

# Estimating Divergence Dates from Molecular Sequences

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The ability to date the time of divergence between lineages using molecular data provides the opportunity to answer many important questions in evolutionary biology. However, molecular dating techniques have previously been criticized for failing to adequately account for variation in the rate of molecular evolution. We present a maximum-likelihood approach to estimating divergence times that deals explicitly with the problem of rate variation. This method has many advantages over previous approaches including the following: (1) a rate constancy test excludes data for which rate heterogeneity is detected; (2) date estimates are generated with confidence intervals that allow the explicit testing of hypotheses regarding divergence times; and (3) a range of sequences and fossil dates are used, removing the reliance on a single calculated calibration rate. We present tests of the accuracy of our method, which show it to be robust to the effects of some modes of rate variation. In addition, we test the effect of substitution model and length of sequence on the accuracy of the dating technique. We believe that the method presented here offers solutions to many of the problems facing molecular dating and provides a platform for future improvements to such analyses.

## Introduction

An increasingly common aim of molecular phylogenetic studies is to estimate the dates of divergence of lineages (e.g., Adachi and Hasegawa 1995; Arnason et al. 1996; Hedges et al. 1996; Wray, Levinton, and Shapiro 1996; Cooper and Penny 1997). If sequences have a uniform rate of molecular evolution across different taxa, a known date of divergence for a given pair of sequences can be used to calculate a rate of substitution (the calibration rate), which can then be applied to dating other nodes within a molecular phylogeny.

However, the use of a “molecular clock” to place absolute dates on lineage divergence times is limited by the ability to accurately reconstruct phylogenetic branch lengths from molecular data, and by the validity of applying a calibration rate from one part of the tree to date other nodes. Accurate inference of branch lengths is essential to molecular dating and is governed by the type and amount of data used, the phylogenetic technique chosen, and the selection of an appropriate model of molecular evolution. Uniform rates of change across a tree cannot be assumed, as lineage-specific rate variation has been demonstrated for many taxonomic groups (e.g., mammals [Bromham, Rambaut, and Harvey 1996], birds [Mooers and Harvey 1994], and plants [Bosquet et al. 1992; Gaut et al. 1992]). Variation in the rate of molecular evolution constitutes a grave problem for molecular clock techniques, so any analysis must incorporate explicit means for dealing with rate-variable data (e.g., Hasegawa, Kishino, and Yano 1989; Takezaki, Rzhetsky, and Nei 1995) or test for rate constancy (e.g., Hedges et al. 1996).

Here, we describe a method for estimating dates of divergence from molecular data which addresses the problems of lineage-specific rate heterogeneity. The method is based on that presented by Cooper and Penny

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(1997) but is performed within a maximum-likelihood framework. This provides a means of testing assumptions of rate constancy among lineages and allows estimation of the confidence intervals for the estimated dates of divergence. The method incorporates a “clock test,” which is used to reject any data for which lineage-specific rate heterogeneity is detected. In addition, the method described is robust to error arising from limited departures from rate constancy.

## Methods

We begin with an aligned set of sequences for the taxa in question and a set of independently derived dates of origin for some of these taxa (e.g., fossil evidence). This alignment is used to construct a tree using the maximum-likelihood procedure described by Felsenstein (1981). A model of nucleotide substitution must be selected that is appropriate to the sequences being analyzed: the one used here is the HKY model of Hasegawa, Kishino, and Yano (1985) coupled with the discrete gamma model (Yang 1994) of site-specific rate heterogeneity (denoted as the HKY- $\Gamma$  model). The parameters of these models (i.e., transition-transversion ratio and shape parameter for the gamma model) may be estimated by finding the values that maximize the likelihood of the tree of all taxa. This results in a tree describing the phylogenetic relationships and a model of substitution including estimated parameter values.

Using the phylogenetic tree obtained in the previous stage or a priori knowledge of the phylogenetic relationships of the taxa, we construct quartets consisting of two pairs of taxa under the following constraints: (1) each pair must be known to be monophyletic with respect to the other pair in the quartet, and (2) we must have an independent estimate of the date of divergence for each pair. A quartet is defined here as a rooted tree consisting of four taxa, two internal nodes, and a root node. We shall label the taxa A, B, C, and D, the internal nodes X and Y, and the root node Z (fig. 1). Node X is the most recent common ancestor (MRCA) of taxa A and B, and node Y is the MRCA of taxa C and D. As

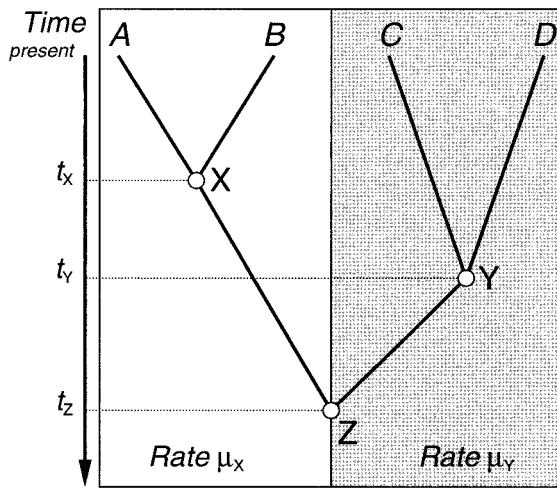


FIG. 1.—A quartet consisting of two pairs of taxa. The first pair (A and B) share a common ancestor (node X) which has a known date  $t_x$ . The second pair (C and D) share a common ancestor (node Y) at a date,  $t_y$ . The root of the quartet (node Z) is at some older date,  $t_z$ . Given a rate of molecular evolution for each of the pairs ( $\mu_x$  and  $\mu_y$ ) we can give expressions for the branch lengths of this rooted tree in substitutions per site. These are:  $AX = BX = \mu_x t_x$ ;  $XZ = \mu_x(t_z - t_x)$ ;  $CY = DY = \mu_y t_y$ ;  $YZ = \mu_y(t_z - t_y)$ .

each pair is monophyletic with respect to the other, the root Z is the MRCA of X and Y.

#### Estimating the Divergence Date

We start with a date estimate,  $t_x$ , that is, the age of node X (i.e., the date of divergence of taxa A and B). Likewise, we have a second date,  $t_y$ , which is the estimated age of node Y (the date of divergence of taxa B and C). The root, Z, has an unknown date,  $t_z$ , which is further back in time than either  $t_x$  or  $t_y$ . We then define two unknown rates,  $\mu_x$  and  $\mu_y$ , which are the rates of molecular evolution in substitutions per nucleotide site per million years (Myr). One rate is assigned to the branches AX, BX, and XZ, and the other is assigned to CY, DY, and YZ. From dates  $t_x$ ,  $t_y$ , and  $t_z$ , we can use  $\mu_x$  and  $\mu_y$  to provide expressions for all six branch lengths of the quartet in units of substitutions per site (fig. 1). With these branch lengths and a suitable model of nucleotide substitution, we can calculate the likelihood of the quartet. We have three unknown parameters,  $t_z$ ,  $\mu_x$ , and  $\mu_y$ , in the branch length expressions which must be estimated by finding the values that maximize the likelihood of the quartet. As we only allow two rates, we shall refer to this as the two-rate model. It is also possible to assume that the rates  $\mu_x$  and  $\mu_y$  are equal, a model we shall denote as the one-rate model. Both the two-rate and one-rate models are referred to as rate-constrained models.

#### Testing the Constancy of Rates

The hypothesis of rate constancy is tested using a likelihood ratio test (Felsenstein 1981), where the test statistic is the difference in log likelihood ( $\Delta$ ) between a rate-constrained model (see fig. 1) and a model which has no constraints on branch lengths. The unconstrained model is an unrooted tree with five branch lengths, each

with its own rate. To obtain a null distribution against which to test this statistic, we simulate a set of quartets of nucleotide data under the constrained hypothesis using the same model of nucleotide substitution including the maximum-likelihood estimates of any substitution model parameters (Goldman 1993; Hillis, Mable, and Moritz 1996; Huelsenbeck, Hillis, and Jones 1996). These simulated data sets are then analyzed under the constrained and unconstrained models in the same way as the real data, and a distribution of log likelihood differences is obtained. If  $\Delta$  is greater than the 95th percentile of this distribution, then the constrained hypothesis may be rejected as significantly worse than the unconstrained hypothesis.

#### Estimating Confidence Intervals

After rejecting all quartets for which rate heterogeneity is detected by the test described above, we are left with a subset of our original quartets, which have been found to be consistent with the hypothesis of either the one-rate or two-rate model. For these, we obtain the 95% confidence intervals on the estimate of the divergence date by finding the two values either side of the best value for which the likelihood is 1.92 less than the maximum likelihood. The value of 1.92 corresponds to half the  $\chi^2$  95% critical value for one degree of freedom. The procedure is based on the assumption that twice the likelihood ratio is distributed as a  $\chi^2$  with degrees of freedom equal to the number of constrained parameters. This assumption is an expectation of general maximum-likelihood theory (e.g., see Felsenstein 1981) and, in addition, is shown here and elsewhere (Yang, Goldman, and Friday 1995) to be reasonable by simulations. A program, written in C, for performing the analyses described here is available from the authors' web site: <http://evolve.zoo.ox.ac.uk/QDate/QDate.html>.

#### Simulations

We did an extensive set of simulations in order to examine the conditions under which the quartet method will produce biased estimates of the date and the power of the test of rate constancy to exclude such dates from the analysis. These simulations were performed using a program called Seq-Gen (Rambaut and Grassly 1997), which uses an explicit model of nucleotide substitution to evolve sequences along phylogenetic trees.

#### The Effect of Substitution Model

First, it is important to consider the effect of choosing an inappropriate model of nucleotide substitution for the estimation of the divergence date. A poor choice of substitution model can severely affect the estimation of branch lengths (Fukami and Tateno 1991; Ruvolo et al. 1993; Gaut and Lewis 1995; Yang 1996). To test this assertion, we generated 500 sets of 4 sequences using a model quartet and a range of values for the parameters of the models of substitution. In each case, we repeated the simulations for two different rates of substitution: (1) a slow rate, in which there were 0.1 expected substitutions per site between a member of one pair of the quartet and a member of the other; and (2) a fast rate,

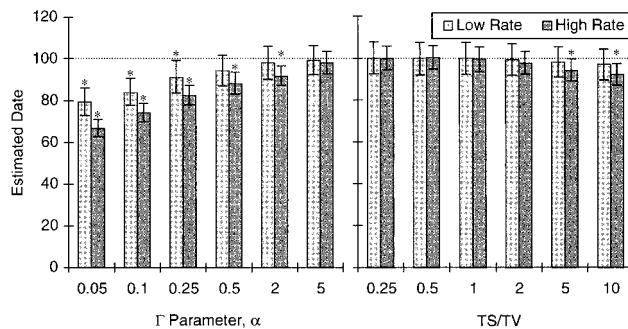


FIG. 2.—*a*, Estimated date for simulated quartets constructed using a range of gamma ( $\Gamma$ ) rate heterogeneity and reconstructed assuming rate homogeneity. *b*, Estimated date for simulated quartets constructed using a range of transition-transversion ratios (TS/TV) and reconstructed assuming an equal rate of transitions and transversions (TS/TV = 0.5). In both studies, the sequences were 3,000 nucleotides in length. The actual date is 100.0 in arbitrary units of time (dotted line). The low rate is  $10.0 \times 10^{-4}$  substitutions per site per unit time; the high rate is  $25.0 \times 10^{-4}$  substitutions per site per unit time. For each treatment, 500 simulated data sets were generated. The error bars encompass 95% of the estimates, and the asterisks denote those cases where this range does not contain the actual date.

in which 0.5 substitutions were expected per site over the same distance.

The first parameter to be studied was a measure of the site-specific rate heterogeneity using the discrete gamma distribution (Yang 1994). This defines a shape parameter,  $\alpha$ , which, as it approaches zero, signifies an increasing degree of rate heterogeneity between sites. Values greater than 1 approximate rate homogeneity (Yang 1996). Ignoring rate heterogeneity between sites can seriously underestimate branch lengths and will result in underestimation of the date of divergence (Adachi and Hasegawa 1995; Yang 1996).

The second parameter studied was the transition-transversion ratio (TS/TV), defined as the relative frequency of transitions to transversions (as there are two possible transversions to each transition, a ratio of 0.5 gives the Jukes-Cantor [1969] model). The rate of transitions can be considerably higher than that of transversions in many genes (e.g., mitochondrial genes), and a failure to accommodate this difference has been shown to cause biases in the estimation of branch lengths (Fukami and Tateno 1991; Ruvolo et al. 1993). To examine the effect of ignoring the transition bias, we simulated quartets with a range of TS/TV values and then estimated the divergence date assuming an equal rate of transitions and transversions.

It is clear from these results (shown in fig. 2) that erroneously assuming uniform rates across sites can cause a serious underestimation of the date of divergence. This bias is exaggerated in the fast-evolving sequences. However, failure to account for a transition bias does not have a strong effect on the estimates of date, except for extreme cases.

#### Rate Variation

To examine the effect of lineage-specific rate variation on the estimation of divergence dates, we simulated the evolution of nucleotide sequences along quartets, under three different modes of rate heterogeneity (see fig. 3). The first (mode A) has a rate for each of the two pairs of taxa, including the branch leading from each pair to the root. Mode B has a single lineage of one pair evolving under the second rate. Mode C has one lineage from each of the two pairs evolving under the second rate. While it is unrealistic to expect two lineages to differ in rate by the same degree, mode C is designed to test the effect of rate variation in both pairs. Clearly, other modes of rate heterogeneity are conceivable, but these three were chosen as a representative sample for the purposes of this study.

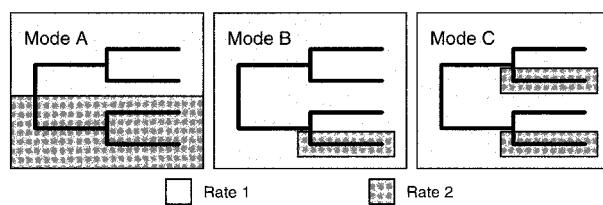


FIG. 3.—The three modes of rate variation modeled in the simulation study. Mode A has one half of the quartet evolving at a different rate than the other half and is the same as the two-rate model used in the reconstruction of quartets. Modes B and C are models in which one tip of one pair or one tip of each pair is evolving at a different rate, respectively.

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For each mode, five degrees of rate variation were used. The first rate was set to a value of 0.1 expected substitutions per site from the root of the quartet to any one of the tips, and the second rate was set to values 0.5, 0.75, 1.0, 1.25, and 1.5 times that of the first. An HKY- $\Gamma$  model of nucleotide substitution was used with a TS/TV of 2.0 and a gamma shape parameter ( $\alpha$ ) of 0.5. The simulated quartets were then used to estimate the dates of divergence using this same model of substitution.

The results show that, for the three modes tested, rate heterogeneity within this range has a limited effect on the maximum-likelihood quartet estimate of divergence date (fig. 4). Under any degree of mode A rate heterogeneity, no effect can be discerned, as the fossil dates can independently calibrate each pair in the quartet. Mode B and C rate heterogeneity both affect the date estimate, with mode C showing a greater effect. As might be expected, sequence length affected only the precision of the estimates, not their accuracy.

#### Statistical Power of the Likelihood Ratio Test

Given the potential for error in date estimation caused by rate heterogeneity between lineages, it is important that the rate-constancy test has reasonable statistical power to reject rate-variable quartets. For each of the quartets simulated above, we performed a likelihood ratio test of the one-rate and two-rate models. The test has considerable power to detect all three types of rate heterogeneity (fig. 5), but its power is dependent on sequence length. Modes B and C are detected less often than mode A for shorter sequences. The two-rate model does not reject the mode A heterogeneity except by chance, because this model is the equivalent of mode A heterogeneity.

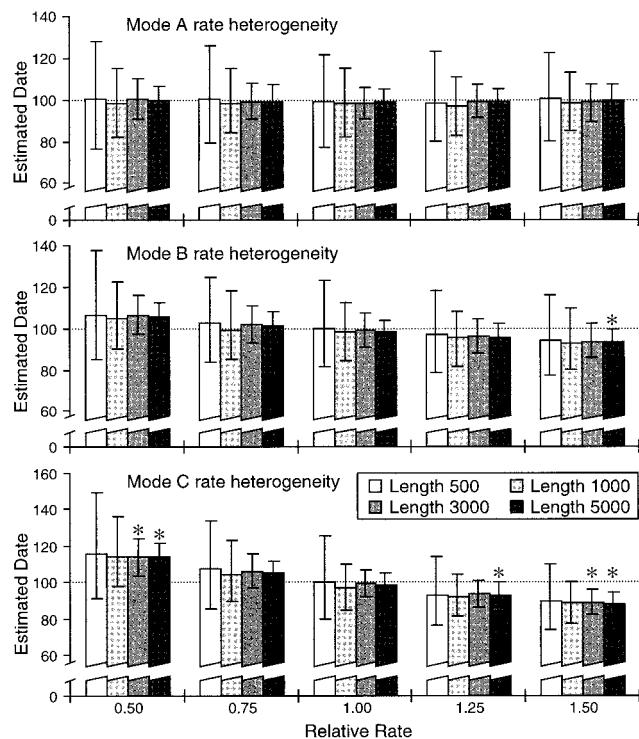


FIG. 4.—The effect of rate variation of modes A, B, and C (see fig. 3) on the estimate of divergence date under the one-rate model. Each treatment consists of 200 replicate simulations of sequences of 500, 1,000, 3,000, and 5,000 nucleotides. The actual date is 100.0 in arbitrary units of time. The first rate is  $1.0 \times 10^{-3}$  substitutions per site per unit time, with the second rate relative to this by factors of 0.5, 0.75, 1.0, 1.25, and 1.5. An HKY- $\Gamma$  model of nucleotide substitution was used (Hasegawa, Kishino, and Yano 1985; Yang 1993) with a TS/TV of 2.0 and a gamma shape parameter of 0.5 (the same model was used in reconstruction). The bars are confidence intervals spanning 95% of the 200 estimates. The asterisks denote those confidence intervals which do not contain the actual date. Under the two-rate model, almost identical results were obtained (data not shown).

#### Confidence Intervals

Simulations were used to test the appropriateness of the procedure for estimating 95% confidence intervals of divergence dates. Sets of sequences with lengths of 500, 1,000, 3,000, and 5,000 nucleotides were simulated along a model quartet, repeating the procedure 200 times for each length. These simulated sequences were then used to estimate the date and confidence intervals repeatedly, using the same procedure as for the real data (table 1).

If the confidence intervals are correctly estimated, then only 5% of them would be expected to fail to contain the divergence date used in the original quartet (for the model quartet, this was 100 in arbitrary units of time). Under this expectation, the 95% confidence limits on a binomial distribution with a sample size of 200 is 2.41%–9.03%. As all the values fall within this range, we can conclude that the procedure is satisfactory. As might be expected, as the sequence length increased, the mean range of the estimated confidence intervals decreased.

#### Example: The Origin of the Modern Bird Orders

To demonstrate the use of this method, we used the data set compiled by Cooper and Penny (1997) for dating the origin of some of the modern bird orders and to test the hypothesis of radiation of these orders at the Cretaceous-Tertiary (K-T) boundary (Feduccia 1995). This data set consists of partial sequences for the 12S and *c-mos* genes for members of 23 families, giving a total of 957 nucleotides each. A tree was constructed by the maximum-likelihood procedure using test version 4.0d52 of PAUP\*, written by David L. Swofford. An HKY- $\Gamma$  model of substitution was assumed, and the maximum-likelihood values for TS/TV and the gamma shape parameter,  $\alpha$ , were estimated separately for the 12S and *c-mos* sequences. The tree (not shown) was similar to that described by Cooper and Penny. For the 12S sequences, we estimated TS/TV to be 11.46 and  $\alpha$  to be 0.16. For the *c-mos* gene, these parameters were 4.41 and 0.35, respectively. Both values of  $\alpha$  suggest a high degree of site-specific rate heterogeneity. These values were then used in the estimation of dates using quartets.

Pairs of taxa with fossil-derived dates of origin (table 2) were combined into quartets. To prevent conflicts due to uncertain phylogeny, for each quartet, only one pair was used from each of the three groups of birds. This resulted in 2 quartets estimating the pheasant-seabird divergence, 7 estimating the pheasant-ratite divergence, and 13 estimating the seabird-ratite divergence, a total of 22 quartets. The constancy of rates was tested using the likelihood ratio test, as described above, with 500 replicate simulations. The quartets were run under both the one-rate and two-rate models.

The results are shown in figure 6. The only quartet to be rejected as not fitting the one-rate model was the guinea fowl/chicken versus ostrich/emu. Under the two-rate model, this quartet was accepted and the rate of the ostrich-emu pair was estimated to be 0.58 times that of the guinea fowl-chicken pair. Interestingly, for this quartet, the estimates of divergence date under the one-rate and two-rate models differed only by 2.9 Myr, suggesting that the rate variation detected was of mode A.

While examining these results, we should keep in mind that the 22 estimates are not independent, because the quartets share lines of descent, i.e., branches of a molecular phylogeny. We cannot, therefore, combine the estimates or confidence intervals to produce a single estimate of the date of divergence. However, we may consider the minimum and maximum of the confidence intervals and use these to reject specific hypotheses about the date of divergence. The most recent dates of all of these estimates are considerably older than the K-T boundary, so it can be concluded that the molecular data are incompatible with the divergence of these orders at the K-T boundary. Instead, an earlier divergence of these orders is suggested.

#### Discussion

The maximum-likelihood quartet method presented here has four main advantages. First, by using two fossil

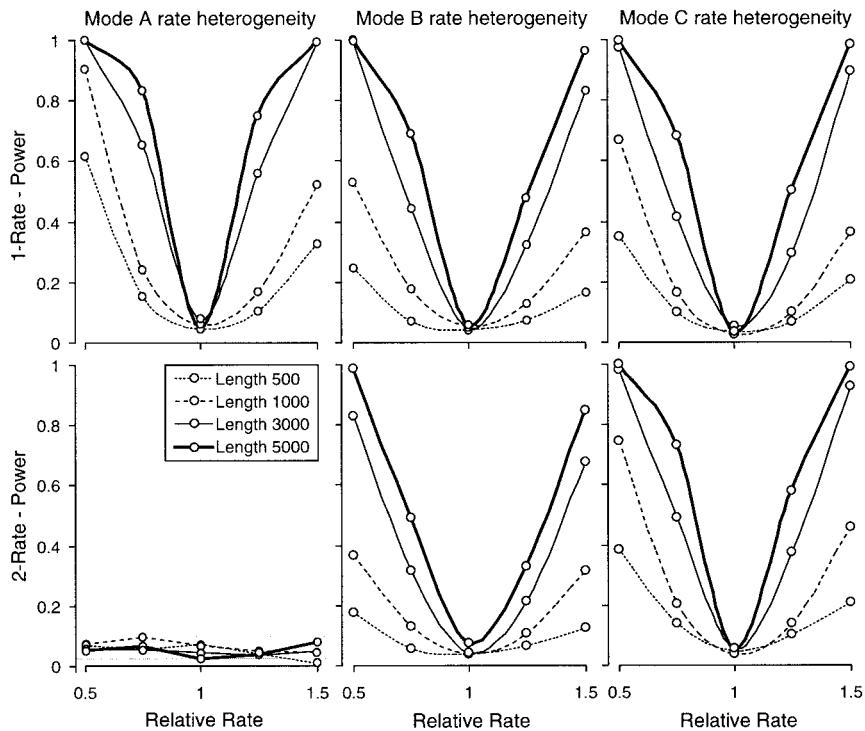


FIG. 5.—The statistical power of the quartet parametric bootstrap test to reject a false null hypothesis of rate constancy. The three types of rate variation (modes A, B, and C; see fig. 3) are simulated for 200 replicates of sequences of lengths 500, 1,000, 3,000, and 5,000 nucleotides. The values of the second rate relative to the first were 0.5, 0.75, 1.0, 1.25, and 1.5. These simulations were reconstructed under the one-rate (top row) and two-rate models (bottom row). The lines were fitted by eye. At a relative rate of 1.0 (rate constancy), all the treatments converge to a correct type I error rate (the chance of rejection of a correct null hypothesis) of 5%. As expected, with mode A rate heterogeneity and a two-rate model (bottom left), the null hypothesis is correct and is rejected at a rate of 5%. In this graph, the horizontal lines represent the 95% confidence limits for a binomial distribution with an expectation of 5% and a sample size of 200. Two points fall outside these limits, but this would be expected out of a total of 20 points.

dates, it can accommodate rate heterogeneity between taxa under the two-rate model. Second, we use a likelihood ratio test to reject quartets for which the assumptions of the two-rate model are violated, therefore largely avoiding the effect of rate heterogeneity on date estimates. Third, simulations indicate that the method is robust to moderate departures from the model for both rate heterogeneity and the substitutional process. Finally, we are able to use the maximum-likelihood framework to obtain confidence intervals of the date estimate under any of the models used.

Confidence intervals are an important part of any molecular dating technique, as the sources of error inherent in molecular clock analyses prevent precise dat-

ing. If the uncertainty is adequately expressed as confidence intervals (which should reflect the error arising from both the phylogenetic reconstruction process and the stochasticity of the molecular clock), then molecular date estimates are ideally suited to hypothesis testing, as it is possible to ask if molecular data is consistent with a given date of divergence. Here, we illustrate the use of molecular dates with confidence intervals to test hypotheses with the example of the timing of the origin of bird families. We show that molecular dates are incompatible with a radiation of bird families at or near the K-T boundary.

One source of error that is not contained within the confidence intervals is the accuracy of the fossil dates which are used to estimate rate of substitution. Fossil dates represent the first appearance in the fossil record of recognizable members of differentiated phyla. As such, they must underestimate the true date of divergence of two lineages and thus cause an overestimate of the evolutionary rate, making the molecular date estimates too young. This, combined with the asymmetric confidence intervals (table 1), will mean that the quartet method has more statistical power when testing a null hypothesis of a more recent divergence than when testing that of a more ancient divergence.

As a further development, it would be possible to combine estimates obtained from different genes for a

**Table 1**  
Confidence Intervals (CIs) for the Maximum-Likelihood Estimate of Divergence Date

Length	CI Test <sup>a</sup> (%)	Mean Date	Mean CI Range <sup>b</sup>
500 .....	5.5	99.21	46.10 $\pm$ 16.7
1,000 .....	6.5	98.24	30.71 $\pm$ 7.4
3,000 .....	3.5	98.19	17.79 $\pm$ 2.4
5,000 .....	8.5	98.88	13.99 $\pm$ 1.6

<sup>a</sup> The percentage of the simulations for which the estimated confidence intervals did not contain the actual date of 100.0 (in arbitrary units of time).

<sup>b</sup> The mean of the upper confidence interval minus the lower confidence interval, with the errors encompassing 95% of the values.

**Table 2**  
The Pairs of Bird Taxa Used to Construct the Quartets

Pair	Families	Group	Date <sup>a</sup>
Guinea fowl/chicken . . . . .	Numididae/Phasianidae	Pheasants	40
Penguin/shearwater . . . . .	Spheniscidae/Procellariinae	Seabirds	58
Tropic bird/gull . . . . .	Phaethontidae/Laridae	Seabirds	60
Rhea/strich . . . . .	Rheidae/Struthionidae	Ratites	55
Rhea/cassowary . . . . .	Rheidae/Casuariidae	Ratites	55
Rhea/emu . . . . .	Rheidae/Casuariidae	Ratites	55
Rhea/kiwi . . . . .	Rheidae/Apterydidae	Ratites	55
Ostrich/cassowary . . . . .	Struthionidae/Casuariidae	Ratites	43
Ostrich/emu . . . . .	Struthionidae/Casuariidae	Ratites	43
Ostrich/kiwi . . . . .	Struthionidae/Apterydidae	Ratites	43

<sup>a</sup> The dates are in MYA, and details of their references can be found in Cooper and Penny (1997).

particular quartet. This would be done by combining the likelihood framework for the two genes such that they each have independent parameters for their models with the exception of the date of divergence. For this, we would obtain the value which maximized the product of the likelihoods for the two genes. It is not even necessary to have exactly the same taxa represented for each gene, as long as we are certain that the divergence that we are estimating in each case is the same (i.e., the same node in the phylogeny). When combining genes of different natures (e.g., mitochondrial with nuclear or protein-coding with RNAs), it is desirable to allow different substitution models for each gene.

The maximum-likelihood quartet method represents an improvement over previous molecular dating techniques because of the explicit treatment of rate heterogeneity and the production of date estimates with confidence intervals that express the uncertainty in phylogenetic reconstruction of branch lengths, including the stochastic nature of the molecular clock. It can therefore be used to test specific hypotheses concerning dates of divergence. The present analysis is limited by the availability of sequence data and the inability to combine estimates from individual quartets due to nonindependence. We are optimistic that these limitations can be overcome by further developments of methods like the one presented here.

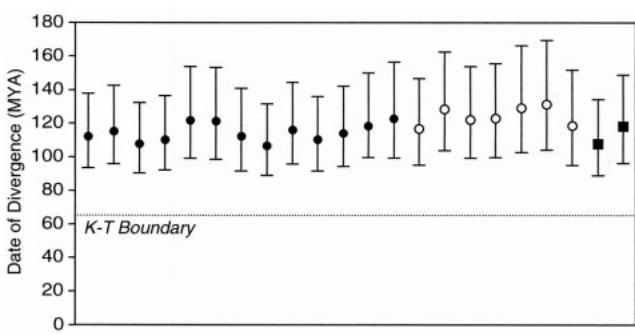


FIG. 6.—The maximum-likelihood estimates of the dates of divergence of some modern orders of birds. The closed circles, open squares, and closed squares denote the seabird-ratite, pheasant-ratite, and pheasant-seabird divergences, respectively. The bars represent the 95% confidence intervals estimated for each quartet, the lower limits of which are all considerably earlier than the K-T boundary (65 MYA).

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