Relaxed Clocks and Inferences of Heterogeneous Patterns of Nucleotide Substitution and Divergence Time Estimates across Whales and Dolphins (Mammalia: Cetacea)

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Abstract

Various nucleotide substitution models have been developed to accommodate among lineage rate heterogeneity, thereby relaxing the assumptions of the strict molecular clock. Recently developed "uncorrelated relaxed clock" and "random local clock" (RLC) models allow decoupling of nucleotide substitution rates between descendant lineages and are thus predicted to perform better in the presence of lineage-specific rate heterogeneity. However, it is uncertain how these models perform in the presence of punctuated shifts in substitution rate, especially between closely related clades. Using cetaceans (whales and dolphins) as a case study, we test the performance of these two substitution models in estimating both molecular rates and divergence times in the presence of substantial lineage-specific rate heterogeneity. Our RLC analyses of whole mitochondrial genome alignments find evidence for up to ten clade-specific nucleotide substitution rate shifts in cetaceans. We provide evidence that in the uncorrelated relaxed clock framework, a punctuated shift in the rate of molecular evolution within a subclade results in posterior rate estimates that are either misled or intermediate between the disparate rate classes present in baleen and toothed whales. Using simulations, we demonstrate abrupt changes in rate isolated to one or a few lineages in the phylogeny can mislead rate and age estimation, even when the node of interest is calibrated. We further demonstrate how increasing prior age uncertainty can bias rate and age estimates, even while the 95% highest posterior density around age estimates decreases; in other words, increased precision for an inaccurate estimate. We interpret the use of external calibrations in divergence time studies in light of these results, suggesting that rate shifts at deep time scales may mislead inferences of absolute molecular rates and ages.

Key words: divergence times, molecular clock, Mysticeti, Odontoceti, rate heterogeneity, uncorrelated relaxed clock.

Introduction

The observation that lineages do not accumulate nucleotide substitutions at a constant rate over time, but instead vary in their rates of molecular evolution, has been well documented across the Tree of Life (e.g., Wu and Li 1985; Britten 1986; Martin and Palumbi 1993; Smith and Donoghue 2008). This heterogeneous pattern of nucleotide substitutions between lineages has presented a significant challenge to the estimation of divergence times, one that often results in large discrepancies among independent molecular divergence time estimates and between molecular divergence time estimates and the fossil record (Hillis et al. 1996; Li 1997; Norman and Ashley 2000; Theodor 2004; Pulquério and Nichols 2006). Not surprisingly, the development of models that relax the strict assumptions of the molecular clock, thereby mitigating discrepancies in age estimates, has been an important focus of research in molecular phylogenetics (e.g., Sanderson 1997; Thorne et al. 1998; Huelsenbeck et al. 2000; Yoder and Yang 2000; Aris-Brosou and Yang 2002; Sanderson 2002; Drummond et al. 2006). Rather than expecting genetic divergence to scale linearly with time, these approaches incorporate various models of nucleotide evolution that guide the expected distribution of molecular rates on a given tree, thereby accommodating lineage-specific rate heterogeneity.

A common framework of many of these models is the assumption that molecular rates are autocorrelated, whereby the rates in daughter lineages are inherited from parent branches (e.g., Sanderson 1997; Thorne et al. 1998; Aris-Brosou and Yang 2002; Drummond and Suchard 2010). The expected distribution of autocorrelated rates varies across these approaches, including minimizing change between branches (Sanderson 1997), allowing descendant branches to evolve their own rates (Huelsenbeck et al. 2000; Aris-Brosou and Yang 2002), or modeling the decay of autocorrelation (Lepage et al. 2007). However, a critique of all autocorrelated methods is that the degree of rate autocorrelation is expected to diminish over large phylogenetic scales or in the presence of incomplete sampling (Ho, Phillips, Drummond, et al. 2005; Drummond et al. 2006;

Ho 2009). Furthermore, the distribution of expected rates under most of these models regard shifts in the rates of molecular evolution as unlikely (but see Huelsenbeck et al. 2000).

Assumptions of shifts in molecular rates over larger phylogenetic scales are not without biological justification as physiological and life history components such as generation time, longevity, metabolic rate, DNA repair mechanisms, or population size are often hypothesized to be correlated with molecular rates of evolution (Gillooly et al. 2005; Lanfear et al. 2007; Nikolaev et al. 2007; Nabholz et al. 2008; Smith and Donoghue 2008; Bromham 2009; Galtier et al. 2009). Additionally, deep time scales further confound the signal of rate inheritance, as even molecular rates between lineages that are strongly correlated will be subject to stochastic processes (Hillis et al. 1996), heterotachy (e.g., Philippe and Lopez 2001; Lopez et al. 2002; Zhou et al. 2010), substitution saturation (e.g., Yang 1996; Xia et al. 2003; Phillips 2009; Brandley et al. 2011), or the extinction of lineages, thereby potentially degrading the signal of rate inheritance while driving the inference of a shift in substitution rate. This suggests that lineage-specific molecular rate heterogeneity may be a pervasive feature of macroevolution. Indeed, varying rates of molecular evolution have been well documented among plant (e.g., Soltis et al. 2002; Goremykin et al. 2004; Smith and Donoghue 2008) and metazoan lineages (e.g., Martin and Palumbi 1993; Bromham 2002; Jiang et al. 2007; Nabholz et al. 2008; Wiens et al. 2008).

In cases where the assumption of rate autocorrelation is violated, recently developed Bayesian methods that do not assume rate autocorrelation a priori are expected to perform better in the presence of lineage-specific rate heterogeneity (Ho, Phillips, Drummond, et al. 2005; Drummond et al. 2006; Drummond and Suchard 2010). For example, the Bayesian relaxed clock model of uncorrelated molecular rates allows for the sampling of disparate molecular rates between adjacent branches of a tree by drawing the rate of molecular evolution for each branch from a single continuous parametric distribution, usually in the form of a lognormal or exponential distribution (Drummond et al. 2006). By relaxing the assumptions of rate inheritance, this model has been shown to be more robust to the decay of rate autocorrelation over time (Ho, Phillips, Drummond, et al. 2005; Drummond et al. 2006).

The Bayesian random local clock (RLC) model, on the other hand, incorporates a model averaging approach in which the rate of molecular evolution for a given branch of a phylogeny is either inherited from parent taxa, as in an autocorrelated model, or allowed to vary thereby incorporating punctuated shifts in rate between taxa or taxonomic groups (Drummond and Suchard 2010). Although technically an autocorrelated model of divergence time estimation, the RLC model departs from strict models of autocorrelation through the use of a prior on the number of rate changes to form a prior distribution over model space spanning the spectrum of clock models from a global clock at one extreme to independent rates on every branch at the other. Although the performance of divergence time methods that incorporate uncorrelated and autocorrelated models has been tested (Ho, Phillips, Drummond, et al. 2005; Drummond et al. 2006; Lepage et al. 2007), it is still unclear how either of these relaxed clock models perform in the presence of punctuated molecular rate shifts between lineages or how the modeling of calibration age priors influences their performance in these situations.

As divergence time estimation is a complex problem requiring the simultaneous integration of neontological and paleontological elements, cetaceans (whales and dolphins) provide a useful study system from which to explore how molecular rate heterogeneity and calibration age priors influence the posterior distribution of age and molecular rate inferences. Cetaceans possess a rich fossil record that provides multiple calibration age priors for divergence time analyses (e.g., Gingerich et al. 2001; Deméré et al. 2005, 2008). In addition, there is strong support for significant molecular rate variation between toothed (Odontoceti) and baleen (Mysticeti) whale lineages (Kimura and Ozawa 2002), with the latter exhibiting the slowest rates of molecular evolution in both the mitochondrial and the nuclear genome relative to all other examined mammalian lineages (Martin and Palumbi 1993; Nabholz et al. 2008; Alter and Palumbi 2009; Jackson et al. 2009; Meredith et al. 2009). This observation has been the basis of studies assessing whether these slow molecular rates are correlated with intrinsic biological traits such as metabolic rate or generation time (e.g., Martin and Palumbi 1993; Lanfear et al. 2007; Nabholz et al. 2008; Jackson et al. 2009). However, a large-scale study that explicitly reconstructs patterns of molecular rate evolution across all cetaceans has not been attempted.

In this study, we employ Bayesian phylogenetic analyses using the RLC and uncorrelated rates relaxed clock substitution models for analysis of nuclear and mitochondrial genome data sets of a large sample of cetacean species to explore patterns of nucleotide evolution across the clade's major lineages. As accurate rate estimation is critical for the accurate inference of divergence times, we couple the inferences made from the empirical data sets with a simulation approach to assess the credibility of incongruent results between models and to investigate the performance of these two classes of Bayesian divergence time estimation models in the presence of clade-specific rate heterogeneity. We also use empirical and simulated data sets to assess the influence of calibration placement and temporal uncertainty of calibration ages on the posterior distribution of molecular age estimates and molecular evolutionary rates to evaluate the robustness of our inferences.

Materials and Methods

Framework Data Sets

We assembled two data sets with data downloaded from Genbank (supplementary table S1, Supplementary Material online). The first data set consisted of the 13 protein-coding genes of the mitochondrial genome (ND1, ND2, COX1, COX2, ATP8, ATP6, COX3, ND3, ND4L, ND4, ND5,

ND6, and CYTB) sampled from 32 species of cetacean and three artiodactyl outgroup taxa (*Sus*, *Ovis*, and *Hippopotamus*). The nuclear gene data set comprised four protein-coding genes (RAG1, PRM1, BDNF, and ATP7A) sampled from 51 cetacean species and included several artiodactyls as outgroup taxa. All molecular data were aligned using MUSCLE v3.7 (Edgar 2004), with alignments adjusted by eye. Individual alignment files were concatenated using Phyutility (Smith and Dunn 2008).

As model choice can influence branch length estimation (Abdo et al. 2005; Emerson 2007), we assessed the fit of potential models of DNA sequence evolution for each gene and potential data partition by comparing sample size corrected Akaike Information Criterion (AICc) (Akaike 1973) scores calculated by MrModeltest v2.2 (Nylander 2004) in conjunction with PAUP v. 4.0b10 (Swofford 2003). To account for the potentially misleading influence of the repeated convergent evolution of nucleotide character states (noise) in divergence time estimates (e.g., Phillips 2009; Brandley et al. 2011), we profiled the probability of signal for each marker and codon position prior to analysis using phylogenetic informativeness plots (Townsend 2007) generated using the phydesign web interface (Lopez-Giraldez and Townsend 2011). Although phylogenetic informativeness plots do not predict a linear relationship between the probability of signal and the probability of resolution, the curvature following the peak of informativeness can be interpreted as increasing the potential of noise contributing to phylogenetic inference (see supplementary fig. S1, Supplementary Material online). Following Townsend and Leuenberger (2011), all markers were screened prior to analysis to ensure that the peak probability phylogenetic informativeness occurred subsequent to the most recent common ancestor (MRCA) of all sampled cetacean species.

Paleontological Data

The molecular dating analyses utilized eight fossil-based calibration age priors that represent both deep and shallow divergences within Cetacea. In most cases, we chose fossil taxa whose relationships have been previously inferred by phylogenetic analysis and were representatives of the oldest known occurrences of a clade to provide minimum age calibrations for subsequent divergence time analysis. The date of separation between Hippopotamidae and Cetacea was set to a minimum of 48 Ma derived from the age of *Pakicetus inachus* (Kuldana Formation, Pakistan), the oldest known and most complete stem cetacean that has been integrated into a phylogenetic analysis (Gingerich and Russel 1981; Geisler and Uhen 2003). Crown Cetacea (Mysticeti + Odontoceti) was assigned a minimum date of 34 Ma as this reflects the appearance of *Llanocetus denticrenatus* (La Meseta Formation, Seymour Island, Antarctica) the age of the oldest crown-cetacean fossil (Mitchell 1989; Fitzgerald 2010). Crown Mysticeti was given a minimum date of 28 Ma based on its oldest known representative, an unnamed balaenid from the Lower Kokoamu Greensand, New Zealand (Fordyce 2002; Sasaki et al. 2005). Although this specimen has yet to be included in a phylogenetic analysis, it has been used in multiple recent studies of cetacean rate and age estimation (Jackson et al. 2009; McGowen et al. 2009; Steeman et al. 2009; Slater et al. 2010). Within crown Mysticeti, relationships between modern and fossil species remain contentious (Bouetel and de Muizon 2006; Bisconti 2007; Steeman 2007; Deméré et al. 2008); however, the MRCA node of *Megaptera novaeangliae* and *Balaenoptera physalus* can reasonably be assigned a minimum age of 4 Ma, correlating to the age of *M. hubachi* (Dathe 1983), a Pliocene fossil from the Bahia de Guayacán Formation, Chile, that is closely related to *M. novaeangliae* (Bisconti 2007; Deméré et al. 2008).

The crown age of Odontoceti was set to a minimum of 23.7 Ma based on the appearance of *Ferecetotherium kelloggi* (Perikeshkul, Azerbaijan), as this physeteroid represents the oldest known crown-odontocete fossil (Mchedlidze 1970). We set crown Ziphiidae, Inioidea (*Inia* + *Pontoporia*), and Phocoenidae + Monodontidae to minima of 12, 11.2, and 10 Ma, respectively. These dates are derived from the minimum age of the oldest fossils known from each respective clade and confirmed by phylogenetic analysis: the ziphiid *Nazcacetus urbinai* (Cerros los Quesos, Pisco Formation, Peru; Lambert et al. 2009), the pontoporid *Brachydelphis mazeasi* (El Jahuay, Pisco Formation, Peru; de Muizon 1988; Hamilton et al. 2001), and the phocoenid *Salumiphocaena stocktoni* (Monterey Shale Formation, California, USA; Barnes et al. 1985; Fajardo-Mellor et al. 2006).

Although cetaceans possess a rich fossil record, the nature of fossilization provides a challenge to the setting of upper bounds to the calibration date estimates (e.g., Marshall 1990; Holland and Patzkowsky 2002; Lu et al. 2006; Marshall 2008). Since the width of the calibration age prior distribution has been shown to influence the posterior distribution of Bayesian age estimates (Inoue et al. 2010; Dornburg et al. 2011), we used the FAc equation of Marshall (2008) to guide the designations of soft upper bounds for the calibration age priors. This method takes the potential taphonomic bias of the fossil record and the sampling intensity of the group into account, thereby providing a conservative distribution of credible ages for each fossil calibration (Marshall 2008) (table 1).

Assessing Effects of Fossil Calibration Placement

To determine how posterior parameter estimates are influenced by the interaction of multiple calibration age priors, we incrementally added fossil calibrations to each data set based on the mean empirical scaling factor rankings of the Bayesian based approach to Marshall's (2008) method of calibration selection (table 1; see Dornburg et al. 2011). For every analysis, we monitored the mean and 95% highest posterior density (HPD) interval of age and molecular rate estimates for the crown Odontoceti and Mysticeti. We further multiplied the 95% upper bound estimated by the FAc of Marshall (2008) by factors of two and four and repeated these analyses to assess how increasing the mean prior age across multiple calibration age priors influences rate and time estimates. These treatments represent the practice

Table 1. Calibration Age Priors Used in This Study.

Clade Calibrated	Minimum Age	FA_{95}	Fossil Used for Calibration	Order Added All/Non-Mysti.
Crown Mysticeti	28.0	40	Undescribed Balaenidae Taxon	1/—
Stem Cetacea	48.6	50	*Pakicetus inachus*	2/1
Crown Cetacea	34.0	43	*Llanocetus denticrenatus*	3/2
Monodon + *Phocoena*	10.0	33	*Salumiphocaena*	4/3
Crown Odontoceti	23.7	38	*Ferecetotherium kelloggi*	5/4
Crown Ziphiidae	12.0	34	*Nazcacetus urbinai*	6/5
Balaenoptera physalus + *Megaptera novaeangliae*	4.0	29	*Megaptera hubachi*	7/—
Inia + *Pontoporia*	11.2	33	*Brachydelphis mazeasi*	8/6

NOTE.—Table depicting calibrated clades, reference fossils, minimum ages, and 95% age brackets based on Marshall's (2008) method of absolute age bracketing. The order the calibrations were added to the analysis was based on Dornburg et al.'s (2011) Bayesian approach to Marshall's (2008) empirical scaling factor, and numbers represent the order calibrations were added to analysis sets that included or omitted crown mysticete calibrations, respectively.

in divergence dating of assigning large temporal uncertainty to the prior distributions of calibration ages.

Tracking how multiple prior age calibrations interact to influence the posterior distribution of molecular rate and age estimates also has implications for using calibration age priors outside the lineage of interest (external calibrations) when dating lineages with a poor fossil record (Heled and Drummond 2011). We performed Bayesian phylogenetic analyses incrementally adding only non-mysticete fossils and tracked the age and rate estimates of the most recent common ancestor for Odontoceti and Mysticeti, respectively. In other words, we estimated the age of crown Mysitceti when this node is not calibrated and instead the age estimate is calibrated only by priors on outgroup node ages. We repeated these analyses initially using only a crown Mysiceti calibration to assess the effect of this calibration strategy on the age estimate of crown Odontoceti. To choose the sequence in which we added these sequential calibration age priors, we again used the Bayesian implementation (Dornburg et al. 2011) of Marshall's (2008) empirical scaling factor, but this time using only noncrown mysticete calibrations in the pool of candidate fossils (table 1). These analyses were also repeated using the same 2× and 4× scalar multiplications of fossil age priors as above to track how calibration age prior uncertainty influences posterior inferences of rates and ages when using only external calibrations in a data set with a heterogeneous distribution of molecular rates.

Analytical Conditions of the Bayesian Phylogenetic Analyses

We used BEAST v. 1.6.0 to infer the marginal posterior distribution of ultrametric trees under a model of uncorrelated but lognormal distribution of rates (UCLN) and a RLC model. For each analysis, we ran four to eight independent Markov chain Monte Carlo runs of 60–100 million generations, sampling every 5000 generations, and assigning a birth–death prior to rates of cladogenesis (Drummond and Rambaut 2007). Convergence was assessed by visual inspection of the sampled likelihoods using Tracer 1.5 (A. Rambaut and A.J. Drummond) and the "compare" command in AWTY (Nylander et al. 2008), with 5–15 million generations of each run discarded as burn in. The effective sample sizes (ESSs) for model parameters were quantified in Tracer 1.5 (A. Rambaut and A.J. Drummond) to ensure proper mixing of each chain, with ESS values above 200 indicating appropriate sampling from the posterior distribution of each parameter. To ensure proper rooting, we constrained the monophyly of Hippopotamidae + Cetacea in each analysis and additionally constrained the monophyly of both the odontocete and the mysticete clades in accordance with the results of numerous phylogenetic analyses (e.g., Irwin and Árnason 1994; Milinkovitch 1995; Gatesy 1997; Montgelard et al. 1997; Messenger and McGuire 1998; Gatesy et al. 1999; Ursing et al. 2000; Geisler and Uhen 2003; McGowen et al. 2009; Steeman et al. 2009; Xiong et al. 2009; Slater et al. 2010). Every analysis was repeated with and without data to assess the influence of the prior on the posterior distribution of age estimates (Drummond et al. 2006).

We computed clade-specific distributions to compare the posterior rate or age estimates under each respective model inferred from each fully sampled data set above, we conducted separate analyses of alignments under both a UCLN and a RLC model containing either only mysticetes or only odontocetes and all prior age calibrations belonging to each respective clade. Molecular rates weighted by branch lengths using the meanRate statistic in BEAST 1.6.0 and Tracer 1.5 as well as the age distribution for the root of the mysticete-only or odontocete-only phylogenies. We assumed that these clade-specific posterior distributions are our best estimate of the rates and ages of crown Mysticeti and Odontoceti.

Rate Heterogeneity Simulations

The accuracy and precision of posterior ages and rate estimates inferred under the UCLN and RLC models in the presence of a punctuated rate shift was tested using a set of simulations. We used PhyloGen (v. 1.1) with the speciation rate set to twice the extinction rate (birth = 0.4, death = 0.2) to generate a set of 100 random ultrametric trees of the same size as our cetacean data set. Out of those 100 randomly generated trees, we drew a single topology where the diversity was partitioned into two distinct subclades of similar diversity to mirror the conditions of the cetacean analysis (fig. 1).

To simulate the effect of a punctuated rate shift, we ensured that rates for each clade were drawn from two distinct lognormal distributions of rates whose parametric shapes were partitioned into two rate classes for each data set. As the disparity of rates inferred between cetacean

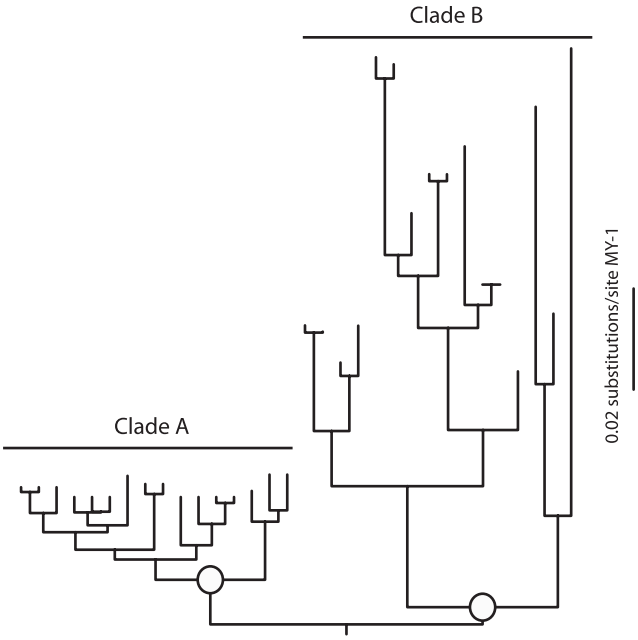

FIG. 1. Graphical representation of simulation design. Two clades of equal diversity yet disparate rates of nucleotide substitution were simulated and calibrated (circles at nodes) using either only a calibration on only the slow clade (clade B) or both clades.

subclades might represent an extreme in vertebrate evolution, our simulation setup was partitioned into two types of rate shifts, each comprising 50 data sets. Set one utilized the rates inferred from separate analyses of the "faster" Odontoceti ($\mu = -6.86$, $\sigma = 0.83$) and "slower" Mysticeti ($\mu = -8.06$, $\sigma = 0.50$) for the parametric shapes. In contrast, set two utilized the same rate parameters for the faster clade but increased the molecular rate parameters in the Mysticeti distribution by an order of magnitude when drawing rates for second clade ($\mu = -7.82$, $\sigma = 0.50$). For both sets, we simulated single-gene alignment of 1,000 nt in length using SeqGen (v. 1.5.3) with the simulated topology above under a general time reversible (GTR) model of nucleotide substitution with parameters set to reflect the inferred parameters of our cetacean data set.

Each simulation data set was analyzed using both a UCLN and a RLC model in BEAST v. 1.6.0 using a GTR + G model to mirror the same analytical conditions as the empirical analyses. Simulated data sets were subjected to two external age calibration strategies: 1) calibrating only the crown node of the slow rate clade (clade A, fig. 1) or 2) calibrating the crown node of both slow and fast rate clades (fig. 1). External age priors were modeled using a lognormal distribution with a mean of 1 and a standard deviation (SD) of 0.5 with the offset ensuring the median value of the 95% prior age interval reflected the known age. To assess how increasing temporal uncertainty influences the posterior sample of molecular rate and absolute age estimates, the width of the initial credible prior age interval was doubled and quadrupled and all analyses repeated.

Shifting Rates of Molecular Evolution in Cetaceans

We reexamined the hypothesis of Kimura and Ozawa (2002) that odontocetes and mysticetes possess disparate distributions of molecular rates in their both mitochondrial and nuclear genome. To examine shifts in the rate of molecular evolution in cetaceans at a finer scale, we tracked the inferred number of rate shifts in the RLC analyses that contained our complete fossil calibration set and calibration prior intervals reflecting Marshall's FAc95 for both the mitochondrial DNA (mtDNA) and the nuclear DNA data sets. The inferred number of rate shifts in each case was compared with the prior probability density of rate shifts inferred in the absence of sequence data. If the posterior probability for 0 rate changes was higher than the prior probability, this was seen as evidence for a global clock, whereas posterior probabilities that largely excluded 0 rate changes were seen as strong rejections of the molecular clock hypothesis.

Results

Clock Models and Cetacean Evolution

The rate distributions of the pruned data sets containing only odontocete or mysticete lineages confirmed the hypothesis of Kimura and Ozawa (2002) that these subclades contain disparate rates of molecular evolution (fig. 2). Under the UCLN model, the estimated mean rate of nucleotide evolution in mysticetes was 2.20×10^{-4} substitutions/site/My (95% HPD: 1.76×10^{-4}, 3.29×10^{-4}) for the nuclear data set and 5.63×10^{-3} (95% HPD: 4.54×10^{-3}, 6.66×10^{-3}) for the mtDNA genes. In both data sets, these rate estimates were three to four times slower than the rate estimates of odontocetes, whose mean rates were estimated as 9.10×10^{-4} (95% HPD: 6.68×10^{-4}, 1.18×10^{-3}) for the nuclear data set and 1.38×10^{-2} (95% HPD: 1.23×10^{-2}, 1.54×10^{-2}) for the mtDNA genes (fig. 2). These results were congruent with the clade estimates in the RLC framework. Odontocete mean rates were estimated with a mean of 1.01×10^{-3} (95% HPD: 6.77×10^{-4}, 1.47×10^{-3}) for the nuclear data set and 1.62×10^{-2} (95% HPD: 1.44×10^{-2}, 1.18×10^{-2}) for the mtDNA. Mysticete substitution rates were estimated with a mean of 3.02×10^{-4} substitutions/site/My (95% HPD: 2.14×10^{-4}, 3.94×10^{-4}) for the nuclear data set and a mean of 6.78×10^{-3} substitutions/site/My (95% HPD: 6.04×10^{-3}, 7.46×10^{-3}) for the mtDNA genes.

Assuming the above distributions are the best estimates of molecular rates in the two clades, we use these as clade-specific distributions to assess the performance of the molecular clock models under the varying calibration schemes. Analyses of the full mitochondrial genome or nuclear gene data sets (i.e., all cetaceans and outgroups) were unable to recover these target rate distributions with high precision using the UCLN model (figs. 3 and 4, supplementary figs. S2 and S3, Supplementary Material online). Increasing the width of the calibration age priors (double and quadruple) led to slower estimates of molecular rates (albeit frequently deviating from the target) (fig. 3, supplementary fig. S2,

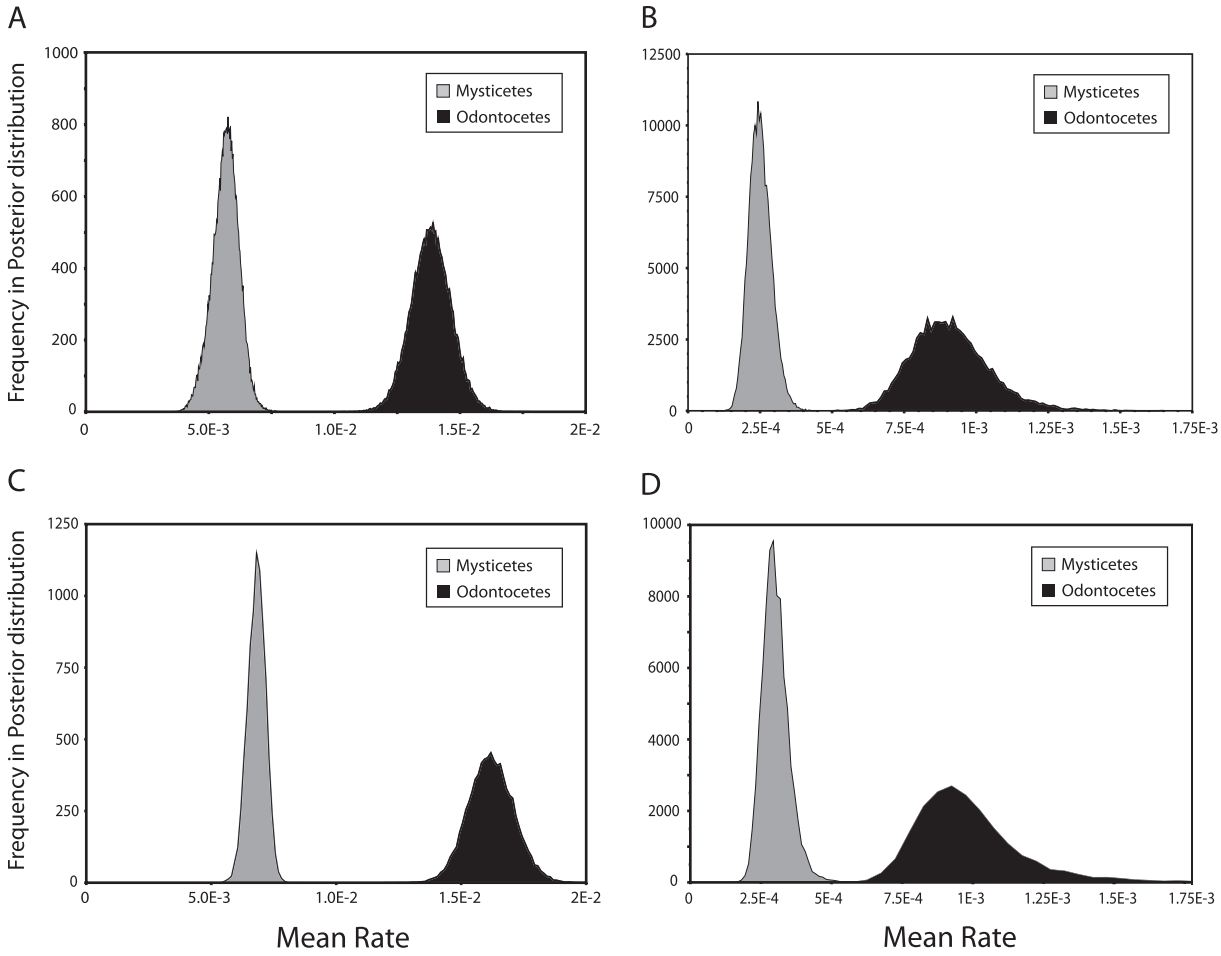

Fig. 2. Molecular rate distributions for crown Odontoceti and Mysticeti in the (*A*) UCLN framework mitochondrial data set; (*B*) UCLN framework nuclear data set; (*C*) RLC framework mitochondrial data set; and (*D*) RLC framework nuclear data set.

Supplementary Material online), as a consequence of larger credible prior age intervals biasing toward older age estimates (fig. 4, supplementary fig. S3, Supplementary Material online) (e.g., Inoue et al. 2010; Dornburg et al. 2011). Regardless of the temporal uncertainty modeled in the prior age calibration, the placement of calibrations had the most profound influence on posterior rate estimates.

In general, calibrating only the mysticete or odontocete cetacean subclades in the UCLN framework led to a tighter 95% HPD interval widths for rate estimates, whereas the presence of taxa from the other subclade in the DNA alignment altered the inferred mean and 95% HPD interval of rate estimates, often away from the target distribution (fig. 3). In contrast to the UCLN results, the RLC framework was more robust to the placement of prior age calibrations that potentially altered posterior rate estimates in the nuclear gene data set. When mysticete calibrations are added first, virtually no change was observed in the posterior rate estimates of crown mysticetes. Likewise, when both the odontocete and the mysticete subclades were calibrated in the UCLN framework the 95% HPD interval of rate estimates width almost tripled in some cases (fig. 3), whereas the nuclear gene based 95% HPD interval of rate estimates either remained consistent or decreased the 95% HPD interval of rate estimates (fig. 3, supplementary fig. S2, Supplementary Material online). This pattern changed when analyzing the mtDNA data set.

Compared with posterior rate estimates, posterior age estimates inferred under the UCLN and RLC models were inversely affected by the modeling of the credible interval. For posterior age estimates, with the width and lower bound of the 95% HPD interval of ages increased when the 95% credible interval of the calibration age prior increased. The addition of precision in the prior age settings resulted in older mean ages and in some cases shifted the accuracy of the mean age outside the target interval (fig. 4, supplementary fig. S3, Supplementary Material online). In the presence of limited prior age constraints, the UCLN model was more influenced by the modeling of calibrations; however, the performance of both models was similar once the number of calibrations was increased.

In both the nuclear and the mtDNA data sets, the estimated mean age of the MRCA of Mysticeti, when calibrated with the crown mysticete fossils, fell within the target mysticete interval (fig. 4). The addition of noncrown mysticete calibrations either did not effect or only slightly increased the uncertainty of these estimates. Increasing the 95% credible interval of prior ages for this calibration resulted in much older age estimates. However, when the crown mysticete prior age calibrations were excluded from

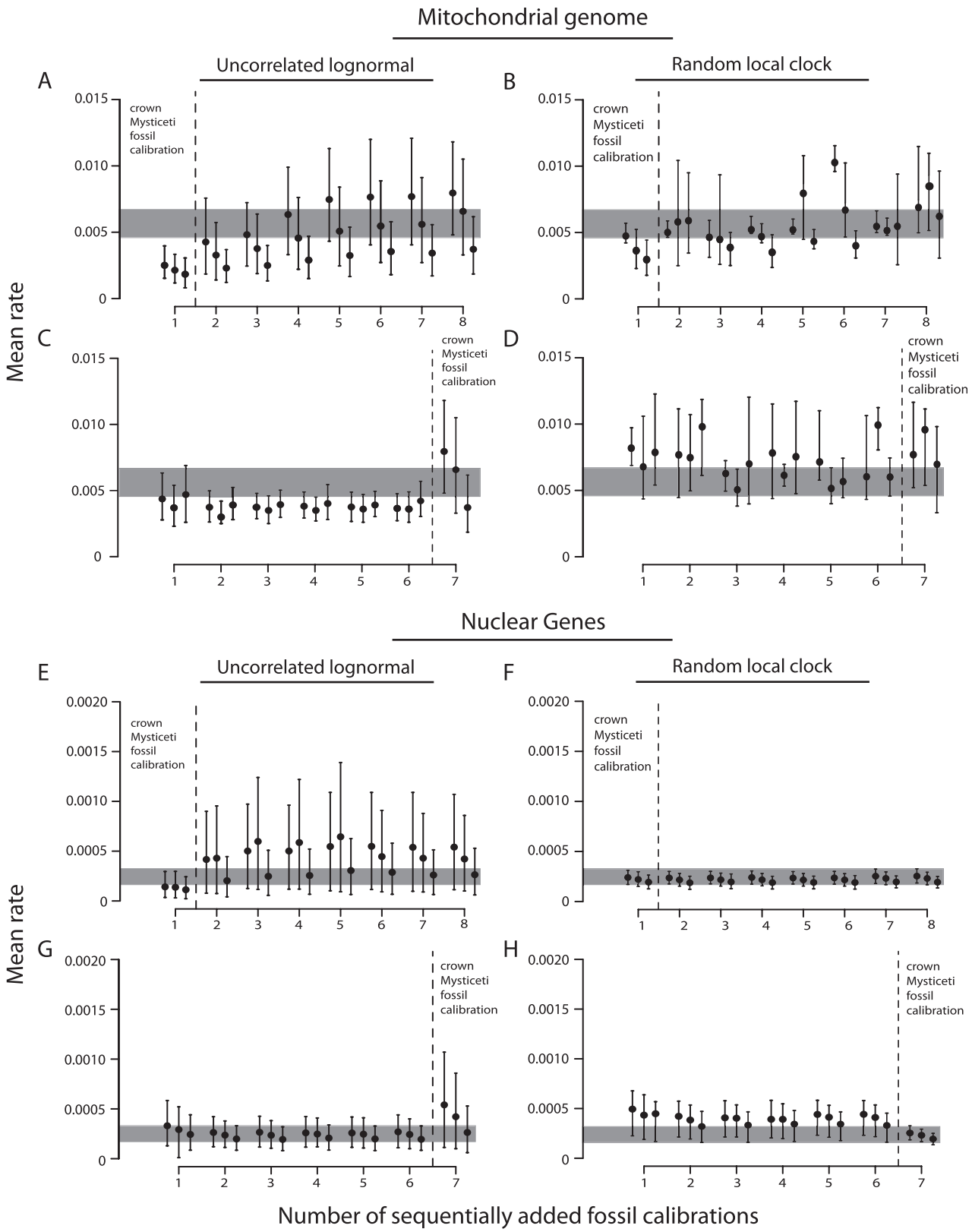

FIG. 3. Molecular rate estimates for crown Mysticeti in the UCLN and RLC frameworks. The *x* axis reflects the number of sequentially added fossil calibration age priors. Circles and solid vertical lines represent the mean and 95% HPD interval of the estimated molecular rate. Each fossil calibration is represented by three values which represent rates estimated using fossil prior age calibrations calculated using Marshall's (2008) FA_{95} and the multiplication of these priors by two and four. (*A* and *B*) the mtDNA data set first calibrating with mysticete fossils, (*C* and *D*) the mtDNA data set first calibrating with noncrown mysticete fossils, (*E* and *F*) the nuclear data set first calibrating with mysticete fossils, and (*G* and *H*) the mtDNA data set first calibrating with noncrown mysticete fossils. Analyses to the right of dotted lines represent the use of both mysticete and odontocete fossil calibrations.

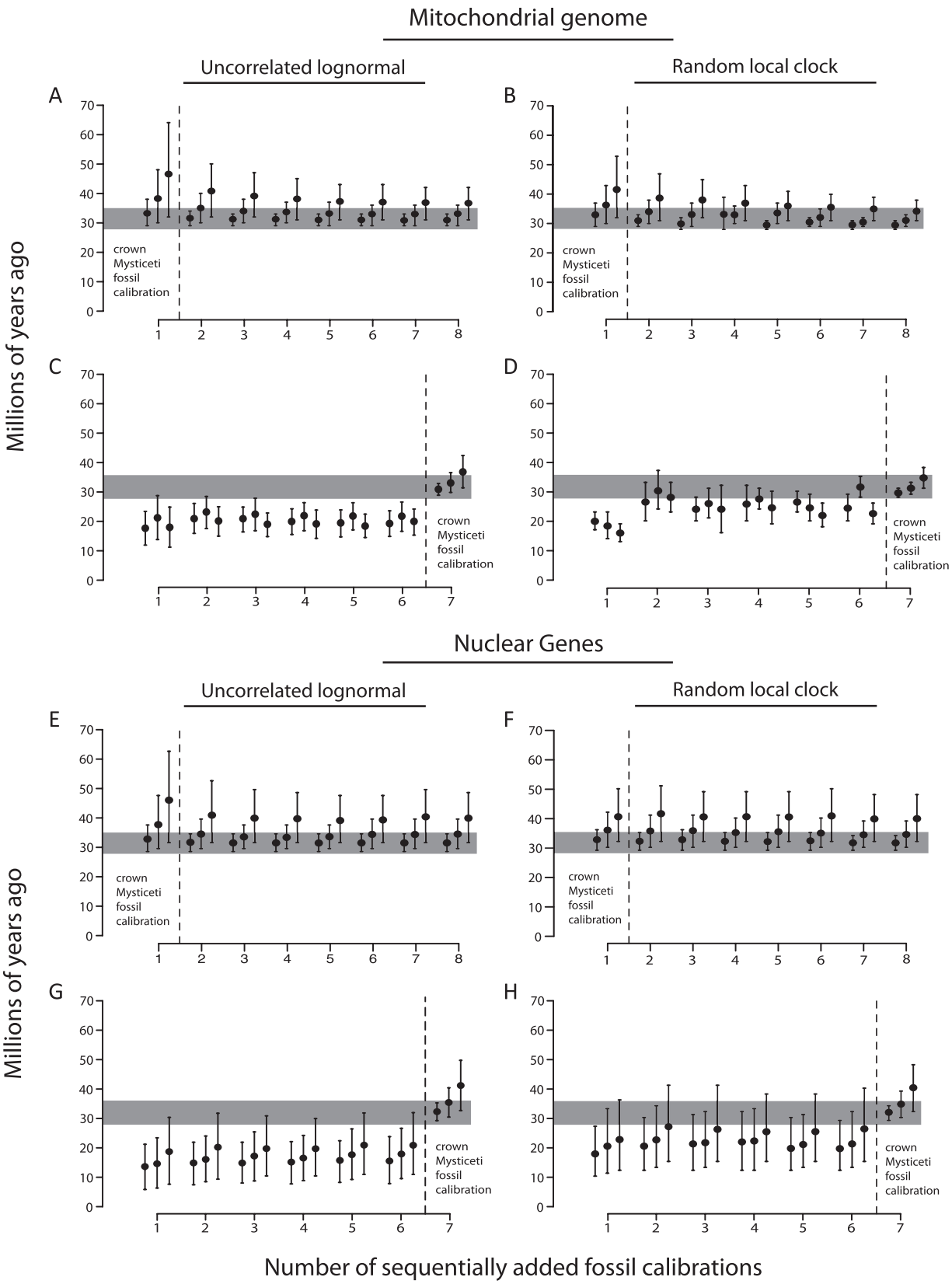

Fig. 4. Molecular age estimates for crown Mysticeti in the UCLN and RLC frameworks. The *x* axis reflects the number of sequentially added fossil calibration age priors. Circles and solid vertical lines represent the mean and 95% HPD interval of the estimated molecular rate. Each fossil calibration is represented by three values which represent rates estimated using fossil prior age calibrations calculated using Marshall's (2008) FA_{95} and the multiplication of these priors by two and four. (*A* and *B*) the mtDNA data set first calibrating with mysticete fossils, (*C* and *D*) the mtDNA data set first calibrating with noncrown mysticete fossils, (*E* and *F*) the nuclear data set first calibrating with mysticete fossils, and (*G* and *H*) the mtDNA data set first calibrating with noncrown mysticete fossils. Analyses to the right of dotted lines represent the use of both mysticete and odontocete fossil calibrations.

the analysis, the 95% HPD interval of age estimates for the MRCA of all mysticetes in the nuclear data set was much younger than the target distribution in the UCLN framework; in most cases, the two intervals were mutually exclusive (fig. 4). Except in the presence of single fossil calibrations, crown mysticete ages estimated in the RLC framework almost always overlapped with the target distribution, although the distribution means are younger as in the ULCN analysis.

Rate Heterogeneity Simulations

Results of simulations showed that the UCLN model estimated 95% HPD intervals that accurately span the true rates nearly 100% of the time, whereas the RLC only overlaps with the target rates in approximately 75% of the simulations (table 2). However, the increased accuracy of the UCLN model was achieved at the cost of lost precision. Compared with the rate estimates of the RLC, the coefficient of variation for UCLN generated mean rate estimates that were nearly always two orders of magnitude higher. The presence or absence of prior age calibrations on the crown age of both simulated subclades also did not have much of an influence in the coefficient of variation of posterior rate estimates, mirroring the 95% HPD interval of odontocete nucleotide substitution rates under different prior treatments (supplementary figs. S2 and S3, Supplementary Material online).

The importance of precision in molecular rate estimates for the purpose of divergence time estimation was highlighted by the contrasting ability of the UCLN and RLC models to infer the true time of divergence in the presence of a rate shift with an absence of prior age information. Despite near 100% accuracy in rate estimates, the UCLN model performs with very low accuracy in divergence time estimates in both simulation sets. Moreover, the uncertainty in the temporal range of the calibration age priors had a pronounced effect on the age inference, similar to the empirical results in other studies (e.g., Inoue et al. 2010; Dornburg et al. 2011). In contrast, the RLC model was less influenced by the increased temporal uncertainty, maintaining a similar degree of accuracy in all but the most extreme prior manipulations (table 2). Results of our simulations highlight that clade-specific rate shifts represent a model misspecification that will tend to mislead posterior rate time estimates in the UCLN frameworks when only one of the clades is calibrated as the differences in rates across the two clades are not easily detected. In the absence of additional prior information, the UCLN model can fit the data with a smaller SD of the lognormal distribution of rates, however, the addition of prior age calibrations in both subclades forces the model to accommodate the two mutually exclusive rate distribution by fitting a very large SD of the lognormal distribution of rates across sites (table 2).

Shifting Rates of Molecular Evolution in Cetaceans

Regardless of the calibration strategy, we found evidence for upwards of ten punctuated changes in the molecular clock rate in the cetacean mtDNA data set (fig. 5). Our results suggest rate change estimates to be influenced by the calibrations present, with a mean of 12 changes inferred in the absence of mysticete calibrations and a mean of 15 in the presence of mysticete prior age calibrations. However, the 95% HPD interval of rate change counts substantially overlapped between all prior manipulations and never dropped below ten, rejecting the model's prior expectation of a strict molecular clock. We found substantially fewer rate changes in the nuclear genes. Although still substantially updating the prior expectations (supplementary figs. S4 and S5, Supplementary Material online), we infer between 3 and 5 shifts, with a median of 3. These shifts in nucleotide substitution rate were congruent with the rate shifts inferred for the mitochondrial genomes, again highlighting the shift between the odontocete and the mysticete clades.

These results further substantiate Kimura and Ozawa's (2002) observation of rate differences between odontocetes and mysticetes but suggest nucleotide evolution to be more complex within each subclade. For the mitochondrial genome, we provide evidence of at least seven local clocks within Odontoceti, with the clade comprising the river dolphin families Iniidae and Pontoporiidae evolving at a mean rate of 1.83×10^{-2} substitutions/site/My (95% HPD: 1.52×10^{-2}, 2.2×10^{-2}) that is over double the mean rate of 8.5×10^{-3} substitutions/site/My (95% HPD: 1.80×10^{-3}, 1.16×10^{-2}) inferred for crown odontocetes (fig. 5). Likewise, the tempo of nucleotide evolution within Mysicetes was inferred to be equally complex, with at least six local clocks supported in our analysis, including the shift between humpback whales (*M. novaeangliae*) and fin whales (*B. physalus*), the latter having a mean molecular clock rate of 1.1×10^{-2} substitutions/site/My (95% HPD: 9.0×10^{-3}, 1.2×10^{-2}), that is, 50% faster than that of its sister taxon 7.7×10^{-3} substitutions/site/My (95% HPD: 5.6×10^{-3}, 9.5×10^{-3}) (fig. 5).

Discussion

We find strong evidence for multiple novel punctuated shifts in nucleotide substitution rates between cetacean lineages, suggesting patterns of molecular evolution in this group are more complex than previously suggested. Based on empirical analysis of the cetacean data sets and simulations, we find that altering taxon-sampling strategies in the UCLN framework influences posterior rate estimates that reflect the global distribution of rates based on the taxa present in the DNA alignment. Our simulations highlight that imprecise rate estimates can lead to poor posterior age inferences in Bayesian divergence time analyses when the UCLN model is misspecified, particularly in the absence of prior age calibrations for focal nodes. We find the RLC model to be better equipped for handling punctuated rate shifts over broad macroevolutionary time scales. Moreover, the RLC framework appears to be more robust to systematic biases induced by prescribing large temporal uncertainty to the calibration age prior. Although we use cetaceans as a case study, our results are applicable to relaxed clock dating analyses of any subset of the Tree of

Table 2. Accuracy and Variance of Posterior Age and Rate Estimates for Clade B under Simulation Conditions (50 simulation replicates per condition).

	Posterior Age Estimates				Posterior Rate Estimates			
	UCLN		RLC		UCLN		RLC	
Analyzed Model	SD/ Coeff. Var.	Acc.	SD/ Coeff. Var.	Acc.	SD/ Coeff. Var.	Acc.	SD/ Coeff. Var.	Acc.
Set A., calibration A	11.1/0.186	0.51	16.6/0.327	0.84	0.0004/0.443	1.00	0.0007/0.677	0.78
Set A., calibration A and B	0.255/0.008	1.00	3.84/0.126	1.00	0.249/4.57	0.96	0.0005/0.423	0.76
Set A., calibration A, double prior age interval	12.84/0.194	0.40	20.84/0.372	0.84	0.0877/5.08	0.96	0.0007/0.697	0.74
Set A., calibration A and B, double prior age interval	1.00/0.029	1.00	0.716/0.022	1.00	0.026/3.89	1.00	0.0005/0.428	0.80
Set A., calibration A, quadruple prior age interval	0.0469/0.0011	0.24	0.0273/0.00067	0.84	0.525/6.34	0.98	0.0001/0.327	0.76
Set A., calibration A and B, quadruple prior age interval	0.341/0.0089	0.00	0.9918/0.0247	0.60	0.428/4.90	0.96	0.0001/0.365	0.84
Set B., calibration A	10.89/0.187	0.44	14.05/0.271	0.70	0.001/0.721	1.00	0.0005/0.578	0.78
Set B., calibration A and B	0.299/0.009	1.00	0.466/0.015	1.00	0.002/0.854	1.00	0.0009/0.635	0.66
Set B., calibration A, double prior age interval	11.2/0.183	0.3	17.14/0.299	0.62	0.044/4.56	0.98	0.0004/0.573	0.78
Set B., calibration A and B, double prior age interval	1.26/0.035	1.00	1.70/0.051	0.98	0.723/5.95	0.94	0.0007/0.551	0.78
Set B., calibration A, quadruple prior age interval	0.046/0.001	0.12	0.0378/0.0009	0.78	0.960/40.27	1.00	0.0001/0.223	0.82
Set B., calibration A and B, quadruple prior age interval	0.321/0.008	0.00	1.03/0.026	0.44	0.853/38.01	1.00	0.0001/0.235	0.86
Accuracy summary (not calibrated/calibrated)		0.34/0.66		0.77/0.84		0.99/0.98		0.77/0.78

NOTE.—Accuracy (Acc.), measured as the proportion of times the 95% HPD interval overlapped the known age and distribution of rates in each simulation set, SD, and the coefficient of variation (Coeff. Var.), for posterior rate and age estimates between the UCLN and the RLC models under different simulation conditions for the MRCA of clade B. Calibrations and clade names correspond to figure 1. Set A corresponds to molecular rate simulations based on the empirical rates estimated from odontocetes and mysticetes independently. Set B corresponds to simulations where the molecular rate of clade A was doubled to reduce the rate disparity between clades. Bold values indicate more than a 10% increase in accuracy between models.

Life that exhibits punctuated shifts molecular evolutionary rates within a specific subclade or subset of branches.

Patterns of Molecular Evolution in Cetaceans

It is well established that cetaceans, in particular baleen whales (Mysticeti), possess rates of molecular evolution that are slow relative to other mammals (e.g., Martin and Palumbi 1993; Bininda-Emonds 2007; Nabholz et al. 2008; Jackson et al. 2009; Meredith et al. 2009; Ho and Lanfear 2010; this study). The factors that underlie these slow rates are less clear, but longevity, generation time, body size, and metabolic rate are often invoked as the most likely correlates to slower molecular rates in baleen whales (Martin and Palumbi 1993; Jackson et al. 2009). The disparate rates of nucleotide evolution between these sister clades also complement some evidence that odontocetes and mysticetes diverged in both ecology and body size early in their evolutionary history (Fordyce and Barnes 1994; Fordyce 2003; Steeman et al. 2009; Slater et al. 2010). Our inference of over ten local clocks best fitting the tempo of mitogenome evolution within cetacean subclades corresponds with both the hypothesis that patterns of rapid phenotypic diversification may extend beyond this initial divergence to the earliest branching lineages of crown odontocetes (Slater et al. 2010) and the hypothesis of diversification in body size beginning in the Late Oligocene based on fossil evidence (Fitzgerald 2006).

Although crown cetaceans many have undergone rapid diversification along ecomorphological axes in their early history, there are differing accounts of associated diversification rate shifts during this time period that may also bear on our inferred shifts in nucleotide substitution rates. Steeman et al. (2009) detected a distinct signature of lineage diversification rate shift near the base of crown Cetacea and correlated this diversification to global changes in currents and climate derived from the opening of the Circum Antarctic Current (e.g., Pastene et al. 2007; Steeman et al. 2009). As patterns of molecular evolution are influenced by a host of factors, ranging from generation time (e.g., Smith and Donoghue 2008), body size (e.g., Martin and Palumbi 1993; Mooers and Harvey 1994; Bromham 2002; Jackson et al. 2009), mating system (Bromham and Leys 2005), DNA repair efficiency (e.g., Drake et al. 1998; Baer et al. 2007), population size (Woolfit and Bromham 2003), and metabolic rate (e.g., Nabholz et al. 2008; Welch et al. 2008), the oceanic changes during Cenozoic had the potential to influence cetacean molecular evolution (Steeman et al. 2009; Marx and Uhen 2010). Alternatively, Slater et al. (2010) did not detect a shift in diversification rates and posit that rates of cladogenesis and morphological innovation were coupled, but extinction eroded the signature of the early bursts of lineage diversification (e.g., Nikaido et al. 2001). If lineage turnover is high in this clade (e.g., Nikaido et al. 2001; Slater et al. 2010), then

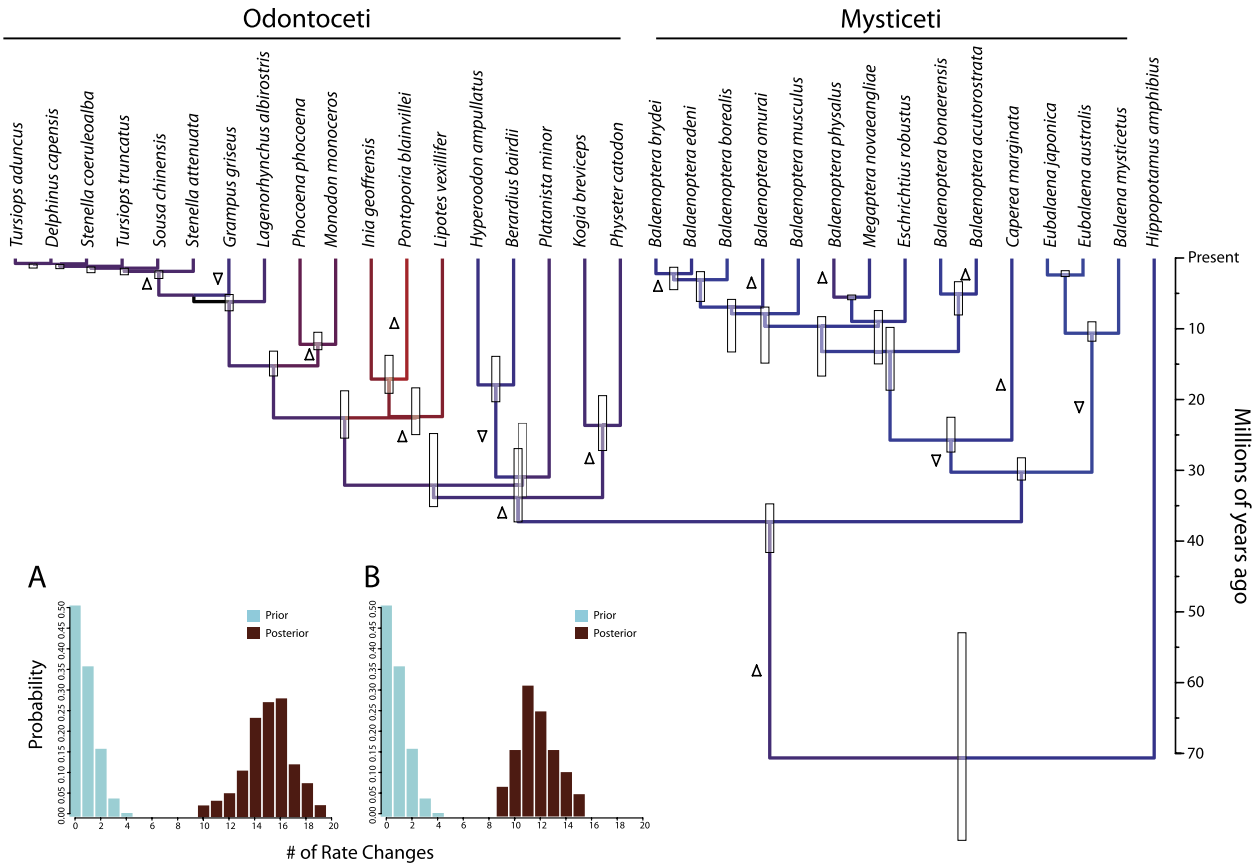

FIG. 5. Divergence times and inference of RLCs in the cetacean mitochondrial genome. Light 95% HPD interval bars indicate posterior probabilities greater than 0.95. Branch lengths are colored to indicate substitution rates with blue branches indicating slow and red indicating fast rates of nucleotide substitution. Arrows represent inferred local clock changes, with the arrows indicating the direction of the rate shifts. (*A*) Compares the prior and posterior distributions of local clock changes across the cetacean tree when all calibrations are employed. (*B*) Compares the prior and posterior distribution of rate changes when only non-Mysticeti calibrations are used.

a further breakdown in the pattern of nucleotide substitution rate inheritance between extant taxa is expected.

Although the forces driving molecular evolution in cetaceans are still being investigated, it is important to recognize that cetaceans did not evolve in isolation and have shared biogeographic conditions with taxa from across the Tree of Life. Although the specific life history and ecomorphology of whales and dolphins may be unique, shifts in phenotypes and life histories are often associated with clade definitions across the Tree of Life. This opens up the possibility that shifts in nucleotide substitution rates are a common feature of macroevolution.

Relaxed Clock Models and Punctuated Rate Shifts

Bayesian and maximum likelihood based branch length estimates are the product of interactions between molecular rates and the passage of time. Given no prior information on the timing of cladogenic events in the phylogeny, inferring the mechanism that generated a relatively long branch as a function of a low substitution rate coupled with an older age may be just as probable as inferring a higher rate coupled with a younger age (Felsenstein 1981; Yang 2005). Choosing between competing hypotheses of age and rate requires the integration of external age information (e.g., Near et al. 2005; Yang and Rannala 2006; Rannala and Yang 2007; Rutschman et al. 2007; Marshall 2008; Ho and Phillips 2009; Inoue et al. 2010; Dornburg et al. 2011) and a model of the molecular substitution process (e.g., Ho, Phillips, Drummond, et al. 2005; Lepage et al. 2007; Phillips 2009; Brandley et al. 2011) to decouple branch length estimates of rate and time (see reviews in Rutschmann 2006; Ho 2009).

Investigation of the effects of punctuated shifts in substitution rates on molecular age inferences is generally an unexplored area of relaxed clock models. We find that in the UCLN framework, exclusive molecular rate categories between clades can either bias or mislead the estimation of molecular rate estimates across a tree by inferring distributions of rates that represent an average of the two mutually exclusive rate classes, thereby compromising posterior parameter estimates. This finding is most likely a consequence of shifts in the molecular rate of evolution violating the UCLN model's assumption that the global distribution of substitution rates is reflected by a unimodal distribution. In the worst case, the presence of taxa within a subclade that possess disparate nucleotide substitution rates from other taxa in the DNA alignment may influence the posterior estimates to reflect the average of the distributions of rates between the two lineages, thereby poorly estimating rates and dates for both groups (figs. 3 and 4). For example, when

odontocetes or mysticetes are analyzed individually, the 95% HPD intervals of rate estimates for these clades are mutually exclusive in both the mitochondrial and the nuclear genome regardless of the model employed (fig. 2). However, in the UCLN framework, incorporating taxa that span all of Cetacea in the alignment, regardless of calibration strategy leads to large 95% HPD intervals of rates, that while potentially accurate, lead to a loss of precision that could potentially compromise age estimation and downstream comparative analyses (table 2). Once additional calibrations are added to calibrate the taxa with disparate rates, the interval of posterior rate estimates experiences a 3- to 5-fold loss in precision and in some cases shifts to reflect a distribution intermediate to the two target distributions.

In contrast, the RLC model performs more consistently in the presence of clade-specific rate heterogeneity both empirically and in simulation. This shift in performance is most likely due to the model relaxing the assumption that substitution rates need to vary across an entire tree (e.g., Bromham and Penny 2003), instead allowing for discrete punctuated shifts in nucleotide substitution rates (Drummond and Suchard 2010). In simulation, we find the coefficient of variation of posterior rate estimates in the RLC to be one to two orders of magnitude smaller than for the UCLN, suggesting this model to be far more precise. The more precise rate estimates also led to more accurate age estimates in the absence of fossil information for one clade, even when the temporal uncertainty around calibrations is amplified (table 2).

However, similar to Drummond and Suchard (2010), we encountered severe problems with the mixing of the Markov chains when using the RLC model. Even with multiple runs over 100 million generations, we frequently estimated differing posterior rate and age estimates with similar likelihoods. We hypothesize that, when two or more distinct distributions may explain the rate of a given node equally well, but these alternate states are separated by moves requiring large transitions, the Markov chain is only sampling one of these distributions instead of the entire posterior distribution. Thus, instead of estimating rate distributions broadly, multiple analyses can infer different precise distributions. This is most likely the result of our using the default transition kernels, though fully exploring possible transition kernels that can integrate over clock shifts and associated tree structures is outside the scope of this work and represents a fruitful area of future research (e.g., Hoehna and Drummond 2011).

Rate Heterogeneity and the Fossil Record

Although our age estimates were robust to punctuated rates shifts in the presence of prior fossil-based age information for focal nodes, given the incompleteness of the fossil record for most taxonomic groups, it is far more common for investigator to not be able to calibrate nodes within a clade of interest for studies of molecular divergence times (e.g., Raup 1972; Strauss and Sadler 1989; Marshall 1990). Frequently investigators utilize fossil taxa to calibrate the ages of lineages external to the group of interest (i.e., outgroups or sister clades) and subsequently infer rates and dates of the ingroup nodes. Our results could have significant implications for the practice of using external age calibrations for estimating the evolutionary divergences of clades with a depauperate fossil record.

For example, in contrast to the fossil record of whales, paleontological estimates suggest that only 5% of primate species diversity is preserved in the fossil record (e.g., Tavaré et al. 2002). It is therefore not surprising that the divergence times marking the origin of primates (e.g., Yoder and Yang 2000; Steiper and Young 2006; Chatterjee et al. 2009; Wilkinson et al. 2011) and the rise of various primate subclades (e.g., Rosenberger 2002; Takai et al. 2000; Kay et al. 2008) have long been debated. The sparse nature of the primate fossil record makes employing prior age calibrations external to the origin of several major subclades critical to understanding the tempo and mode of diversification in our closest relatives. Although Drummond and Suchard (2010) demonstrated the clock-like nature of several loci in anthropoid primates, there is evidence of rate shifts between and within major clades and loci (e.g., Goldberg et al. 2003; Tsantes and Steiper 2009). Given our finding of up to 14 local clocks across a subsample of cetaceans, the diversity of life history strategies, physiology, and ecology witnessed across all primates suggests multiple, as yet undiscovered, local clocks could be heterogeneously ticking across the mitochondrial and nuclear genomes of this group, posing a challenge when dating the evolutionary divergences of our closest relatives.

Dating groups with a depauperate fossil record is inherently challenging as predicting the presence of substitution rate shifts a priori in studies of divergence times is not trivial (e.g., Bromham 2009). However, the variation in life history and physiology present at larger temporal scales suggests that marked disparities in molecular evolutionary rates may be common macroevolutionary phenomena (e.g., Soltis et al. 2002; Jiang et al. 2007; Jorba et al. 2008; Nabholz et al. 2008; Smith and Donoghue 2008; Wiens et al. 2008). We stress that it is not always obvious when a data set violates the UCLN model's assumption of a unimodal distribution of rates and emphasize that our findings are not necessarily a broad condemnation of external calibrations.

As the specter of rate heterogeneity has haunted phylogenetic approaches to divergence time estimation since the inception of the molecular clock, the continued development and testing of increasingly sophisticated models that account for the idiosyncratic nature of nucleotide evolution will be critical in our ability to untangle the complex processes that have set the tempo of diversification across the Tree of Life. Although our results illustrate that rate and date estimates are influenced by the outgroup taxa used, the RLC model could be employed to highlight focal nodes to carefully scrutinize if age estimates appear suspect. Although careful scrutiny of model parameter estimates, for example, insuring the SD of the UCLN relaxed clock is lower than the mean rate (Drummond AJ, personal communication), will assist in diagnosing problematic age estimates, congruence of date estimates between the RLC and the UCLN model could be used as evidence for node age

inferences in the absence of a robust fossil record. Future work on model comparison methods that allow for the fit of nonnested relaxed clock models to be compared directly may also be employed to differentiate between competing posterior parameter estimates inferred under the RLC and UCLN frameworks.

Supplementary Material

Supplementary figures S1–S5 and supplementary table S1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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