology and Evolution, Molecular Phylogenetics and Evolution, Journal of Molecular Evolution, Journal of Mammalian Evolution*, and *Journal of Mammalogy.* Our search indicated that several recent molecular clock studies of placental evolution have favored use of the Artiodactyla-Cetacea divergence to calibrate the remaining mammalian timescale, sometimes in combination with other calibration points. Those studies used the whale fossil record to estimate the absolute rate of molecular evolution or to force this rate to fall within a range given a rate-heterogeneous molecular dataset. One example is the recent timescale provided by Springer et al. (2003). Although six independent fossil records were used to estimate rates across the placental tree, removing the Cetartiodactyl constraint showed the largest shift in divergence time when estimated solely by the remaining five constraints, a sevenfold increase in time estimation error, and yielded younger times for most other nodes. The removal of the Cetartiodactyl calibration indicates that this constraint may be incongruent with other fossil constraints. Alternatively, evolutionary rate shifts could be especially extensive on the branch leading to Cetartiodactyla, and may not have been corrected for entirely by Bayesian analyses (Thorne et al. 1998). Both alternatives merit further investigation.

Documentation of the dating methodology used in calibration fossils or errors associated with molecular clocks is not yet standard procedure. To do so, we must go beyond the often-cited secondary literature. Hence we revisit the primary literature in order to ascertain the details of the earliest Cetartiodactyl fossils and their corresponding stratigraphic horizons. These details include character descriptions for diagnosis of particular fossils, stratigraphic correlation, and dating methods for the stratigraphically important rocks. From this literature we assess likely minimum and maximum ages for the Artiodactyla-Cetacea divergence and their uncertainties. In this context, our age reestimate of this split is used here to reset the placental timescale using a conservative approach.

Our approach is conservative in its use of minimum fossil age or upper bound (Marshall 1990b; see also Eizirik et al. 2001), and in accounting for both genetic and calibration error. Within this type of study, erring toward more recent lineage divergence times is considered conservative. The probability of branching at the K-T boundary (65.0 million years ago [MYA]) is tested at the 5% level by compounding individual errors into a more realistic standard error (SE) based on both genetic and paleontologic uncertainty using $\sqrt{(\Sigma[\text{individual errors/mean estimate}]^2)}$ instead of simple summation (van Tuinen and Hedges 2001). We provide broader, more realistic 95% confidence intervals (CIs) around the new point estimates (± 1.96 SE) and compare these to CIs based on either genetic or paleontologic error alone. These analyses are performed on the most recent and largest (multilocus) available placental dataset (Springer et al. 2003; their supplementary data). Because difficulty in assigning phylogenetic error to fossils that provide maximal ages impedes statistical resolution, we promote the use of minimum molecular divergence time estimates, following Eizirik et al. (2001). We have assumed minimal topologic error for the early history of Placentalia (*sensu* Murphy et al. 2001).

Results and Discussion

Molecular-Based Calibration

Since 1993 the Artiodactyla-Cetacea calibration point has been employed in more than 30 molecular studies using different approaches, definitions, and time estimates. A genetics-based estimate of 60 MY (no error given) for the cow-whale split is commonly used as a calibration point in both mammal and avian mitochondrial genome clocks. Initially estimated at 57 MYA, based on a 0.05%/MY 12S rRNA rate (Arnason et al. 1991), this divergence was later tentatively reset to 60 MYA by calibrating conservative cytochrome *b* substitutions of many cetacean taxa with the early Oligocene (32–34 MYA) fossil record of extant whales (Arnason and Gullberg 1996). Based on these analyses, Arnason et al. (1991) have applied the Artiodactyla-Cetacea divergence as an internal reference (A/C-60) to date the larger mammalian mitogenomic timescale. Initially it was uncertain whether the 60 million year estimate of the Artiodactyla-Cetacea ancestor referred to the Cetacea-*Hippopotamus* or Cetacea-Ruminantia divergence (Arnason et al. 1991). Subsequently it has been placed at the ruminant-whale

divergence and used to date the younger hippo-whale divergence at 54 MYA, the older Suidae/Tylopoda-whale divergence at 65 MYA, and to reset the Odontocete-Mysticete (crown Cetacea) divergence at 33 MYA (Arnason et al. 2000). Use of molecular-based anchor points has been recently criticized (Smith and Peterson 2002), and is ultimately dependent on fossils and not immune to calibration error.

Fossil-Based Calibration

An alternative calibration method using the Artiodactyla-Cetacea divergence is based directly on the Cetartiodactyl fossil record, with a variety of approaches taken. For example, the Artiodactyla-Cetacea divergence has been used in combination with either the minimum age (Eizirik et al. 2001), the midpoint of the minimum and maximum ages (Yoder and Yang 2000), or the maximum age (Delsuc et al. 2001) inferred from the whale fossil record at the time of publication. These ages (ranging between 49 and 63 MY) have been applied to both the Cetacea-*Hippopotamus* node (e.g., Nikaido et al. 2001b) and the Cetacea-Ruminantia node (Wray et al. 1996) to date the timescale of whales, bats, insectivores, primates, rodents, afrotherians, xenarthrans, and the initial metazoan diversification. The variety and trends observed among users of the Artiodactyla-Cetacea calibration (Figure 1) can be traced back to a combination of differing nodal definition, usage of minimum or maximum fossil ages or molecular estimates, and recent finds of older fossils within this lineage (Bajpai and Gingerich 1998).

Phylogeny of Artiodactyla-Cetacea

Molecular data unambiguously support the *Hippopotamus* as the living artiodactyl sister group of Cetacea (e.g., Matthee et al. 2001; Nikaido et al. 1999). This view currently is incongruent with morphological and fossil interpretation, which supports a sister relationship of Cetacea (sometimes plus the mesonychid ungulates; O'Leary and Uhen 1999) to a monophyletic Artiodactyla (Thewissen et al. 2001b). However, recent advances have been made toward reconciling both scenarios and indicate that the long-claimed mesonychid-whale grouping is likely due to convergence in dental morphology (Naylor and Adams 2001; Thewissen et al. 2001b).

Another recent advance has been the exciting find of artiodactyl-type anklebones in early hind limb–retaining whales (Gingerich et al. 2001), supporting their artiodactyl ancestry. The specific ankle morphology in these early whales is observed in few other groups, including anthracotheres and other early artiodactyls, but excluding mesonychids. Anthracotheres have been considered ancestral to hippos and may provide the fossil link supporting the molecular scenario (Gingerich et al. 2001), albeit currently with little additional support (Thewissen et al. 2001b). It is possible that cladistic analyses on morphology may not support a whale-hippo grouping because of the highly derived states found in modern and extinct hippos and whales. Fossils could fill
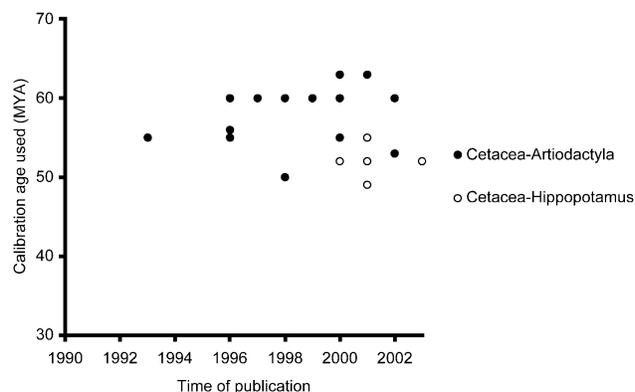

Figure 1. The variety of reported Artiodactyla-Cetacea divergence times from 1990 to 2003 by time of published studies that use this node for molecular rate estimation. Ages denoted by white circles refer to the whale-*Hippopotamus* divergence, black circles refer to the whale-ruminant divergence. Divergence times were taken from the following publications: [whale-ruminant] Martin and Palumbi (1993), Arnason and Gullberg (1996), Ledje and Arnason (1996), Smith et al. (1996), Wray et al. (1996), Xu and Arnason (1996), Xu et al. (1996), Arnason et al. (1997), Härlid et al. (1997), Janke et al. (1997), Springer (1997), Springer et al. (1997), Ursing and Arnason (1997), Arnason et al. (1998), Cao et al. (1998), Hiendleder et al. (1998), Kim et al. (1998), Ursing and Arnason (1998), Arnason et al. (1999), Penny et al. (1999), Arnason et al. (2000), Cao et al. (2000), Huchon et al. (2000), Mouchaty et al. (2000), Wang and Lan (2000), Yoder and Yang (2000), Delsuc et al. (2001), Kumar and Subramanian (2002; [whale-*Hippopotamus*] Koji-Lum (2000), Nikaido et al. (2000), Eizirik et al. (2001), Nikaido et al. (2001a,b), Springer et al. (2003).

those gaps, but the fossil record of Hippopotamidae traces back to only the Middle Miocene and is too poor on the branch from the Cetacea-Artiodactyl split to document their early evolution (O'Leary and Uhen 1999). Compared to hippos, the earliest history of whales is better understood from fossils of Pakicetidae, arguably the most primitive known whale lineage (Thewissen et al. 2001b).

Fossil Record of the Cetacean and Artiodactyl Terminal Branches

The oldest fossil directly relevant to the evolution of whales is *Himalayacetus subathuensis* (Figure 2) (Bajpai and Gingerich 1998) from a marine limestone near the base of the Subathu Formation in the Lesser Himalaya Range, India. *Himalayacetus* is known from a partial dentition and two molars that show similarities to *Pakicetus*, and hence is placed in Pakicetidae [but see Thewissen and Williams (2002) for alternative placement]. Although this fossil is frequently cited as the oldest whale, at 53–54 MYA, its phylogenetic placement and age are somewhat ambiguous. Furthermore, the cetacean synapomorphy, the involucrum of the middle ear (Thewissen 1998), is not preserved in this fossil. Thus, although likely
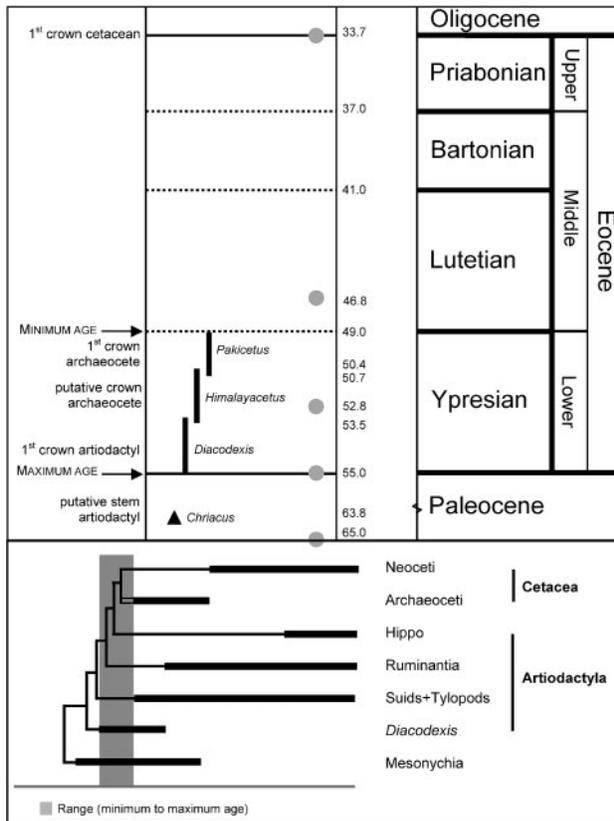
Figure 2. Geological distribution of fossils (black triangle) relevant to the divergence of Cetacea and Artiodactyla. Directly dated geological boundaries are shown with solid line and grey circle. Ages for all other geological boundaries are based on a combination of interpolation, biozonation and paleomagnetics. Note that only three geological calibrations are available for the Eocene timescale [based on Berggren et al. (1995)]. Synchrony between the Kuldana and Subathu formations has not been firmly established, yielding some uncertainty (thick black lines) on the age of the earliest archaeocetes (see text for discussion). A cetartiodactylan phylogeny is shown for reference below, modified from O'Leary and Uhen (1999) with relative fossil depth (thick bars).

a whalebased on the teeth and jaw, cladistically *Himalayacetus* must remain a tentative Cetacean. The material is fragmentary; hence thorough cladistic analysis is difficult (O'Leary and Uhen 1999; Thewissen et al. 2001b). The age of *Himalayacetus* initially was determined from a combination of correlating patterns in local sea level transgression and regression with those described globally, and biochronologically placing the fossil stratum within a benthic foraminifera zone of known age (Bajpai and Gingerich 1998).

According to the same dating techniques and assuming synchrony between the Kuldana and Subathu Formations on the south side of the Himalayas, the previously oldest fossil whales, pakicetids, are younger in age (47.9 MY) than initially

presented (49.5 MY) (Bajpai and Gingerich 1998). However, the use of benthic, instead of planktonic, foraminifera in biochronological dating is controversial, and the upper Subathu sequence may not have been deposited at the same time as the *Pakicetus*-bearing Kuldana-Kalakot Formation (Thewissen et al. 2001a). *Nummulites atacicus* was used to constrain *Himalayacetus* to Subathu zones 2–4, but is also found less abundantly in Subathu zones 5–7 (Mathur 1978), yielding a minimum age of *Himalayacetus* only slightly older than *Pakicetus* if synchronous. Mathur later discontinued the use of *Nummulites atacicus* as a chronological biomarker, and alternative benthic foraminiferan interpretation indicates an age associated with shallow benthic zone 10 (Thewissen et al. 2001a), which correlates with Subathu zone 5 or the geologically more standard nannoplankton zone NP12 or magnetic Chron 23–22 (Bajpai and Gingerich 1998). Absolute ages of these and other magnetic and bioformation zones have been determined either directly with the $^{40}Ar/^{39}Ar$ method or using interpolation from several Cenozoic time points (Berggren et al. 1995). The minimum age of Subathu zone 5 corresponds to 50.4 MYA ($\pm$0.44 MY), with error deriving from the $^{40}Ar/^{39}Ar$ measurement (0.1% SE of the mean) (Berggren et al. 1995:132), from the calibrations used to estimate the $^{40}Ar/^{39}Ar$ decay rate (1.7 MY of 520.4 MY), and from biozonation error (0.4 MY of 50.4 MY). Biozones are used in biostratigraphy to define zonal limits and refer to biotic events (species first and last occurrences).

According to molecular analyses, the oldest artiodactyl fossil that can be accurately placed after the split from whales must come from the hippo branch. If mesonychids belong within Artiodactyla after the hippo-whale split (*sensu* Rose 2001), this would make the oldest mesonychid *Dissacus*, from the San Juan Basin in the Torrejonian 2, the oldest fossil within crown Artiodactyla-Cetacea (62.5–61.3 MYA) (Berggren et al. 1995; Janis et al. 1998) (Figure 2). Such an interpretation would yield a 13 million year ghost line-age on the Cetacea branch and implies a 50 million year fossil gap on the hippo branch. However, considering the new phylogenetic interpretation of mesonychids based on non-dental and ankle morphological data (Gingerich et al. 2001), mesonychid fossils cannot be used with certainty in dating the crown Artiodactyla-Cetacea divergence. Thus the age of this divergence is likely better approached by the age of the earliest undisputed archaeocetes.

Minimum and Maximum Artiodactyla-Cetacea Age

The maximum age of the Artiodactyla-Cetacea divergence based on the oldest fossil artiodactyls coincides with the earliest Eocene (55.0 $\pm$ 0.56 MYA) (Figure 2), but this may be pushed into the Paleocene if the molecular data are correct. *Diacodexis metsiachus* is the oldest artiodactyl bearing an artiodactyl-type ankle (Rose 1982). It is found in the earliest Eocene (Wasatchian) Willwood Formation, Bighorn Basin (Rose 1982). Specifically *Diacodexis* is based on several skeletal fragments, including nearly complete hind limbs, from a paleosol unit about 501 m from the bottom of the Willwood Formation (Bown et al. 1993). This unit can be

dated to 53.3 ± 0.62 MYA using a combination of paleomagnetic stratigraphy and the sedimentation rate of the Willwood paleosol units (Tauxe et al. 1994) estimated from calibration at the bottom of the formation (55.0 ± 0.56 MY: Paleocene-Eocene boundary) and at the lower boundary of C24n.1 (52.8 ± 0.3 MY based on ^{40}Ar/^{39}Ar). However, other less complete *Diacodexis* material has been reported from the earliest Eocene in North America (Janis et al. 1998) and Europe (Thewissen JGM, personal communication).

The possible ancestors to hippos, anthracotheres, date back to the Late Middle Eocene of Asia (Pondaung fauna), North America (Duchesnean), and Europe (Bartonian) (McKenna and Bell 1997), which have been dated in North America at 39.5–37.1 MYA (Janis et al. 1998). Thus the fossil record of Anthracotheriidae postdates the earliest archaeocetes and cannot be used for estimation of the maximum stem Artiodactyla-Cetacea age. Molecular data instead support a Paleocene age for crown Artiodactyla (Arnason et al. 2000), a view supported by condylarth fossils that may be on the stem leading to Artiodactyla. For example, *Chriacus*, an arctocyonid lineage resembling early artiodactyls in its postcranial elements (Rose 1996), is found in deposits as old as 62.5–63.8 MYA (Janis et al. 1998). Unfortunately, cladistic resolution of the archaic Paleocene mammals is limited and the nature of artiodactyl origin from archaic ungulates remains enigmatic (Rose 1996).

The oldest fossils that display an involucrum in the tympanic region (Thewissen 1998) provide a minimum age of 49.0 MYA for the Artiodactyl-Cetacea divergence ("Minimum age" in Figure 2). The first certain fossil cetaceans include the earliest members of Pakicetidae from the redbeds of the lower Kuldana Formation (*Pakicetus inachus*, *Ichthyolestes*, *Nalacetus*) (O'Leary and Uhen 1999; Thewissen et al. 2001b). Most of these fossils are based on limited dental material, but recently uncovered postcranial and cranial material (Thewissen et al. 2001b) suggests a terrestrial lifestyle and artiodactyl affinities. Several ages have been given to Pakicetidae ranging from 55 MYA to 48 MYA (Bajpai and Gingerich 1998; O'Leary and Uhen 1999). The lower Kuldana Formation has been considered of Early Eocene (Ypresian) or earliest Middle Eocene (Lutetian) age, with the latter placement the most commonly and conservatively accepted age (49.0 ± 1.74 MY) (Bajpai and Gingerich 1998; Berggren et al. 1995). This minimum age is based on radiometric interpolation between the earliest and latest Eocene, and a combination of known magnetostratigraphic reversals linked to foraminifera zonation. Gingerich and Uhen (1998) used 49.5 MY to account for pakicetids occurring slightly below the Ypresian-Lutetian border. This corresponds well with placing the Kuldana Formation at the Wasatchian-Bridgerian boundary (49.7–50.7 MYA) (Berggren et al. 1995:309) based on mammalian biostratigraphical correlation (Thewissen et al. 2001a).

Minimum Age Approach

Likelihood (Gingerich and Uhen 1998) and other probabilistic (Marshall 1990a) statistical methods can be used to put confidence intervals on the age of oldest undisputed fossils, from which a maximum age is inferred based on that known fossil record. However, an age distribution of several fossil horizons in combination with small stratigraphic ranges is needed to limit the lower bound of the confidence interval, information that often is not available. When available, as in the history of Cetacea, established maximum ages cannot guarantee that fossils of even older age will never be found (Bajpai and Gingerich 1998). Therefore there are both benefits and limitations to the application of statistical likelihood of estimated maximum ages. Phylogenetic error is difficult to quantify, but its uncertainty is often greater than the geological error and can err in either direction. This is illustrated well with the use of either the age of the mesonychid or of the *Diacodexis* lineage as the upper bound for Artiodactyla-Cetacea. This phylogenetic error can be limited by providing conservative molecular divergence times (see also Eizirik et al. 2001), which are grounded with phylogenetically undisputed fossils. Furthermore, genetic divergences often significantly precede fossil ones in the literature, and repeated testing of these comparisons would be more powerful with use of conservative molecular timescales because the nature of the fossil record makes these scenarios one-tailed.

The whale-artiodactyl divergence highlights the benefits of a diagnostic and continuous fossil record combined with a well-dated early Cenozoic timescale, even though its fossil record is applicable only to one terminal branch and not to the branches immediately preceding the divergence. The uncertainty of taxonomic placement leads to phylogenetic error, particularly if fossils considered for calibration lack diagnostic characters or are placed several nodes further up or down the tree. As a result, the absolute range of minimum to maximum calibration age may exceed 10% of the age of the oldest undisputed fossils, as is the case even in often-used calibrations. This extensive range in time obviously affects evolutionary timescales if calibrated with these fossils. This uncertainty is mirrored by the varied Artiodactyla-Cetacea calibration ages found in the reviewed molecular clock literature (Figures 1 and 4), but can be wide ranging even when focusing on single fossil lineages (Figure 4). Instead, we promote the application of minimum ages in clock calibration using well-diagnosed fossils that derive from well-dated deposits. Resulting timescales will consist of minimum ages that may be in need of future revision, but they benefit from the ability to provide realistic CI's around the individual point estimates.

Molecular Time and Rate Estimation Error

Although genetic error in the concatenated multilocus dataset is smaller than that in single-locus data (3′ UTR, mtRNA only) (Springer et al. 2003), it does outplay fossil error relevant to the minimum age of oldest pakicetid fossils. However, when we partition error by uncertainty due to molecular rate estimation (calibration error consisting of fossil age uncertainty and branch-length error of the calibrated/constrained node) and due to molecular time estimation (branch-length error of the node to be dated), it becomes
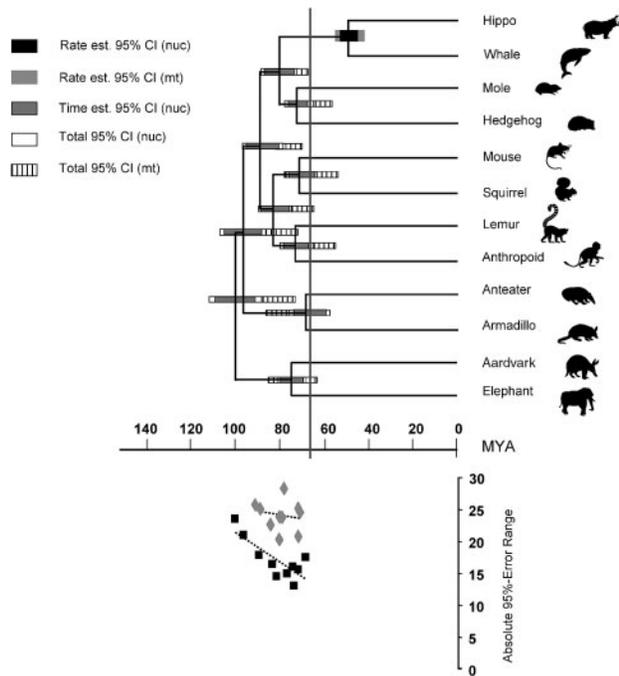
Figure 3. Effect of calibration uncertainty and genetic error on evolutionary timescale interpretation. The phylogeny and point estimates are based on the nuclear data from Springer et al. (2003). Also shown are 95% CIs separately for nuclear and mitochondrial rRNA data, and separately for rate estimation error (fossil plus calibration genetic error), time estimation error (genetic error of node to be dated), and the compounded total clock error (see text for discussion). Below the tree we show the positive correlation between age of nuclear time estimates and their individual error range, and the apparent lack of such a correlation for mitochondrial-based time and error range estimates for ordinal and superordinal nodes.

Figure 4. Comparison of uncertainty in evolutionary divergence times relevant to Artiodactyla-Cetacea. The age uncertainties of Ruminantia-whale and *Hippopotamus*-whale are based on the observed variation in molecular literature (see text), 95% CI is the range discussed in this article (from maximum age + 1.96 SE to minimum age − 1.96 SE). Also shown for comparison are the age uncertainty of the lineages Pakicetidae, *Himalayacetus* (Bajpai and Gingerich 1998; Thewissen et al. 2001b), *Diacodexis* (Tauxe et al. 1994), and the minimum to maximum age range for the whale-artiodactyl divergence estimated by likelihood analyses in Gingerich and Uhen (1998) (minmax-P), and Bajpai and Gingerich (1998) (minmax-H).

clear that uncertainty in rate estimation is extensive. The addition of fossil error alone alters the statistical significance in context with the K-T boundary for some lineages (primates, Eulipotyphla), but this is more pronounced when comparing the addition of molecular rate estimation error to molecular time estimation error only (Figure 3).

We note that studies that have accounted for molecular clock error have based their CIs thus far on the genetic error of the dated node. Springer et al. (2003) noted the decreased resolution from time estimation error for Rodentia. However, despite these statistical limitations, even our most conservative time estimation approach on the concatenated (16 + kb) data yielded no overlap with the K-T boundary for the 95% CI of the following clades: Placentalia, Boreoeutheria, Euarchontoglires, Laurasiatheria, and Afrotheria, although the most recent time estimate for the latter lineage is only at 67.0 MYA. A Cretaceous molecular age for the early placental evolutionary history appears to be a robust genetic phenomenon when based on several nuclear loci. Analyses based on the mitochondrial data alone (Springer et al. 2003) generally yielded younger ages, which in combination with
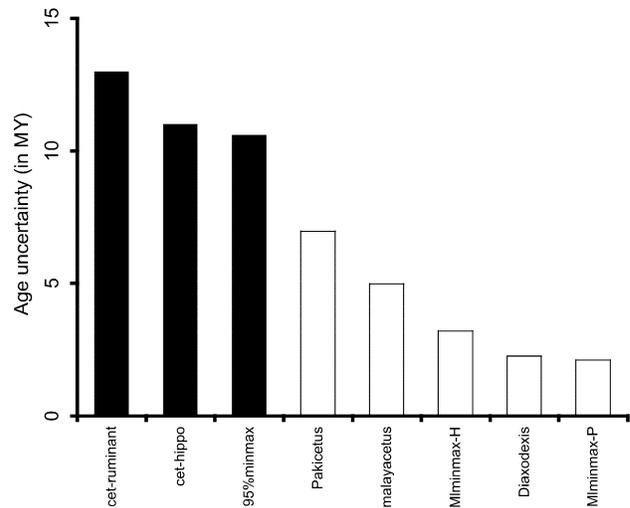
larger genetic error statistically supported Cretaceous diversification for only the basal clades Placentalia, Laurasiatheria, and Arboreoeutheria. The absolute size of the error range around time estimates increases with the age of these time estimates (Figure 3) when based on either nuclear data only ($r = 0.79$) or on the entire concatenated nuclear plus mitochondrial rRNA dataset ($r = 0.80$), but is not correlated with age when based on mitochondrial rRNA data alone ($r = 0.17$). This finding suggests that either among-lineage rate heterogeneity varies more in the mitochondrial genome, or that sampling more characters indeed reduces genetic error.

Conclusion

Pakicetids are the first unambiguous archaeocetes in the fossil record, indicating that the most conservative, or minimum, estimate for the crown Artiodactyla-Cetacea split should be based on the stem age of Cetacea (Figure 2) (*Pakicetus*, 49.0 ± 1.74 MY). *Himalayacetus* may well lie on the stem to Cetacea, following the divergence of whales from artiodactyls. However, its fragmentary condition and age uncertainty make it currently too ambiguous for calibrating the remaining placental tree. Nevertheless, if additional findings confirm the current phylogenetic status of *Himalayacetus* (*sensu* Bajpai and Gingerich 1998) and better resolve the age of the lower

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Subathu Formation or provide more primitive archaeocetes, the minimum age for the divergence of crown Cetacea-*Hippopotamus* divergence may be adjusted back slightly to the earliest Eocene (50.4–53.5 MYA) (Figure 2) as suggested by molecular analyses (Arnason et al. 2000). The fossil record of hippos and anthracotheres, likely sister groups to whales, is currently inadequate to provide better resolution. Similarly the nature and age of the immediate stem to the Artiodactyla-Cetacea divergence is uncertain, and therefore the oldest known artiodactyl *Diacodexis* (which is likely not part of the immediate stem to the divergence of whales from artiodactyls) provides a liberal, or maximum, estimate (55.0 $\pm$ 0.56 MY) (Figure 2) for the Artiodactyla-Cetacea split. The current range for the whale-artiodactyl split is 55.0 ($\pm$0.56) $-$ 49.0 ($\pm$1.74) MYA, with the lower bound preceding the upper bound by 12.2%, about 4% more than in the bird-mammal calibration (van Tuinen and Hadly 2004). This range, lower bound, and upper bound and accompanying error will likely be revised in the future.

The search for errors in the whale-artiodactyl fossil-based divergence exemplifies various techniques employed in dating fossils. These include the radiometric $^{40}K/^{39}Ar$, $^{40}Ar/^{39}Ar$ methods on volcanic ash bounding the fossil strata, biozonation involving foraminifera and mammals, eustatic changes in sea level and magnetic polarity reversal of rocks, global stratigraphic correlation, and interpolation between geological strata. All these methods contain some error, and with the exception of magnetic stratigraphy, do not directly date the fossil. Thus there is intrinsic benefit to calibrating molecular clocks with fossils deposited in strata that are tightly constrained in age. For example, these could include fossil deposits tightly bound on either side by volcanic ashes, or that lie at a major geological boundary, as these are often the emphasis of direct radiometric dating. Many fossil ages currently used to calibrate placental clocks do not derive from such strata, hence calibration error may be significant even for phylogenetically undisputed fossils.

Our analyses are performed on the most recent and largest public placental dataset (Springer et al. 2003), and our conclusions can only be generalized to that dataset. In particular, the effect of chosen tree topology on age estimates is extensive when using instead the tree obtained from complete mitochondrial genomes, a conflict that awaits resolution. The use of more loci, particularly those that need minimal correction for rate heterogeneity and linearity, will aid in increasing the precision in time estimation. Increased accuracy will come only with more optimal calibration points. Assessing molecular divergence time accuracy requires knowledge of paleontology, including phylogenetic uncertainty of important fossils and geochronologic processes used to date fossil deposits. With time estimation becoming routine in molecular studies, we propose to acknowledge and account for all quantifiable uncertainties.

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