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# Accommodating Heterogenous Rates of Evolution in Molecular Divergence Dating Methods: An Example Using Intercontinental Dispersal of *Plestiodon* (*Eumeces*) Lizards

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*Abstract.*—Identifying and dating historical biological events is a fundamental goal of evolutionary biology, and recent analytical advances permit the modeling of factors known to affect both the accuracy and the precision of molecular date estimates. As the use of multilocus data sets becomes increasingly routine, it becomes more important to evaluate the potentially confounding effects of rate heterogeneity both within (e.g., codon positions) and among loci when estimating divergence times. Here, using *Plestiodon* lizards as a test case, we examine the effects of accommodating rate heterogeneity among data partitions on divergence time estimation. *Plestiodon* inhabits both East Asia and North America, yet both the geographic origin of the genus and timing of dispersal between the continents have been debated. For each of the eight independently evolving loci and a combined data set, we conduct single model and partitioned analyses. We found that extreme saturation has obscured the underlying rate of evolution in the mitochondrial DNA (mtDNA), resulting in severe underestimation of the rate in this locus. As a result, the age of the crown *Plestiodon* clade was overestimated by 15–17 Myr by the unpartitioned analysis of the combined loci data. However, the application of partition-specific models to the combined data resulted in ages that were fully congruent with those inferred by the individual nuclear loci. Although partitioning improved divergence date estimates of the mtDNA-only analysis, the ages were nonetheless overestimated, thus indicating an inadequacy of our current models to capture the complex nature of mtDNA evolution in over large time scales. Finally, the statistically incongruent age distributions inferred by the partitioned and unpartitioned analyses of the combined data support mutually exclusive hypotheses of the timing of intercontinental dispersal of *Plestiodon* from Asia to North America. Analyses that best capture the rate of evolution in the combined data set infer that this exchange occurred via Beringia ∼18.0–30 Ma. [Bayesian; Beringia; divergence dating; molecular rate; partitioning; rate heterogeneity; relaxed molecular clock; saturation; Scincidae.]

Chronology is central to analyses of historical biogeography, conservation biology, or any endeavor to discern the rate at which a structure, behavior, or physiology has evolved. However, the fossil record is far from complete and researchers who seek to infer the timing of a historical event must often rely on molecular divergence date methods. Until recently, confidence in molecular divergence date estimates was frequently compromised by the methods' inability to incorporate error in calibrations, phylogeny, branch lengths, and other model parameters (e.g., Graur and Martin 2004; Near et al. 2005; Yang and Rannala 2006). Recent developments of Bayesian divergence date methods (Thorne and Kishino 2002), and uncorrelated "relaxed" molecular clocks that permit lineages to evolve at different rates of evolution drawn from a single continuous distribution (Drummond et al. 2006), have done much to remedy these deficiencies. In addition, instead of using a calibration point estimate (e.g., a specific node is exactly $x$ million years old), a variety of prior probability distributions can be used to accommodate uncertainty in the age of the node. Because divergence date estimation is dramatically affected by the age of the calibration, incorporating this age uncertainty is critically important to estimating accurate divergence times.

Despite these enormous methodological advances, there remains a little-studied obstacle to inferring molecular dates—the potentially confounding influence of heterogenous evolutionary characteristics within (e.g., codon positions) and among loci. Much research and discussion has addressed the theoretical and practical problems with gene trees and species trees (e.g., Maddison 1997; Edwards et al. 2007) and analyzing data sets separately or combined (Kluge 1989; Bull et al. 1993; de Queiroz 1993; Chippindale and Wiens 1994) in standard phylogenetic analyses. Much of the discussion has focused on the realization that unlinked genes may have different coalescent histories and may either evolve at different overall rates (e.g., mitochondrial DNA [mtDNA] vs. nuclear DNA) or are best described by different evolutionary models and parameters.

In a pioneering paper, Thorne and Kishino (2002) explored the effects of different strengths of autocorrelation among loci when estimating Bayesian divergence

ages and determined that indeed different loci infer different molecular ages and that incorporating this information is important to inferring divergence times. Phillips (2009) demonstrated that genes or gene partitions that evolve at extremely high rates may accumulate so many hidden substitutions that it is difficult to estimate the underlying process that created the data. As a result, divergence times may be severely over- or underestimated if the underlying rate of evolution is under- or overestimated. This should be particularly acute in quickly evolving genes that become "saturated" (i.e., numerous nucleotides have undergone multiple substitutions). Indeed, Jansa et al. (2006) found a large discrepancy between divergence dates estimated from nuclear DNA and saturated mtDNA data sets, with the latter being much older.

As data sets with many more loci become the standard in molecular dating studies, the need for an additional thorough exploration of the methodology dealing with these data becomes essential. How can we accommodate the rate of evolution among subsets or partitions of the data (e.g., genes, codon positions)? In this study, we assess the extent to which accounting for rate heterogeneity both within and among loci using partition-specific modeling affects molecular divergence dating using *Plestiodon* (formerly *Eumeces*; Brandley et al. 2005; Smith 2005) lizards in the family Scincidae as a model system.

### *The Biogeography of* Plestiodon

*Plestiodon* is a clade of ∼43 species of lizards in the family Scincidae (skinks) that have a disjunct distribution in East Asia and North America similar to many plants, fungi, and other animals. Given their distribution on both the Eurasian and American continents, one hypothesis is that the current distribution of *Plestiodon* reflects the separation of Laurasia 200 Ma. However, the date of this separation is much too old to explain this geographic distribution as it is both contemporaneous with the origin of crown Squamata and predates the origin of the entire scincid family by 100 myr (e.g., Wiens et al. 2006; Hugall et al. 2007; Conrad 2008). Furthermore, no *Plestiodon* species currently inhabit Europe and Central Asia, nor is there any fossil evidence that they did so in the past.

Although the current distribution of *Plestiodon* cannot be explained by continental vicariance, geological and climatic history may have shaped the biogeographic history of the genus in other ways. Throughout the Tertiary, there have been at least two well-characterized, major terrestrial connections between Eurasia and North America—the Transatlantic Thulean and Transpacific Beringia land bridges. These land continuities have played an important role as migration routes between the two former Laurasian continents (see Wen 1999; Sanmartín et al. 2001 for reviews of numerous organisms).

During the Early Tertiary, Europe and North America were connected via the Thulean land bridge. This connection, and warm climate in the Eocene (∼56–33.5 Ma), facilitated significant biotic exchange of both plants (Tiffney 1985a; Manchester 1999) and animals (McKenna 1975, 1983a, 1983b; Janis 1993). However, geological and fossil mammal evidence indicates that this connection was permanently severed ∼49 Ma (McKenna 1975, 1983a). Although other connections between Europe and North America may have existed (the DeGeer and Greenland-Faeroes bridges), the cold climate and short day length of the region (McKenna 1983a, 1983b; Tiffney 1985b; Sanmartín et al. 2001; Burbrink and Lawson 2007) probably prohibited its use by ectothermic animals such as lizards. Thus, if the Thulean bridge was the route by which early *Plestiodon* migrated between Eurasia and North America, this must have occurred prior to ∼49 Ma.

The other potential dispersal route between Eurasia and North America is Beringia. Although this connection was more or less permanent since the Mesozoic, climatic factors have likely limited the migration of terrestrial animals to specific geological time periods (McKenna 1983b). The Eocene age (∼56–33.5 Ma) is generally characterized as one of the earth's "hothouse" periods when global temperatures were warm enough to permit plants and animals to inhabit high latitudes (Wolfe 1978; Tiffney 1985a; Potts and Behrensmeyer 1992). During this time, a belt of boreotropical forest stretched from East Asia to North America (Wolfe 1975, 1978; Tiffney 1985a) thus providing suitable habitat for *Plestiodon* to inhabit either side of Beringia. The global climate began to cool in the middle Eocene and by the Eocene/Oligocene boundary ∼33.5 Ma (Berggren et al. 1992; Liu et al. 2009), temperatures had cooled 8.2 ± 3.1 °C in a span of ∼400,000 years (Zanazzi et al. 2006) thus radically affecting the distribution of Holarctic plants and animals (Wolfe 1978, 1985, 1987; Tiffney, 1985a, 1985b; Miller 1992; Potts and Behrensmeyer 1992; Prothero and Berggren 1992; Janis 1993).

Thus, one hypothesis is that Beringia permitted dispersal between Eurasia and North America during the Eocene but ceased at the Eocene/Oligocene boundary. Alternatively, there was also a period of warming in the Late Oligocene (∼26–27 Ma; Zachos et al. 2001); this period is especially notable as it coincides with the hypothesized dispersal of ratsnakes from Asia to America (Burbrink and Lawson 2007), animals with somewhat similar ecological requirements to *Plestiodon*. Dispersal was also possible during the Early to Middle Miocene (18–13 Ma), and at times during the Pliocene, when temperatures had once again risen to permit temperate forest at high latitudes (Potts and Behrensmeyer 1992).

Here, we employ Bayesian phylogenetic divergence dating analyses of an eight-locus DNA data set, sampled for almost every species of *Plestiodon*, to evaluate how heterogeneous processes of DNA evolution affect divergence date estimation. Furthermore, we use these data to reconstruct the geographic origin of extant *Plestiodon* and determine if faunal interchange between Asia and America occurred via the Transatlantic Thulean bridge (>49 Ma) or via Beringia during the Middle to Late

Eocene (prior to 33.5 Ma), Late Oligocene (25–28 Ma), Miocene (18–13 Ma), or Pliocene (5.3–1.6 Ma).

## Materials and Methods

### *Taxon and Character Sampling*

The DNA data set included 62 individuals representing 37 of ~43 recognized species of *Plestiodon* and 25 outgroups (Appendix SI, available from http://www.sysbio.oxfordjournals.org). DNA was isolated from tissue using Qiagen DNeasy™ columns. Using standard polymerase chain reaction (PCR) and sequencing techniques (up to 40 cycles: 30 s at 94 °C, 30 s at 60 °C, 60 s at 72 °C), we collected DNA data for nine independently evolving loci: mtDNA (ND1, transfer $tRNA^{LEU}$, $tRNA^{ILE}$, and $tRNA^{GLN}$ totalling 1227 total base pairs [bp]), BDNF (653 bp), MKL1 (903 bp), PRLR (570 bp), PTGER4 (468 bp), R35 (682 bp), RAG1 (2728 bp), and SNCAIP (483 bp) nuclear-encoded loci (see Appendix SII and Townsend et al. 2008 for primer information) for a total of 7714 base pairs in the combined locus data set. We were unable to obtain reliable sequences for MKL1 and PTGER4 from our sampled gerrhosaurid, *Gerrhosaurus major*; in this case, we substituted sequences of another gerrhosaurid, *Cordylosaurus subtesselatus*. PCR products were cleaned using ExoSap-IT (USB Corp.). Purified templates were dye labeled using BigDye (ABI) and sequenced on an ABI 3077 automated DNA sequencer. Nucleotide sequences were examined and aligned by eye. This process was relatively straightforward for the protein-coding genes (BDNF, MKL1, mtDNA ND1, PRLR, PTGER4, R35, RAG1, and SNCAIP) due to their codon reading frames. MtDNA tRNAs were aligned according to their secondary structure, and regions in which homology was uncertain due to multiple insertions and deletions were excluded from subsequent analysis. The size of the final combined locus data set for phylogenetic analysis was 7667 bp. All sequences were deposited into GenBank (HM160578-161336).

### *Divergence Dating Analyses*

*Bayesian phylogenetic analyses*.—All phylogenetic analyses were conducted using BEAST v1.4.8 (Drummond and Rambaut 2007). The best-fit model of sequence evolution for each partition (and combined data) was estimated using the Bayesian information criterion, BIC = $2 \times \Pr(D|H)$ + (number of free model parameters) × (ln number of characters) (Schwarz 1978; Appendix SIII). Bayesian phylogenetic analyses were performed for each of the eight loci and combined data. Three general sets of analyses were performed for the combined DNA data set. The first used a single model for the entire combined loci data set (the "unpartitioned" analyses). The second set of analyses employed separate DNA evolution models and parameters for the third codon position and the combined first and second codon positions of each gene for a total of 16 partitions (the "1+2" analyses; the mtDNA tRNAs were included in the ND1 second codon partition). The third set of analyses used partition-specific models and parameters for each codon position of each gene, and a single partition for the mtDNA tRNAs, for a total of 25 partitions (the "codon position" analyses). We used a randomly generated coalescent starting tree, a birth–death tree prior on rates of cladogenesis, uncorrelated lognormal relaxed molecular clock, and the program's default prior distributions of model parameters (with the exception of general time reversible substitution rates in which we used a uniform [0,100] distribution). We also used age distributions of the most recent common ancestor of the three clades used for calibration (see below). Analyses for each locus were run for $5 \times 10^7$ generations, and the combined data were run for $10^8$ generations; all analyses were sampled every 10,000th generation. To determine convergence, we constructed cumulative posterior probability plots for each clade using the "cumulative" function in Are we there yet? (AWTY) (Nylander et al. 2008). Stationarity was assumed when the cumulative posterior probabilities of all clades stabilized. To decrease the chance of reaching apparent stationarity on local optima, we conducted at least 4 separate analyses for each locus and 10 for the combined data; posterior probability estimates for each clade were then compared between the analyses using a scatter plot created by the "compare" command in AWTY. If posterior probability estimates for clades were similar in the analyses, the results were combined. Posterior probabilities (PP) $\geq$0.95 are considered statistically significant clade support (Huelsenbeck and Rannala 2004).

We used Bayes factors to determine whether applying partition-specific models significantly improved explanation of the data (see Brandley et al. 2005). The Bayes factor measures the amount by which one's opinion is changed after viewing the data. This can be interpreted as the change in odds in favor of a hypothesis and can be measured as the change in odds from the prior to the posterior (Lavine and Schervish 1999) or as the relative success of two hypotheses at predicting the data (Kass and Raftery 1995). The Bayes factor was determined by calculating the marginal likelihood for both the partitioned and the unpartitioned analyses using Tracer v.1.4 (Suchard et al. 2001; Rambaut and Drummond 2007). The difference in these ln-transformed marginal likelihoods was compared with the table provided by Jeffreys (1935, 1961) and further modified by Raftery (1996). Based on these tables, we consider a 2ln Bayes factor >10 as strong evidence for a hypothesis (Brown and Lemmon 2007).

*Calibration age constraints*.—Potentially useful fossil evidence of the earliest existence of *Plestiodon* is a fossil *Eumeces sensu lato* from the South Dakota Brule formation (Oligocene: Orellian), which is ~33.5–32 Ma (Skinner 1951; Kepferle and Culbertson 1955; Prothero et al. 1983; Hoganson and Lammers 1992; Hoganson et al. 1998). However, there has been sufficient taxonomic uncertainty associated with this fossil to render it of dubious value for both calibration purposes and evidence that *Plestiodon* inhabited North America at that time. The specimen was first described by

Gilmore (1928) as *Exostinus serratus*, a xenosaurid genus (McDowell and Bogert 1954) (xenosaurids are not closely related to scincids; Townsend et al. 2004; Hugall et al. 2007). Later, Estes (1965) remarked that this specimen ". . . is not referable to *Exostinus*, but is actually an *Eumeces*-like skink, and will be discussed elsewhere," yet never subsequently discussed this determination. However, the use of this fossil, and other pre-Pleistocene fossils, as calibration age constraints cannot be justified because despite numerous efforts (Kingman 1932; Taylor 1935; Griffith et al. 2000), no researcher has identified unambiguous skeletal characters that diagnose the genus *Plestiodon* let alone subclades within the genus. Moreover, all fossils were described as *Eumeces*, but *Eumeces sensu lato* is not monophyletic (Griffith et al. 2000; Schmitz et al. 2004; Brandley et al. 2005). Finally, the pre-Pleistocene fossil material that does exist consists entirely of fragmentary skeletal material (such as a single dentary bone) thus making any endeavor to positively identify a *Plestiodon* fossil speculative at best.

Therefore, we employ "external" calibration age constraints (i.e., fossils of lineages outside *Plestiodon*; outgroup lineages). However, even this is a difficult task given the "very poor fossil record of Scincidae" (Evans 2003). Instead, we used three fossil calibration age prior distributions from non-scincid fossil taxa whose phylogenetic placement in the squamate tree was recently inferred (Conrad 2008). The age of crown Episquamata (represented here as *Anniella, Aspidoscelis*, *Basiliscus*, and *Bipes*) was calibrated using the age of the earliest stem "anguimorph" fossils, *Becklesius, Dorsetisaurus*, *Paramacellodus,* and *Pseudosaurilius* (148 Ma; Conrad 2008). We chose a lognormal distribution so that the earliest possible sampled age corresponds to 148 Ma and the older 97.5% credible interval (CI) encompasses the earliest age of crown Squamata (180 Ma; mean = 0, standard deviation = 1.769; Wiens et al. 2006; Hugall et al. 2007). The age of the divergence between Amphisbaenia (*Bipes biporus*) and Teiidae (*Aspidoscelis*) was calibrated using the age (Albian–Cenomanian boundary) of the earliest teiioid (Polyglyphanodontidae) fossils (e.g., *Bicuspidon*; Nydam and Cifelli 2002; Conrad 2008). We chose a lognormal distribution so that the earliest possible sampled age corresponds to 96 Ma and the older 97.5% CI encompasses the earliest age of crown Episquamata (148 Ma; mean = 0, standard deviation = 2.016; Wiens et al. 2006; Hugall et al. 2007). The age of Scinciformata (represented here by skinks, Gerrhosauridae, and Xantusiidae) was calibrated using the age (Berriasian) of the fossil *Sakurasaurus* (Evans and Manabe 1999; Conrad 2008). We chose a lognormal distribution so that the earliest possible sampled age corresponds to 138 Ma and the older 97.5% CI encompasses the earliest age of the root (151 Ma; mean = 0, standard deviation = 1.309; Wiens et al. 2006; Hugall et al. 2007). We therefore enforced the monophyly of these clades in accordance with recent phylogenetic analyses that have inferred these relationships (Townsend et al. 2004; Hugall et al. 2007). Because *Sakurasaurus* is a fragmentary fossil, we also conducted additional analyses removing it. Note that these prior probability age distributions are quite large, spanning ~40 myr, thereby reflecting the uncertainty of when these clades radiated.

Because "saturation" is known to result in misestimation of the evolutionary process of DNA substitution and potentially misestimation of divergence dates (Jansa et al. 2006; Phillips 2009), we assessed saturation in the rapidly evolving mtDNA data set. For the individual codon positions and combined data, we plotted uncorrected "*p*" distances against Jukes-Cantor (JC) + Γ corrected distances (assuming $\alpha = 0.5$). If the JC + Γ corrected distances are larger than the uncorrected distances, we interpret this as evidence that these data include hidden substitutions.

To insure that there is sufficient phylogenetic information to inform the posterior age distributions (i.e., inferred ages are not solely influenced by our prior calibrated age constraints), we conducted an additional BEAST analysis enforcing these calibration age constraints, but with no DNA data. We compared the shape and mean of the posterior age distribution of crown *Plestiodon* from this "priors-only" analysis to that from the analyses of each locus and the combined data. If the distribution of divergence dates estimated from data differs from the priors-only distribution in both shape and mean, we conclude that these estimated dates are influenced by the data rather than only the prior age calibration constraints.

### *Ancestral Area Reconstruction*

To determine the geographic origin of crown *Plestiodon*, we used Bayesian character state reconstruction analyses. We chose this method over competing methods (such as DIVA, Ronquist 1996; LaGrange, Ree and Smith 2008; Lemey et al. 2009) primarily because it is a relatively simple hypothesis to test (there are a limited number of possible regions that where *Plestiodon* could conceivably exist and few possible dispersals), and the method used here permits calculation of Bayesian posterior probabilities, thus providing an assessment of confidence, for each of the geographic regions for every node in the phylogeny. Moreover, likelihood-based character state reconstruction methods have been shown to be a robust method to infer ancestral geographic areas (McGuire et al. 2007; Clark et al. 2008).

Given that the closest living relative of *Plestiodon* is not known with confidence, we included potential sister taxa including other genera formerly included in *Eumeces sensu lato*, and lineages that primarily inhabit Africa, Madagascar, and the Seychelles (see Appendix SIV). We coded each species as being distributed in Africa (0), Asia (1), or North America (2) and inferred the ancestral area state of crown *Plestiodon* using Markov chain Monte Carlo (MCMC) in the program BayesTraits v1.0 (Pagel et al. 2004). The analysis included all the trees in the posterior distribution of the fully partitioned analysis of the combined data. We ran four MCMC analyses each for $1.1 \times 10^7$ generations sampled every 1000 generations. To achieve a desired proposal acceptance rate of 15–40%, we tried a variety of values for the rate parameter proposal mechanism in addition to

hyperpriors on the character state transition rates. Our final analyses used a rate parameter proposal mechanism of 0.1 and an exponential [interval 0,30] hyperprior on the character state transition rate. We discarded the first $10^6$ generations as burn-in and calculated the posterior probability of the ancestral area state of the root of *Plestiodon* from the remaining samples. If the posterior probability for the reconstructed state is $\geq$0.95, we interpret that as statistically significant evidence that crown *Plestiodon* originated in that region. We interpret posterior probabilities <0.95 as equivocal and a failure to distinguish between the competing hypotheses.

## RESULTS

The results of the cumulative analyses in AWTY indicate that discarding the first 20–30% of generations is sufficient to insure convergence of the Bayesian analyses. All results of the biogeographical reconstructions and divergence date estimates are calculated from these remaining trees. For reference, the phylogenetic interrelationships and estimated divergence times for *Plestiodon* species estimated by the codon position partitioned analyses of the combined data are provided in Figure 1. (The full tree, including outgroups, is provided in Appendix SIV). The effective sample sizes

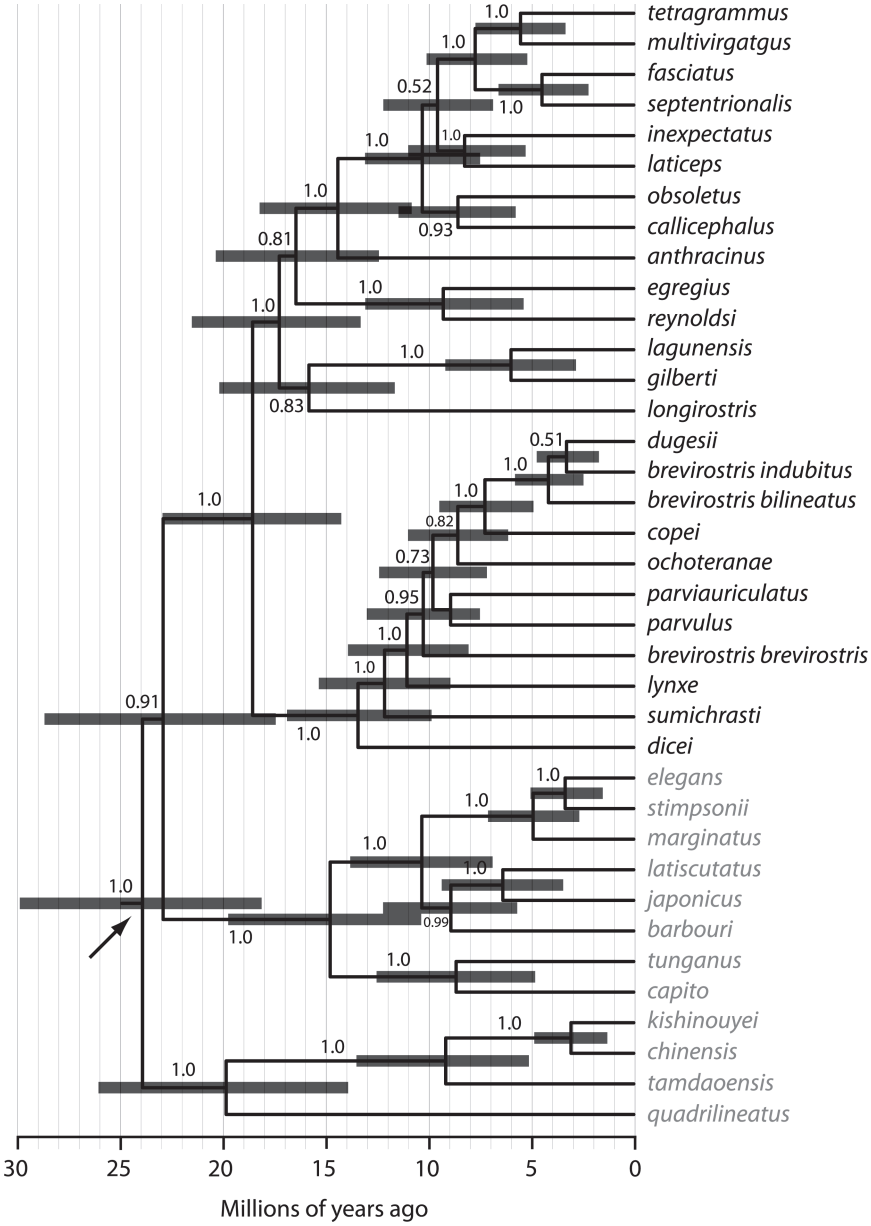


FIGURE 1. Chronogram of *Plestiodon* species inferred from a partitioned Bayesian analyses of the combined data set (mtDNA, BDNF, MKL1, PRLR, PTGER4, R35, RAG1, and SNCAIP). The chronogram was pruned to exclude non-*Plestiodon* taxa. Branch lengths represent the mean values of the posterior distribution. Taxa shaded in black are distributed in North America, and taxa shaded in grey inhabit Asia. The grey boxes indicate the 95% CI of the node age posterior probability distribution. The arrow indicates the focal node for the divergence dating analyses and biogeographic reconstruction.

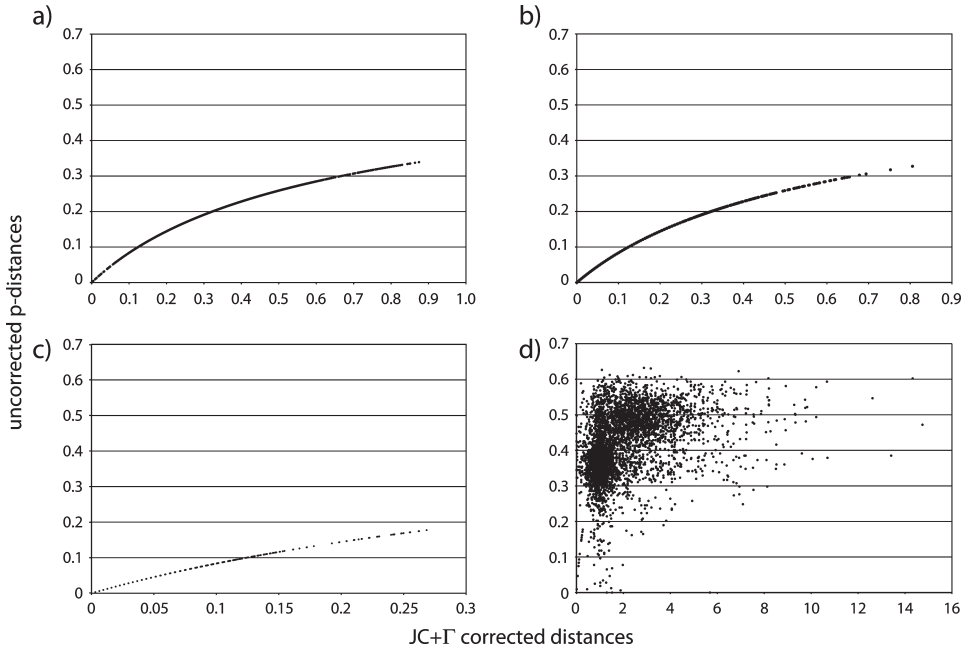


FIGURE 2. Saturation plots of the (a) entire ND1 mtDNA gene, (b) ND1 mtDNA first codon positions, (c) ND1 mtDNA second codon position, and (d) ND1 mtDNA third codon position.

(ESS) for the divergence date estimates were >100 for all analyses. Saturation plots suggest an abundance of hidden substitutions in the mtDNA data, especially in the third codon position (Fig. 2).

### *Divergence Date Estimates from Individual Loci and Effects of Partitioning*

The analysis evaluating only the effect of the calibration age constraint priors (i.e., the "no data" analysis) infers a lognormal distribution with a mean age of crown *Plestiodon* of 140.1 Ma (95% CI = 138.0–151.4) that strongly contrasts to the much younger normal distributions of dates inferred by the data analyses (below). Thus, we conclude that these estimated dates are driven by the data rather than only the prior age calibration constraints.

Bayes factors strongly suggest that partitioned models are a better fit to the data for seven of the eight loci (Table 1). For the SNCAIP data, the fully partitioned model was not strongly better than the 1+2 model. However, for the most part (see exceptions below), the mean divergence age estimates of crown *Plestiodon* are quite similar (~25 Ma) for most of the loci regardless whether these ages were calculated from partitioned or unpartitioned analyses (Table 2 and Fig. 3). BDNF and SNCAIP are two exceptions in that they infer older mean ages (~37 and ~40 Ma, respectively) and whose age distributions extend to much older ages (although the younger bound of the distribution is similar to the other nuclear loci). However, compared with the other loci, the divergence date distributions inferred by the mtDNA data set are drastically different. In the unpartitioned analysis, the estimated mean age of divergence for the mtDNA data is 62.6 Ma, and the 95% CI (46.3–80.8 Ma) excludes the distributions of most other genes; this is best visualized in Figure 3. The partitioned analyses of the mtDNA data set estimates a younger posterior age distribution (1 + 2 mean age = 38.6 Ma, 95% CI = 29.1–50.4 Ma; codon position mean age = 40.0 Ma, 95% CI = 29.5–52.4 Ma), but these estimates are nonetheless much older than distributions inferred by the other loci.

Even more striking are the differing results of the combined data analysis when we apply the partitioning schemes. The age distribution estimated from the unpartitoned analysis resides in between the distributions estimated by the nuclear loci and mtDNA (Fig. 3). However, when the heterogeneous characteristics of DNA evolution are modeled using either the 1 + 2 or the codon position partitioned models, the age distribution estimated from the combined data analysis strongly favors ages inferred by a majority of the loci analyzed separately.

Additional analyses of the combined data set using the unpartitioned and codon position model excluding the fragmentary fossil *Sakurasaurus* (not shown) estimated date distributions slightly older than those of the fully calibrated data set (unpartitioned mean age = 43.0 Ma, 95% CI = 39.4–51.3 Ma; codon position mean age = 26.7 Ma, 95% CI = 19.3–34.8 Ma).

### *Biogeographic Reconstruction*

The four Bayesian ancestral state reconstruction analyses all converged on a similar posterior distribution (mean $-\ln L = 12.6$) and the proposal acceptance rate

TABLE 1. 2ln Bayes factors comparing the performance of the unpartitioned and partitioned analyses

| Locus | Ln marginal likelihood | | | 2Ln Bayes factor | | |
|---|---|---|---|---|---|---|
| | Unpartitioned | 1 + 2 partitioned | Codon position partitioned | 1 + 2 partitioned versus unpartitioned | 1 + 2 partitioned versus unpartitioned | Codon position partitioned versus 1 + 2 partitioned |
| BDNF | −2921.2 | −2811.2 | −2802.2 | 220.0 | 237.8 | **17.8** |
| MKL1 | −7216.9 | −7102.0 | −7057.9 | 229.8 | 318.0 | **88.2** |
| mtDNA | −27719.6 | −27461.9 | −26737.6 | 223.8 | 853.0 | **629.2** |
| PRLR | −7000.3 | −6983.9 | −6973.1 | 32.8 | 54.4 | **21.6** |
| PTGER4 | −3175.8 | −2997.9 | −2986.4 | 355.8 | 378.8 | **23.0** |
| R35 | −7015.6 | −6925.6 | −6877.3 | 180.0 | 276.6 | **96.6** |
| RAG1 | −23296.0 | −22805.4 | −22775.0 | 981.0 | 1042.0 | **60.8** |
| SNCAIP | −3569.8 | −3467.4 | −3468.2 | 204.8 | **203.0** | 1.7 |

Notes: Marginal likelihoods were calculated using the method of Suchard et al. (2001) using Tracer 1.4 (Rambaut and Drummond 2007). The Bayes factors represent relative evidence in favor of the partitioned analyses; 2ln Bayes factors $\geq$10 are interpreted as strong evidence for the partitioned analysis (Kass and Raftery 1995). Values in bold indicate that the partitioning strategy is considered optimal for that gene. Note that the codon position partitioning strategy is strongly better than all other strategies with the exception of SNCAIP.

was 16.7% suggesting adequate mixing. These analyses infer significant support for an Asian origin of crown *Plestiodon* (PP = 0.968).

## DISCUSSION

### *Accounting for High Rates of Evolution Dramatically Affects Divergence Time Estimates*

The posterior age distributions of crown *Plestiodon* inferred by 7 of 8 genes (BDNF, MKL1, PRLR, PTGER4, R35, RAG1, and SNCAIP) are largely congruent regardless of whether partitioned models are used (Fig. 3). This is interesting because it suggests that the parameters that partitioned models are modeling better (as evidenced by marginal likelihoods and Bayes factors, Table 1) are also parameters that do not significantly impact divergence date estimates for these loci. The very slowly evolving BDNF and SNCAIP genes infer older ages, but this is probably due to the very low phylogenetic signal in these data sets; this imprecision results in large posterior age distributions that subsequently "pull" the mean age into older ages when compared with the other nuclear loci.

However, there are enormous differences between the ages inferred by the mtDNA data when compared with the nuclear data (Table 2 and Fig. 3) and between the unpartitioned and 1 + 2 and codon position partitioned analyses of the combined data. In the case of the unpartitioned analysis, the posterior age distribution of the combined data is essentially a compromise between the two age extremes of the mtDNA and nuclear loci; yet, the distribution estimated by the partitioned analyses strongly favor an age of crown *Plestiodon* congruent with a majority of the loci. Moreover, the 95% CI of the age distribution estimated by the unpartitioned model excludes the 95% CIs of the 1+2 and codon position age distributions (Table 2 and Fig. 3).

What accounts for the nonoverlapping incongruent age estimates of the mtDNA and the unpartitioned and partitioned combined analyses? When divergence date estimates of two or more data sets are extremely different, the most obvious culprit may be disparities in the estimated tree topology among loci. For example, if the node in question (in this case, crown *Plestiodon*) is not monophyletic with respect to presumably "old" lineages, or if there is large uncertainty in the placement of clades used as calibration age constraints, estimated ages may be radically different. However, *Plestiodon* is monophyletic with statistically significant support in every analysis of every locus (not shown), regardless of partitioning scheme. Moreover, the monophyly of the clades used for fossil calibration were constrained (Appendix SIV), and therefore topological incongruence cannot explain the very different age distributions inferred by these analyses.

A more plausible explanation for the nonoverlapping age distributions is the failure to adequately model the underlying evolutionary process that created these data,

TABLE 2. Mean ages and 95% confidence intervals of age posterior distributions estimated from unpartitioned and partitioned Bayesian analyses of individual loci and combined data assuming a lognormal relaxed molecular clock

| Data set | Unpartitioned | | | 1 + 2 partitioned | | | Codon position partitioned | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean age (myr) | Lower 95% CI | Upper 95% CI | Mean age (myr) | Lower 95% CI | Upper 95% CI | Mean age (myr) | Lower 95% CI | Upper 95% CI |
| BDNF | 36.6 | 15.8 | 61.2 | 36.9 | 15.0 | 62.7 | 37.0 | 15.5 | 63.3 |
| MKL1 | 26.4 | 14.4 | 40.5 | 26.5 | 14.7 | 41.7 | 26.3 | 14.7 | 40.7 |
| mtDNA | 62.6 | 46.3 | 80.8 | 38.6 | 29.1 | 50.4 | 40.0 | 29.5 | 52.4 |
| PRLR | 27.2 | 16.4 | 39.2 | 26.9 | 16.7 | 39.0 | 27.2 | 17.0 | 39.3 |
| PTGER4 | 23.4 | 10.7 | 39.6 | 22.7 | 11.3 | 37.3 | 22.5 | 10.8 | 37.3 |
| R35 | 23.2 | 14.4 | 32.8 | 22.1 | 13.7 | 31.4 | 24.1 | 14.9 | 34.2 |
| RAG1 | 24.4 | 16.2 | 33.3 | 24.0 | 16.2 | 33.3 | 24.2 | 16.6 | 33.1 |
| SNCAIP | 39.9 | 18.7 | 66.8 | 39.9 | 19.6 | 65.6 | 40.4 | 18.5 | 65.5 |
| Combined data | 39.8 | 32.9 | 46.9 | 22.4 | 16.7 | 28.8 | 23.6 | 18.0 | 29.6 |

especially the rate of substitution in the mtDNA data. The failure to adequately model the evolutionary process of DNA evolution may lead to systematic over- or underestimation of divergence dates depending on the placement of calibrations (see Ho et al. 2005, 2007; Ho and Larson 2006; Phillips 2009). However, modeling this process in genes that have a high substitution rates is difficult because they are prone to accumulate hidden substitutions. This "saturation" (i.e., when many sites have undergone multiple substitutions) obscures the true evolutionary process (see also Jansa et al. 2006; Phillips 2009). Saturation plots of our mtDNA data set indicate that the nucleotides have undergone multiple substitutions to varying degrees, with the third codon position indicating massive saturation (Fig. 2d).

Figure 4 shows the mean rate and 95% CI for the unpartitioned, 1 + 2 partitioned, and codon position partitioned analyses of each locus (and combined data). The estimated rate for most of the loci (BDNF, MKL1, PRLR, PTGER4, R35, RAG1, and SNCAIP) is the same in both unpartitioned and partitioned analyses, and indeed, the posterior age distributions are also essentially the same between the unpartitioned and the partitioned analyses (Fig. 3). However, the mean rates estimated from the partitioned analyses of the mtDNA and combined data set are much higher than those estimated by the unpartitioned analysis (Fig. 4). Because the partitioned analyses are expected to better model the underlying parameter distributions of a complex data set (i.e., our best estimate of the actual rate of evolution), these results show that the unpartitioned analyses are severely underestimating the overall rate of evolution in both the mtDNA and the combined data sets.

In a molecular divergence dating analysis, estimating our variable of interest (time) is directly related to how well we are estimating the other variable (rate of evolution). Thus, over- or underestimating the rate of evolution will lead to under- or overestimation of divergence dates, respectively. For example, in the case where hidden substitutions are underestimated, there will be more substitutions than expected by the model, especially in the terminal branches, and ages of the internal nodes will be estimated to be too old. This is precisely the pattern seen in our data.

Further evidence that poorly modeling high rates of evolution may result in overestimated ages is seen in the age distributions of the partitioned analyses of the combined and mtDNA data. In the unpartitioned analysis, the posterior age distribution of the combined data is essentially a compromise between the two age extremes of the mtDNA and nuclear loci. The use of a partitioning scheme that separately models the third codon position (i.e., either the 1 + 2 or codon position partitioning scheme) is better able to estimate the overall rate and rate heterogeneity among loci results in an age distribution more congruent with all the nuclear loci. Moreover, when better modeling evolutionary rate, the age distribution of the partitioned analysis of the mtDNA shifts from one with a 95% CI of 46.3–80.8 Ma to a much younger distribution (~29–52 Ma; Fig. 3).

Thus, the drastically different divergence date distributions estimated by the mtDNA data set are likely the result of systematic underestimation of the evolutionary rate due to the difficulty in modeling this rate in saturated data; in the case of the third codon position, the degree of saturation has obliterated much of the evidence of the evolutionary process. This difficulty is made more extreme because our available fossil calibrations used to estimate the rate are "deep" in the phylogeny where saturation is expected to be most severe. In other words, we are trying to capture the rate of evolution of the mtDNA data in the part of the tree with the least amount of reliable signal. However, the application of partition-specific modeling does help here because it essentially isolates the third codon position thereby permitting better modeling of the global rate of evolution. We attempted to test this further by conducting additional analyses deleting the third codon position; however, these analyses inferred large flat posterior distributions indicating a lack of signal in the data (not shown). These results are important because it demonstrates that, as we come closer to estimating the "true" rate of evolution of the combined data set using partitioned models, the date distributions inferred by the combined data become congruent with the seven nuclear genes; that is, with this data set, the current divergence date estimation models are adequate to effectively capture the rate of evolution and infer

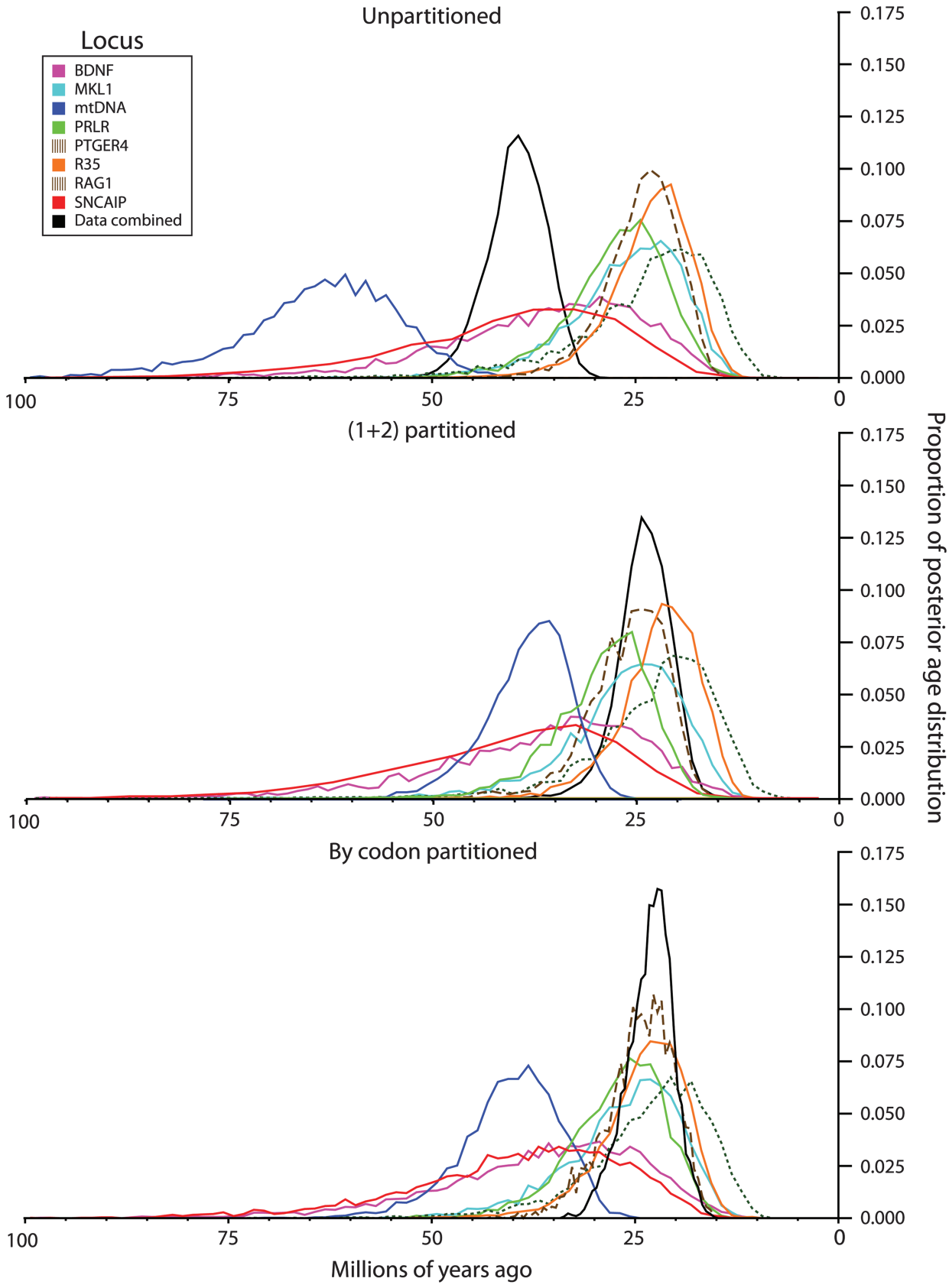


FIGURE 3. Age posterior probability distributions of crown *Plestiodon* estimated from analyses of each gene and combined data using three different partitioning strategies.

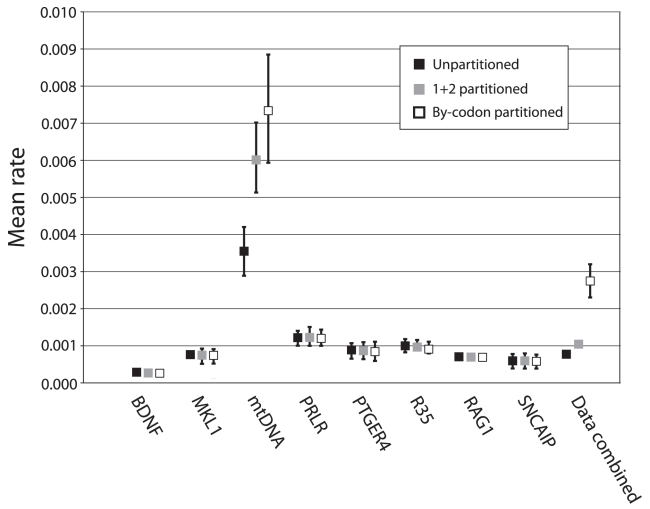


FIGURE 4. Posterior probability distributions for the mean rate of evolution of estimated from analyses of each gene and combined data using three different partitioning strategies. Bars indicate the 95% CI.

corroborating dates. Indeed, in data sets with very low rates of evolution (e.g., the individual nuclear genes), even a simple unpartitioned model was sufficient.

Our analysis also corroborates a recent study by Phillips (2009) that also demonstrated large overestimation of divergence dates for the mitochondrial genome as a result of inadequately modeling the underlying rate of evolution. This is particularly notable because mtDNA (including complete mitochondrial genomes) has been used extensively to estimate deep divergence dates among numerous vertebrate lineages (e.g., Xiong et al. 2009; Zhang and Wake 2009), sometimes with results that are far different than the fossil record (e.g., Yamanoue et al. 2006; Yamanoue et al. 2009; Inoue et al. 2009; Phillips 2009).

When estimating divergence dates from mtDNA, or combined mtDNA and nuclear DNA, it is critical that the analysis incorporates the best estimate of the rate of evolution through the use of partitioned models, when available and internal calibrations; indeed, two other divergence data analyses using a more extensive fossil record for age calibration found essentially no difference between ages estimated with and without the use of partition-specific modeling (Alfaro et al. 2007; Poux et al. 2008). In addition, researchers should assess saturation, especially in third codon positions of mitochondrial genes. Although there is no objective metric for "too saturated," results such as those in Figure 2 will at least encourage the researcher to view with suspicion the divergence dates estimated from those data. Finally, these results bespeak a need for the incorporation of more advanced models of DNA evolution in divergence dating analyses, including model averaging (Green 1995; Huelsenbeck et al. 2004; Dornburg et al. 2008), mixture models (Lartillot and Philippe 2004), simultaneous partition and phylogeny estimation (Huelsenbeck and Suchard 2007), and the use of different rate distributions for different subsets of the data.

### *Partitioned Analyses Discriminate between Competing Hypotheses of Intercontinental Dispersal*

The Bayesian reconstructions of ancestral area of crown *Plestiodon* strongly support an Asian origin of *Plestiodon*. When did this occur, and more importantly, via what terrestrial connection between Eurasia and North America? These two questions are intimately related because the two potential colonization routes, the Transatlantic Thulean and Transpacific Beringia land bridges, were likely habitable by ectothermic organisms only during distinct time periods.

Estimating divergence dates using better modeling of the heterogeneous processes of DNA evolution among and within genes allows us to discriminate among competing hypotheses of when and how early *Plestiodon* migrated between Asia and America. For the remainder of the discussion, we focus on the results of the partitioned analyses of combined data as it is the hypothesis of *Plestiodon* history derived from the most data (Fig. 1).

The age posterior distributions of crown *Plestiodon* in both the unpartitioned (mean age = 39.8 Ma, 95% CI = 32.9–46.9 Ma) and the partitioned (mean age = 23.6 Ma, 95% CI = 18.0–29.6 Ma) analyses exclude the possibility that the Thulean bridge facilitated biotic exchange of at least one crown *Plestiodon* lineage between Eurasia and America because this route closed ∼49 Ma. Instead, both analyses support migration through Beringia. The age distributions inferred by each analysis coincide with two distinctly different time frames during which the environment of Beringia was hospitable to ectothermic organisms.

The age distribution estimated from the unpartitioned analysis coincides with the global "hothouse" climatic conditions of the Late Eocene, when the two continents shared a contiguous belt of boreotropical forest via Beringia (Wolfe 1975; Tiffney 1985a) and terminates near the Eocene–Oligocene boundary when global temperatures drastically cooled (Zanazzi et al. 2006). In contrast, the age distribution estimated by the partitioned analysis coincides with a warming period in the Late Oligocene (∼26–27 Ma; Zachos et al. 2001). Moreover, the results of the partitioned analysis are congruent with a recent molecular study exploring the colonization history of another clade of squamate reptiles inhabiting Asia and North America (ratsnakes; Burbrink and Lawson 2007).

These results are particularly important because both the unpartitioned and the partitioned analyses infer plausible time frames for dispersal. Thus, failing to model the heterogenous parameters of DNA evolution would lead to the plausible, yet incorrect conclusion that the intercontinental dispersal of *Plestiodon* occurred before the Eocene–Oligocene boundary.

Finally, we note that these results cannot determine with certainty that biotic exchange of *Plestiodon* between Asia and America occurred only once. It is possible that there were multiple faunal exchanges between the continents during the Late Eocene, or even the Middle Miocene and Pliocene, but those lineages became

extinct. Unfortunately, addressing this question may be impossible given the extremely poor *Plestiodon* fossil record.

## Conclusions

We demonstrate that time-calibrated phylogenetic analyses may be severely affected by inadequately capturing the rate of evolution due to saturation in the mtDNA even when combined with five other nuclear loci that collectively infer a different age estimate. This effect is especially strong given that the available fossil calibrations we used to estimate the evolutionary rate are "deep" in the tree where saturation is expected to be most severe in the mtDNA data. This confirms previous studies that also implicated model misspecification of model parameters (e.g., Phillips 2009) or saturation (e.g., Jansa et al. 2006) as the potential source of inaccurate date estimates.

Yet, the application of models that both attempt to account for rate heterogeneity among data partitions (partitioned models) apparently could not fully account for the evolutionary dynamics of the mtDNA set (although the divergence date estimates from the partitioned analysis of mtDNA were approaching congruence with the nuclear loci; Fig. 3). Although it is clear that trying to account for the heterogenous evolution of data partitions improved our estimates, it was not sufficient to avoid severely biasing our estimates of divergence dates in the mtDNA. However, we note that our study attempted to evaluate the effects of rates and rate heterogeneity among *data partitions* and not necessarily severe rate heterogeneity among *lineages*, and that some of this bias may be attributable to the latter.

Nonetheless, these results are promising because they show that better incorporating heterogeneous evolution of DNA results in improved congruence of divergence time estimates among different loci. In fact, the statistically incongruent age distributions inferred by the partitioned and unpartitioned analyses support mutually exclusive hypotheses of the timing of intercontinental dispersal of Plestiodon between Asia and North America. In other words, failing to account for rate heterogeneity among data partitions would provide strong support for the presumably incorrect hypothesis.

## Supplementary Material

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

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