Robust Time Estimation Reconciles Views of the Antiquity of Placental Mammals

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Background. Molecular studies have reported divergence times of modern placental orders long before the Cretaceous–Tertiary (K–T) boundary and far older than paleontological data. However, this discrepancy may not be real, but rather appear because of the violation of implicit assumptions in the estimation procedures, such as non-gradual change of evolutionary rate and failure to correct for convergent evolution. Methodology/Principal Findings. New procedures for divergence-time estimation robust to abrupt changes in the rate of molecular evolution are described. We used a variant of the multidimensional vector space (MVS) procedure to take account of possible convergent evolution. Numerical simulations of abrupt rate change and convergent evolution showed good performance of the new procedures in contrast to current methods. Application to complete mitochondrial genomes identified marked rate accelerations and decelerations, which are not obtained with current methods. The root of placental mammals is estimated to be ~18 million years more recent than when assuming a log Brownian motion model. Correcting the pairwise distances for convergent evolution using MVS lowers the age of the root about another 20 million years compared to using standard maximum likelihood tree branch lengths. These two procedures combined revise the root time of placental mammals from around 122 million years ago to close to 84 million years ago. As a result, the estimated distribution of molecular divergence times is broadly consistent with quantitative analysis of the North American fossil record and traditional morphological views. Conclusions/Significance. By including the dual effects of abrupt rate change and directly accounting for convergent evolution at the molecular level, these estimates provide congruence between the molecular results, paleontological analyses and morphological expectations. The programs developed here are provided along with sample data that reproduce the results of this study and are especially applicable studies using genome-scale sequence lengths.

INTRODUCTION

Despite great progress over the past decade, the evolutionary history of placental mammals remains controversial. While a consensus is emerging on the topology of the evolutionary tree [1–5], although with occasional disagreement [6–8], divergence times remain uncertain. The age of earlier nodes and in particular the root, remain especially uncertain in the absence of definitive placental fossils deeper into the Cretaceous [4–6,9]. Both paleontological and morphological studies suggest that the radiation of placental orders and super orders occurred close to the Cretaceous–Tertiary (K–T) boundary about 65 million years ago (mya) [9,10].

In contrast, molecular studies have suggested markedly older origins for many superordinal groups and that some extant orders diversified before the K–T boundary. The root of living placental mammals has been reported to be in the range of 100–140 mya [4,11–15] even with application of rate-adjustment techniques [4,15]. The molecular consensus of an old root is becoming strong enough that it may become dogma without a close examination of the assumptions being made in such analyses. The true age of the orders and superorders has important implications for determining the overall paleoecology and biogeography of placental mammals during a period that included the breakup of continents and the extinction of terrestrial dinosaurs. Reconciling the different paleontological and molecular divergence time estimates has important implications for basic methodologies that are central to evolutionary study.

The strength of molecular divergence time studies is their potential to draw information from very long aligned sequences of many species. It is widely assumed that a huge amount of sequence data and the approximate rate constancy of sequence evolution [16] make molecular estimates more reliable than those based solely on fossil data. Such analyses on a genomic scale are generally anticipated to be decisive because they are expected to be free of stochastic noise [17,18].

However, molecular studies acknowledge the problem of misspecification of the model of sequence change, which may result in seriously biased estimation. The relationships among placental orders do vary according to the data used and the taxa sampled [1–8,13–15,19–22]. Most methods of phylogenetic inference rely strongly on probabilistic models of sequence evolution, and neither directly detect nor correct for convergent evolution [23]. When left uncorrected, such “homoplasy” may attract lineages and underestimate certain pairwise distances. This in turn distorts branch (edge) length estimates, which are of primary importance for divergence time estimation. While some methods will detect

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potential convergent evolution, for example Hadamard conjuga-
tions and SplitsTree [24]. Kitazoe et al. have developed
multidimensional vector space (MVS) representation methods to
both detect convergent evolution among estimated distances and
to also correct for this bias [25–27].

A further problem facing molecular dating is the evolutionary
rate constancy, or lack of constancy, over long periods. Since its
proposal in 1965 [29], the molecular clock hypothesis has been
one of the most hotly debated subjects in evolutionary biology. It
is now widely accepted that a molecular clock tends to hold well
for closely related organisms, but breaks down with increasing
evolutionary divergence. To adjust for the inevitable fluctuation
of evolutionary rates, nonparametric [17,19], local clock [29,30], and
hierarchical Bayesian [31] methods have been developed. These
methods are robust against stochastic fluctuations of evolutionary
rates [31–33]. However, they may cause serious problems when
pronounced transient changes of rate occur, because they overly
smooth such rate changes. In this article, we show the magnitude
of such problems using clear worked examples.

To improve the detection of, and robustness to, abrupt rate
changes, we have developed a new procedure that minimizes the
local variability of the inverse of the evolutionary rate. Just as the
effective size of fluctuating populations is represented by the
harmonic mean over time, the mean evolutionary rate among
lineages is expressed better by the harmonic mean. This approach
is especially useful when branch lengths measured in the expected
number of substitutions per site (the products of rates and times)
are estimated accurately, and there are either rapid transient
changes of rate (hence large rate heterogeneity) or a general bias
towards a speed up or slow down in rates through time.

Using this new procedure, an analysis of 69 mitochondrial
protein sequences (3660 amino acid sites in total) from placental
mammals identified a rapid acceleration of evolutionary rate for
the lineage directly leading to the common ancestor of Supraprimates and an even more marked one for the lineage
leading to Laurasiatheria. This acceleration was followed closely
by a strong deceleration, which persisted in nearly all lineages of
Laurasiatheria. In contrast, almost all lineages of Afrotheria and Xenarthra seem to have retained rates similar to that of the root.
This view is in marked contrast to current rate-change penalty
functions. The robustness of the new procedure is assessed using simulations that show the types of change that most concern
biologists; speedups or slowdowns through time, transient rate
changes, and rate changes that do not follow a normal or
transformed normal distribution, as well as stochastic variation.
A revised estimate of the origin of placental mammals is as young as
84 mya, which is much more recent than current estimates using
molecular data. The inferred age of deeper splits in the placental
tree are compared with the rate of occurrence of new species from
the North American fossil record. These two sources of data are
far more congruent than is suggested by using current, possibly
strongly misleading, dating methods.

RESULTS

Revised rates and times of mitochondrial protein
sequences from placental mammals

Mitochondrial protein sequences are used widely in phylogenetic
studies and have been particularly popular in studying placental
mammals. A desirable feature of these data is relatively long
sequences, good taxon sampling and very little missing data.
Following alignment, we retained 3660 amino acid sites present in
all of 62 placental mammals plus seven outgroup taxa (Table S1
gives the accession numbers of protein sequences). In contrast,
large nuclear protein datasets of mammals with diverse taxon
sampling [e.g., 3, 4] show a large proportion of missing data. Such
missing heterogeneous data may lead to complex systematic errors
in distance estimation [34]. This is particularly relevant since MVS
analyses work best with low stochastic noise (hence relatively long
sequences), diverse taxon sampling with a considerable proportion
of taxa showing minimal convergent evolution, and minimal bias
of the input distances from sources such as missing data.

We adopted a standard two-step procedure to estimate
divergence times. The first step is to estimate the phylogenetic
tree with unconstrained branch lengths in units of expected
numbers of substitutions per site. Given the problems with
convergent evolution in mitochondrial data [4,5,22,25], we used
the MVS procedure to correct the input distances for convergent evolution. A variant of the core set approach [27] to MVS
was used in this instance (Materials and Methods). Fixing the topology
to the tree obtained by MVS, which is very similar to trees
obtained by previous authors using varied data sets [2–5], branch
lengths were reestimated using maximum likelihood (ML) in
the program PAML [35]. We used the JTT [36]+Gamma model with
(α = 0.5) for these analyses. The second step uses the tree and its
branch lengths, some fossil constrained nodes, and a penalty or
cost function to dampen rate changes, to infer divergence times of
all nodes and evolutionary rates along all branches.

Three cost functions, F_ADD, F_LOG, and F_IR, were applied to the
MVS and ML trees. These functions penalize the fluctuation of
rates, and do this on either a linear scale (here called the ADD
function), on the log rate (LOG), or on the inverse rates (IR),
respectively (see equations (1), (2), and (4) in Materials and Methods). The tree estimated by MVS and F_ADD is denoted MVS-
F_ADD and so on (while ML-F_ADD indicates use of ML branch
lengths).

To calibrate these trees, we used eight fossil constraints, all
taken from previous studies (see Figure 1 caption) [4,15]. The
divergence times were estimated by then minimizing each cost
function subject to these constraints (see Materials and Methods).
The CI was calculated using a likelihood interpretation (see
Materials and Methods) of the cost function residual analogous to
the method used in Multidivtime [31]. This captures the error
caused by deviations of the tree’s branch lengths away from their
expected values and includes the unpredictability of rate changes,
which might mimic Brownian motion, for example. It will also
incorporate variability arising from ancestral polymorphism.
Polymorphism will cause fluctuations of the edge lengths of the
tree when the analysis has multiple nodes constrained by fossil or
other data [c.f. 13]. Generally, the time at the most recent
common ancestor of sequences from different species is older than
the time at speciation. The extent of this difference varies among
internal nodes due to both the stochastic nature of the coalescent
and factors governing its expected magnitude, such as population
size. The cost function does not take account of this bias, but the
CI does include the variance arising from ancestral polymorphism
at internal nodes.

The MVS-FIR analysis (Figure 1A) estimated the age of the root
at 84.2 mya with a 95% confidence interval (CI) of 80.7–88.4 mya
(Table 1). This time is much more recent than the result of
the ML-FIR analysis (Figure 1B), i.e., 122.2 (95% CI, 112.2–
144.7) mya. Note that, ML-FIR is giving results consistent with
that reported in earlier studies [1–3, 12–15], indicating that the
younger root for placentals returned using the function F_IR (see
below) is due to the method and not the data. The difference
between the F_LOG and the F"LOG function in table 1 is explained in
Materials and Methods. It is the F"LOG method that most closely
approximates the Brownian motion assumed by Multidivtime.
Figure 1 shows the estimated ancestral rates across the tree, while Figure 2 represents the inferred evolutionary rates going from the root to a terminal. The single instance of root to fin whale is shown in figure 2, while figure S1 traces the evolutionary rates inferred by different methods along seven representative lineages. The MVS-FIR tree identified an abrupt acceleration of evolutionary rate near the common ancestor of both Supraprimates and Laurasiatheria, then a very strong acceleration in just the ancestral lineage of Laurasiatheria (Figures 1A and 2A). This acceleration was followed closely by a strong deceleration along nearly all lineages of Laurasiatheria. In contrast, almost all lineages of Afrotheria and Xarathra have retained rates close to that of the root. There are also sporadic later accelerations, for example that among hedgechogs and Moon rat (Figure 1A).

The cost function FIR detected an acceleration-deceleration pattern near the base of Laurasiatheria using both the MVS and ML branch lengths (the red lines of Figure 2), whereas the function FLOG showed a far more flat prediction of generally lower evolutionary rates that led to older root times (the green lines of Figure 2). Even more extreme, the cost function FADD inferred gradually decreasing rates in all deep branches of the MVS and ML trees (the blue lines of Figure 2), and the root time tended to infinity. Table 1 summarizes the root time values estimated by various models (all the node divergence times from these models are listed in Table S2).

Table 1 also compares the inferred times with those obtained from r8s, a popular program for estimating absolute rates [17, 18]. This program has two options of for its cost functions, FADD and FLOG (equation (3) in Materials and Methods). It also takes account of stochastic noise caused by the finite length of the sequences used to derive the branch lengths using a penalized-likelihood approach. The fitting to the estimated branch lengths is expressed by the log likelihood, while the weight for the cost function is estimated using cross validation. In our mitochondrial dataset, with its long sequences, the root time values changed little due to the cross validation effect (the reason is explained in Materials and Methods). Further, root divergence time estimates returned by r8s do not show confidence intervals in table 1, since r8s only assesses the sampling variance of node times due to stochastic errors in branch lengths due to finite sequence length (it does this via the bootstrap) and does not include the effect of stochastic variation of evolutionary rate. This source of error remains as sequence lengths go to infinity, and will be dominant with long sequences.

Agreement with fossil data
Figure 3 represents the chronological distribution of the internal node density for the MVS-FIR tree (Figure 1A) compared with the rate of new species appearing in the well-studied North American fossil record. To avoid potential bias of divergence times in the molecular tree due to species sampling, the scaling constant was determined by a least-squares fit to the fossil data in the period 85–50 mya.

The MVS-FIR analysis reconciles molecular with fossil data in two ways. First, the chronological distribution of the internal node density for the MVS-FIR tree (Figure 1A) compared with the rate of new species appearing in the well-studied North American fossil record. To avoid potential bias of divergence times in the molecular tree due to species sampling, the scaling constant was determined by a least-squares fit to the fossil data in the period 85–50 mya.

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The combination of MVS and FIR is the best way to analyze this data, but it does reframe the discussion of the relationship between the fossil and molecular times into one where the molecular dates are being looked at far more critically.

Second, and more indicatively, the cost function FIR resolves incongruence among the fossil constraints/inferred divergence times in different parts of the molecular tree. The best fossil constraints in Laurasiatheria suggest much older times than constraints in other parts of the tree [4,37]. The MVS-FIR, MVS-FLOG, and MVS-FADD trees with all constraints give the root as 84.2, 105.0, and \( \infty \) mya, respectively (Table 1). Removing the whale–hippo constraint gives 82.4, \( \infty \), and 91.2 mya; removing the horse–rhino constraint gives, 82.8, 105.0, and \( \infty \) mya; and removing both sets of constraints gave 82.9, 87.2, and \( \infty \) mya. Thus, in this case only the FIR tree is insensitive to “constraint sampling”.

Finally, there is a good fossil calibration for tarsier [4] that was withheld because it is not used in both references 4 and 15. It suggests that human and tarsier split around 50–60 mya and the molecular trees suggest the human-loris split was not much earlier (probably \(<5\) myr earlier). Despite our use of whale–hippo and horse–rhino calibrations, the MVS-FIR tree (Figure 1) gives a human-loris split close to this age, in contrast to earlier studies [4,15,33].

### Sensitivity and robustness of FIR assessed by simulations

We highlight two distinct properties of the new function FIR with the help of evolutionary simulations and worked examples. The first property is its ability to detect a transient acceleration of evolutionary rate. Such an effect might be caused by a burst of positive selection and/or a bottleneck in population size. The second is to assess the effects of both stochastic fluctuations and systematic bias on the robustness of estimated times. Here, we model bias in the form of either a general slowdown or a general acceleration of evolutionary rate across the whole tree.

![Figure 2. Evolutionary rate changes from the root to the fin whale.](image)

Figures A and B, respectively, trace the estimated rates along edges on analyses using MVS and ML branch lengths. The root time of the FADD analysis in Figure A and B was set at a large value (400 mya) because the numerical calculation continues towards an infinite root time. doi:10.1371/journal.pone.0000384.g002

![Figure 3. Comparing molecular and fossil records of placental diversification.](image)

The blue squares show the rate of the appearance of new species based on the fossil record [adapted from 3]. At present, such quantitative data are limited to the well-studied North American record. The red circles represent the chronological distribution of node density (or splits) on the MVS-FIR tree (Figure 1A). The green triangles represent split frequency on the ML-FLOG tree (Figure 1B). The node density is given by the number of nodes in an 8 myr sliding window. A scaling constant for the molecular frequencies was calculated via a least-squares fit to the fossil data in the period 85–50 mya. doi:10.1371/journal.pone.0000384.g003

### Table 1. The age of the root of placental mammals

| Tree model | Cost function | F_{IR} | F_{ADD} | F_{LOG} | F'_{LOG} | F_{ADD}(r8s) | F'_{LOG}(r8s) |
|------------|---------------|--------|---------|---------|---------|-------------|---------------|
| MVS        | 84.2(80.7, 88.4) | Infinity | 105.0(97.0,117.0) | 91.8(84.5,104.7) | 160.5 | 91.1 |
| ML         | 106.4(102.4,110.6) | Infinity | 122.2(112.2,144.7) | 112.0(102.0,121.2) | 122.2 | 111.6 |

The age of the root using MVS and ML branch lengths was estimated after minimizing various penalty functions with the same fossil constraints. All times are in mya, and 95% confidence intervals were estimated by the sum-of-squares method described in Materials and Methods. The times estimated by the r8s program use cross-validation and penalized likelihood methods with essentially the same penalty functions [18], but do not report comprehensive errors.

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We first modeled a strong instantaneous acceleration as an analogue to what is inferred by FIR to have occurred ancestral branch leading to Laurasiatheria. We simulated a 32-taxon symmetric tree in which a molecular clock holds except for an abrupt elevation (by a factor of 10) of evolutionary rate along a short internal branch (the red line in Figure 4A). The true root time was set to 80 mya, and the times at the internal nodes are 48, 56, 64, and 72 mya. The deep internal branch (the red line in Figure 4A) is given an evolutionary rate ten times that of the remaining edges. The various cost functions were minimized subject to two calibrated nodes (the red numbers 1 and 2 in Figure A), using the exact branch lengths of this example as input data. The cost functions FIR, FLOG, and FADD inferred the weighted trees of Figures B, C and D, respectively. Figures E–G show the trace of evolutionary rates along the lineages from the root to taxa numbers 5, 9, and 25 of Figure A, respectively. The inferred age of the root for each cost function is shown with an arrow. The function FIR recovered the original pattern of rate change, whereas the other two functions inferred far more gradual changes, which resulted in a substantial overestimation of the root time.

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Table 2. Estimated age of the root in the presence of an abrupt acceleration then deceleration of ancestral rates

| True value | Cost function |
|------------|---------------|
| 80         | FIR FADD FLOG F'LOG FADD(r8s) F'LOG(r8s) |
| 81.3       | 161.1 114.5 101.7 160.5 108.7 |

These estimates are from a worked example with an abrupt acceleration then deceleration (the red line of Figure 4A) which occurs deeper in the tree than the calibration points. The true root age is 80.0 mya. The estimated values are made by minimizing the cost functions (that also appear in table 1), but upon the weighted constrained tree of figure 4A.

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We next simulated stochastic rate fluctuations by themselves, plus either a prevailing slowing down or acceleration of rates through time. Such simulations are distinct from a Brownian-type process, and are used to gauge the general robustness of the functions. To impose rate fluctuations on the same basic tree as.

Figure 4. A worked example of the effect of a transient elevation of evolutionary rate upon estimated divergence times. In this worked example using a symmetric 32-taxon tree (Figure A), a global molecular clock holds, except for a short-term increase in evolutionary rate along one branch (the red line in Figure A). The true root time was set to 80 mya, and the times at the internal nodes are 48, 56, 64, and 72 mya. The deep internal branch (the red line in Figure A) is given an evolutionary rate ten times that of the remaining edges. The various cost functions were minimized subject to two calibrated nodes (the red numbers 1 and 2 in Figure A), using the exact branch lengths of this example as input data. The cost functions FIR, FLOG, and FADD inferred the weighted trees of Figures B, C and D, respectively. Figures E–G show the trace of evolutionary rates along the lineages from the root to taxa numbers 5, 9, and 25 of Figure A, respectively. The inferred age of the root for each cost function is shown with an arrow. The function FIR recovered the original pattern of rate change, whereas the other two functions inferred far more gradual changes, which resulted in a substantial overestimation of the root time.

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Reduced bias in branch length estimation by the MVS model

The MVS model was shown previously to recover the correct tree in a simulation with two strongly convergent lineages [26] that were grouped erroneously by standard methods (including the neighbor joining (NJ) [38] and ML [35] methods). Here, we examined a different question; this is how well the MVS method recovers the true branch lengths, which are of primary importance when estimating divergence times. We simulated the evolution of a sequence of 10^4 amino acid sites, following the tree depicted in Figure 5. The model of amino acid substitutions used was the JTT [36]. Convergent evolution was then imposed on this data. This was done by sharing parts of the sequences among three ingroup clades plus the outgroup. The red lines in Figure 5 indicate which lineages shared sequence and were therefore subject to a form of convergent evolution.

Pairwise distances were then estimated from the terminal sequences obtained above using the same JTT model. A modified MVS core-set procedure [27] was able to recognize that the tree consisted of three groups of eight sequences which had additive distances within each group and the outgroup. The MVS procedure then converted the distances within the three ingroups into three sets of perfectly additive distances and sequentially combined them to form a single core set (Materials and Methods). The final MVS tree was obtained by modifying the distances between this single ingroup core set and the outgroup following the rules described in Materials and Methods.

The branch lengths recovered by the MVS model reproduced the true values accurately (Figure 6). The distribution around the diagonal line in Figure 6 represents the stochastic fluctuation of the estimated distances; that is, the magnitude of this fluctuation did not change after the simulation was rerun without convergent evolution. In contrast, the NJ and ML trees returned branch lengths that were affected clearly by convergent evolution. The worst affected branch lengths were ancestral to the groups showing convergent evolution (underestimated) or else were leading to the

Table 3. The age of the root inferred by functions FMR, FLOG, and FADD on simulated data with random auto-correlated rate changes

| Rate range | Cost function |
|------------|---------------|
| FMR | FLOG | FADD |
| 0.25–1.0 | 111.3±11.1 | 125.1±12.7 | 172.0±33.9 |
| 0.5–1.5 | 100.7±4.4 | 103.3±3.9 | 103.6±5.1 |
| 1.0–1.75 | 93.2±2.0 | 94.0±2.1 | 94.3±2.4 |

In these simulations, the length of a branch (on what tree!) is selected randomly proportional to its true duration in time. A rate adjustment (change) factor is then chosen randomly from a uniform distribution and all its descendant branch lengths were then multiplied by this factor. A total of 25 such random rate changes were placed on the tree, then the branch lengths (measured in the product of rate and time) of the weighted tree were passed on to the time estimation procedures. The whole procedure was repeated 600 times to obtain the average root time and standard error. Three different ranges were used for the uniform distribution of rate changes. The first has a range of 0.25 to 1 and represents a strong persistent bias towards rate deceleration. A range of 0.5 to 1.5 gives minimal rate change bias, but retains stochastic fluctuations. Finally a strong acceleration effect is achieved by the use of the range 1.0–1.75. The function FADD gave an age of the root tending to infinity on 139 of 600 samples. These undefined root-time values were set arbitrarily to 200 mya before calculating the mean and standard error.
sister groups of the groups affected by convergent evolution (overestimated).

**DISCUSSION**

We begin the discussion by examining why the current cost functions, FLOG and FADD, overestimated the age of the root in the worked example with strong rate heterogeneity in the form of a short term highly elevated evolutionary rate. It is also important to examine the profile of the cost function around their minimal values for the age of the root. The functions FLOG and FADD showed asymmetric behavior around the estimated root time, even in worked examples with a perfect molecular clock (data not shown), whereas the FIR profile was symmetric and parabolic in shape. The asymmetry seen with FLOG and FADD increased in response to a general bias towards deceleration of rates through time (Figure 7A). In particular, the function FADD showed a monotonic decrease with respect to increasing age of the root, that is, its estimate tended to infinity. Thus, asymmetric behavior of a cost function seems to be a symptom of unstable estimation of the age of the root. A similar strong asymmetry appeared in the profile of the current cost function with respect to the age of the root of the mitochondrial tree of placental mammals (Figure 7B), irrespective of whether MVS or ML branch lengths were used. Comparing Figures 7A and 7B suggests that bias due to deceleration of evolutionary rates as time progresses (from the set of simulations used for Table 3). The true age of the root was 100 mya. Figure 8 shows the results using the MVS tree derived from the mitochondrial sequences of placental mammals. The dotted line indicates the 95% confidence interval of the estimated age of the root using the sum-of-squares approach described in Materials and Methods.

Other approaches to divergence time estimation are under active development. For example, Drummond et al. [39] recently developed a set of Bayesian procedures to jointly estimate divergence times, evolutionary rates, and the tree topology. The uncorrelated variable rate model they use, which assumes independence of evolutionary rates between branches, may also be able to identify instantaneous accelerations. However, the magnitude of rate acceleration may be underestimated because hierarchical Bayes estimates can generally be regarded as shrinkage estimators [40]. An important future direction is to assess the performance of the various estimators in simulations where rate variability is a mixture of an autocorrelated process plus rare, but strong, instantaneous accelerations.

Our refined approaches resolve apparent contradictions between the quantitative molecular and paleontological data of placental mammals. Given such agreement, there is no need for ancillary hypotheses such as the long fuse model [2,5,9,12–15,41] to explain the lack of any positively identified fossils of modern placental mammals prior to about 75 million years ago. In addition, dates of markedly less than 100 mya for the root of placental mammals also bring into question earlier hypotheses that traditional continental drift models explain the geographic distribution of the four major groups of placental mammals [2–5,13,42]. We anticipate that the FIR cost function developed in this paper will provide an improved methodology for a wide range of molecular studies because its robustness to rapid fluctuations of rate is essential to understanding events such as adaptive evolution.
For example, because the acquisition of new molecular functions can be achieved in a few million years after gene duplications [40], the duration of an inflated evolutionary rate may be surprisingly short, and, correspondingly, difficult to detect. We hope our new methods will be beneficial in such situations and will lead to a richer understanding of molecular evolution.

**MATERIALS AND METHODS**

**Autocorrelated variation of inverse rate**

Because the branch lengths of a phylogenetic tree are estimated from the data as the product of the evolutionary rate of a branch and its time duration, these two factors cannot be directly estimated separately. It has become standard to use loose constraints to accommodate uncertainty and it is often wise to exclude constraints if there is no firm basis for them. Information on the age of some internal nodes may be fairly directly available from the fossil record. An example of this is the horse-rhino split [13]. If the molecular clock [16] governs the process of molecular evolution well, we can accurately estimate the times at which nodes given just a single reliable calibration point. However, the assumption of the molecular clock is often rejected by the fit of a clock-like tree to the data.

The most widely used assumption is to model the evolutionary rate changes away from being constant (or away from a clock) to introduce a stochastic process. For example, Sanderson [17] suggested the following cost function be minimized:

\[
F_{ADD} = \sum_{a}(R_a - R_{a0})^2
\]

with rates \(R_a\) and \(R_{a0}\) being from the \(n\)th branch and its ancestral branch \(a(0)\), respectively. The exponent of the cost function \(P_L\) \((2\pi)^{1/2}\sigma^{-1}\exp\left(-|R_a - R_{a0}|^2/2\sigma^2\right)\) implies that this function can be interpreted assumes a priori that rate changes follow a random walk with independent increments randomly drawn from a normal distribution. Likewise, Thorne et al. [31] considered a cost function of the type

\[
F_{LOG} = 2\sum_{a}(\log R_a - \log R_{a0})^2/(T_a + T_{a0})
\]

in the framework of the hierarchical Bayes procedure. Here \(T_a\) is the time interval of the \(n\)th branch (the current version of Multidivtime [31] uses rates at the nodes, instead of average rates along branches, as the free parameters). This can be interpreted as an assumption that the log rate undergoes Brownian motion. That is, independent increments of normal random variables, whose variances are proportional to the average time duration between the pair of branches (that is, the average length in time of the two branches). Recently, Sanderson implemented the unweighted cost function

\[
F'_{LOG} = 2\sum_{a}(\log R_a - \log R_{a0})^2
\]

into his program r8s [18].

However, the estimated rates based on any of the above cost functions may overly smooth the change of evolutionary rates when a pronounced transient change of rate has occurred. In turn, biased estimates of evolutionary rates will lead to biased estimates of divergence times. Here we propose a new cost function, which penalizes the local rate deviation from the harmonic mean. For simplicity, we ignore the stochastic variance of branch length [17,10,31], which goes to zero with increasing long sequences. Denote the branch length and inverse rate of the \(n\)th branch by \(B_n\) and \(Q_n\) (see \(T_n/B_n\), respectively. The local rate variability \((F_{IR,n})\) of two successive branches can be expressed as the variance of \(Q_n\) and \(Q_{n+1}\), around their average value, \(A_n = (Q_nB_n + Q_{n+1}B_{n+1})/(B_n + B_{n+1})\), which is equal to the inverse of the harmonic mean of \(R_n\) (see \(B_n/T_n\) and \(R_{n+1}\) with the weights \(B_n\) and \(B_{n+1}\). That is, \(F_{IR,n} = \left(Q_n - A_n\right)^2B_n(Q_n - A_n)^2B_{n+1}/(B_n + B_{n+1})^2\). Because \(F_{IR,n}\) is rewritten 

\[
F_{IR,n} = \left(Q_n - Q_{n+1}\right)^2W_n\text{ with the weight }W_n = B_nB_{n+1}/(B_n + B_{n+1})^2,\text{ the inverse-rate cost function, }F_{IR}, \text{ is defined as the weighted average of }\left(Q_n - Q_{n+1}\right)^2\text{ over all ancestor-descendant branch pairs in the tree:}
\]

\[
F_{IR} = \sum_n F_{IR,n}/\sum_n W_n = \sum_n (Q_n - Q_{n+1})^2 W_n/\sum_n W_n,
\]

which contrasts with previous expressions in terms of \(R_n\).

Because \(F_{IR}\) places a smaller penalty on abrupt rate changes than do previous models, it can confine an abrupt change close to where it occurred on the tree. In contrast, \(F_{ADD}\) and \(F_{LOG}\), a priori put stress upon the smoothness of evolutionary rate change from one branch to the next, and this may propagate the effect of an abrupt rate change over successive branches. In this article, the variable \(T_n\) (the divergence times) were estimated by minimizing the cost functions (equations 1–4) subject to the (fossil) constraints without introducing other modifiers such as cross-validation.

At first glance, it seems most intuitive to use a penalty such as \((rate_1 - rate_2)^2\); this has been the implicit assumption until now [31] and is the basis of published programs such as r8s and Multidivtime [17,10,31]. It rests on the implicit expectation that any reasonable type of smoothing of changes in rate will give a similar answer.

However, a simple linear form for the difference in rates is misleading, and this is illustrated by considering the general problem of estimating velocity (rate) given distance (in our case branch length). Going from point \(a\) to \(b\) at velocity \(v_1\), then back at velocity \(v_2\), the average velocity \(\langle v \rangle\) is equal not to the standard arithmetic mean, but to the harmonic mean, \(1/\langle v \rangle = (1/v_1 + 1/v_2)/2\). Further, going from point (node) \(a\) to \(b\) at velocity (rate) \(v_1\), and from point \(b\) to \(c\) at velocity \(v_2\), then the distances traveled \((d_{ab}\) and \(d_{bc}\) need no longer be equal so we must use weights. Specifically, \(d_{ac} = (d_{ab} + d_{bc})/(v_1 + v_2)\), where \(v_1 = d_{ab}/t_1\) and \(v_2 = d_{bc}/t_2\). As a result, \(1/\langle v \rangle = (d_{ab}/t_1 + d_{bc}/t_2)/d_{ac}\). This is the same form as the average quantity \(A_n\) used to obtain \(F_{IR}\) (equation 4). The use of the harmonic mean becomes important when \(v_1\) differs greatly from \(v_2\).

**The penalty in hierarchical Bayes and penalized likelihood approaches**

The Bayesian approach estimates the divergence times and evolutionary rates in the form of a posterior distribution, which is summarized approximately as \(P(B|T,R)\) exp\((-\lambda F)\). Here \(B, R,\) and \(T\) are the vectors of the estimated branch lengths, evolutionary rates, and divergence times, respectively, while \(F\) is the cost function. The value of \(\lambda\) expresses the weight for the penalty, and is called a hyperparameter. Introducing the distribution for the hyperparameter is done via a so-called hyper-prior; the hyperparameter is then estimated concurrently with the posterior distribution. The penalized likelihood approach maximizes log \(P(B|T,R) - \lambda F\). The weight for the penalty \(\lambda\) is estimated using cross-validation. When the sequences are long enough, for example concatenated mitochondrial protein sequences, it is often safe to assume that the branch lengths are estimated accurately, that is with minimal stochastic error (but not necessarily without systematic error, something we address in this paper using MVS). Accordingly, it is assumed that the products of rates and times are known exactly. Thus in a situation of long, or very long sequences
additivity and then complete a new core set for the remaining
definitely, we first exclude taxa with the largest deviations from
second step, and when it is difficult to connect two groups
(variance) between groups. At present, the decomposition is done
device (variance) within groups while maximizing the device
index. Here, a revised core-set approach, that is, an extension of
structure. This index enables one to diagnose whether a distance
pairwise distances do not satisfy additivity, MVS provides an index
additivity. The approach developed as part of this particular
branch vectors in a multidimensional Euclidean space [25]. If the estimated
pairwise distances do not satisfy additivity, MVS provides an index
to measure deviations from orthogonality without specifying a tree
matrix is compatible with a tree and to modify the biased distances
by minimal enlargement of the distances between the core set
internal node times reestimated, except for the root time $T_{root}$
sequences. We then locate the excluded sequences on the core set
tree using minimal modification of distances (a parsimony-like
criterion) under the constraint of only enlarging the distances
identified as being biased (thus the only biases are assumed to be
due to attraction).
When analyzing the mitochondrial protein sequences, we
estimated pairwise distances initially under the JTT+Gamma
model with $\alpha = 0.5$. Given moderately high bootstrap support, the
placental tree was decomposed into four major groups: Laur-a
asiatheria, Supraprimates, Xenarthra, and Afrotheria. Laurausiatheria was decomposed into five subgroups, namely Cetartiodactyla, Perisodactyla, Carnivora, Chiroptera, and Eulipotyphyla. Supraprimates was decomposed into two subgroups: Primates and Glires. The core-set analysis began by generating additive distances
within these subgroups, after which the clustering procedure
described above was applied to connect these subgroups. In this
analysis, we analyzed sequences of Xenarthra and Afrotheria
together in a single core-set. This is because a relatively short
internal branch separates them and Xenarthra contains but a single
sequence in this analysis. Finally, we tried to modify the distances
between placental mammals and the outgroups, but we could not
determine the position of the root with confidence because of very
strong attractions between many placental mammals and the
outgroups. The consensus [3–5,15] has been that the most probable
placement of the root separates Afrotheria from the other placental
mammals. The most likely alternative placement for the root is
between Xenarthra plus Afrotheria (Atlantogenata [2,20]) and all
other placentals (Boreotheria). Recent analyses including LINE
sequences and indels [43] support this view. When we place the root
in this position, the divergence times, including that of the root,
change little (results not shown).
Further details of the current MVS procedure of distance
modification are documented in Methods S1. The derived additive
distance matrix was converted to a Newick tree file with the help of
the NJ method, and this was used as the input data for divergence
time estimation by the cost functions.

Log-likelihood profiles of the cost function with
respect to the age of the root
We note that least-squares estimators can be interpreted as ML
estimators when errors in the data follow a multi-normal distribu-
tion. Accordingly, the least-squares residual can be treated as twice
the log-likelihood ratio for the three cost functions $G_j(T_{root})$
($j = \text{IR, ADD, or LOG}$); that is, $G_j(T_{root}) = N \log \{F_j(T_{root})/
F_j(T_0)\}$. Here, $F_j(T_{root})$ gives the minimum residual value with all
internal node times reestimated, except for the root time $T_{root}$, and
it becomes truly minimal at $T_{root} = T_M$. Here, $N$ is the number of
terms in the cost function, which is equal to $2t - 4$, where $t$ is the
number of tips on the tree (here $t = 62$, so $N = 120$). The 95% CI
can be obtained using the threshold of $G_j(T_{root}) = 3.84$. Here, the
minimum for criterion $F_{ADD}$ was arbitrarily set to $F_{ADD}(T_{root})$
at $T_{root} = 200$ because the profile decreased monotonically for all
greater values of the root time. In all evaluations, we avoid putting
arbitrary constraints near the root of the tree (for example, that the
rate at the root is the same as that of one of its descendants)
because while arbitrary constraints may bound the minimum, they
will also create unknown biases. Note that the MVS-FIR profile is
symmetric and parabolic around the estimated root time, whereas
those of $F_{LOG}$ and $F_{ADD}$ are not. We see the same thing even in
worked examples using a perfectly clock-like tree.
Our novel way of estimating CIs jointly takes into account
some, but not all, of the sources of error noted in a previous
publication [13]. These include errors in edge length estimation
(because of a finite sampling of sites), the unpredictability of fluctuating evolutionary rates, and coalescent time. The assumptions of this method, vis-à-vis independence of errors, are the same as those used in programs by Thorne et al. [31]. Note that even if edge lengths have no error due to sampling of sites (e.g., the type of error identified by the bootstrap), they may still show four wide CI, because of the other two factors, which do not go to zero with increasing sequence length. This, in turn, shows that using the sequence bootstrap to infer errors on divergence times [17,18] is not comprehensive and therefore the CIs are too narrow. We expect that this empirical approach to estimating standard errors will become increasingly accurate with increased numbers of internal nodes and calibration points.

Fortran source code and executable versions of the programs used in this study are downloadable from http://www.kochi-ms.ac.jp/~ct_cmis/kitazoe/”. The operation of these programs, using the data in this analysis, is documented in Methods S2.

**SUPPLEMENTARY INFORMATION**

**Methods S1** The procedure of MVS distance modification for placental mammals

Found at: doi:10.1371/journal.pone.0000384.s001 (0.04 MB PDF)

**Methods S2** The operation manual of programs for divergence time and MVS analyses

Found at: doi:10.1371/journal.pone.0000384.s002 (0.04 MB PDF)

**Figure S1** Trace of evolutionary rates along seven lineages in the MVS-FIR and ML-FIR analyses. The ML-FIR analysis (B) showed a flatter peak rate than did the MVS-FIR tree (A) (the black lines of F in the whole lineage) and inferred a longer period at a lower rate of evolution, producing an older root time. If lineages appear to merge with each other, zoom in to follow their exact path.

Found at: doi:10.1371/journal.pone.0000384.s003 (0.68 MB TIF)

**Table S1** Sequences and accession numbers used in this paper. The aligned amino acid sequences of the mitochondrial proteins ND1-ND6, ND4L, CO1-CO3, CTYB, and ATP6, are concatenated giving a total length of 3660 sites.

Found at: doi:10.1371/journal.pone.0000384.s004 (0.09 MB DOC)

**Table S2** Divergence times of the MVS-FIR, ML-FIR, and ML-FILOG analyses. The numbers 1–61 denote the ancestral nodes in Figure 1. The red numbers stand for the positions of the time constraints. Times older than the constraints varied the most, particularly using ML branch lengths.

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**Author Contributions**

Analyzed the data: HK NN YK TO TW YO. Wrote the paper: HK PW YK. Other: Developed the initial theory and developed the simulations: HK YK. Developed the simulations, added biological/theoretical interpretations and revised the manuscript: PW. Developed the Fortran programs: NN TO TW YO.

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