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DATING THE TIME OF ORIGIN OF MAJOR CLADES: Molecular Clocks and the Fossil Record

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■ **Abstract** Molecular and paleontological data provide independent means of estimating when groups of organisms evolved in the geological past, but neither approach can be considered straightforward. The single most fundamental obstacle to developing an accurate estimate of times of origination from gene sequence data is variation in rates of molecular evolution, both through time and among lineages. Although various techniques have been proposed to circumvent this problem, none unambiguously allow the components of time and rate to be separated. Furthermore, problems of establishing accurate calibration points, correctly rooted phylogenies, and accurate estimates of branch length remain formidable. Conversely, paleontological dates fix only the latest possible time of divergence, and so probabilistic methods are required to set a lower boundary on origination dates. Realistic confidence intervals that take preservational biases into account are only just becoming available.

Although molecular and paleontological approaches to dating often agree reasonably well, there are two notable areas of disagreement; when mammal and bird orders originated and when the major phyla originated. The discrepancy in dating bird/mammal ordinal origins probably reflects a global rock-record bias. Paleontological sampling in the Late Cretaceous is still too restricted geographically to draw any firm conclusions about the existence of a pre-Tertiary record for modern orders of bird or mammal from anywhere other than North America. Dating the time of origin of phyla is more complicated, and is confounded by both preservational biases and problems of molecular clock estimation.

INTRODUCTION

Being able to date when major taxonomic groups arose in the past is of critical importance to any discussion about rates and patterns of evolution. Until recently, times of origin could only be estimated by turning to the fossil record with all its

known imperfections. For Darwin, who expected slow and gradual change, the abrupt appearance of major higher taxa in the fossil record was a clear indication of its incompleteness (1859). But more recently, there has been a noticeable shift towards taking the fossil record at face value. Part of the reason for this is that various independent lines of evidence now suggest that the fossil record is really rather well sampled (Donovan & Paul 1998, Foote & Sepkoski 1999). So, for example, the sudden appearance of many new morphological forms at the start of the Cambrian has come to be taken as evidence for there having been an explosive burst of evolution (Gould 1989, Conway Morris 2000, Budd & Jensen 2000).

However, an independent means of estimating times of origin for extant clades, first suggested by Zuckerkandl & Pauling (1962), is to use the amount of genomic evolution as a molecular clock. Although initial results seemed promising (e.g., Wilson et al. 1977), methodological problems quickly became apparent (e.g., Ayala 1986), and it was only when advances in molecular biology made it relatively easy to generate large amounts of sequence data in the mid-1990s that a renewed interest in molecular clocks arose. Whereas paleontological estimates are based on the appearance in the fossil record of the earliest recognizable member of a clade, molecular estimates attempt to date actual divergence times (Figure 1). In many cases, molecular estimates and paleontological estimates are in good agreement (Kumar & Hedges 1998, Michaux & Catzeflis 2000, Adkins et al. 2001), but there are occasional striking mismatches. Sometimes this is clearly a preservational problem, as in the case of scleractinian corals which evolved from soft-bodied ancestors (Stanley & Fautin 2001). In other cases, there is no such obvious explanation. This has sparked a heated debate between molecular biologists and paleontologists about the reliability and relative merits of the two methods (e.g., Easteal 1999 versus Benton 1999). The arguments are fiercest where the two estimates seem most at odds: over the origins of the metazoan phyla and the origins of the ordinal groupings of living birds and mammals. In both cases, molecular clock estimates give much older divergence dates than would be expected from the fossil record (e.g., Cooper & Fortey 1998, Bromham et al. 1999).

Here we review the relative merits and drawbacks of paleontological and molecular methods primarily with reference to the debate over the origins of living bird and mammal orders. Is their sudden appearance after the Cretaceous-Tertiary extinction event, some 65 Ma ago, attributable to a burst of rapid evolution, an artifact of sampling, or a combination of the two? Although we briefly discuss the origin of the metazoan phyla, we choose to focus on bird and mammal origins for two reasons. First, the nature of the fossil record on either side of the critical interval is well documented, and there is a much clearer understanding of both the paleoenvironmental and paleogeographical settings. Second, the taxa in question are better sampled in terms of both numbers of gene sequences and breadth of species coverage.

PALEONTOLOGICAL DATING

Approaches and Problems

The oldest fossil with one or more synapomorphies of a particular clade establishes the time by which that clade must have come into existence. Furthermore, if that date is older than the oldest fossil assignable to its sister group, then it also establishes the time by which that sister group had evolved, even in the absence of any fossil record of that clade (Figure 1). Even with a perfect fossil record, the oldest recognizable member does not pinpoint the time of divergence, only the time at which that lineage acquired its first recognizable synapomorphy. So molecular and morphological approaches are effectively measuring different aspects of phylogeny (see Figure 1 caption).

The major drawback of dating originations on paleontological evidence is that it defines only the latest possible time of divergence and sets no limits to the lower boundary. The fossil record provides a definitive date by which individual clades must have arisen, but this is not necessarily when they arose. If there are biases in the fossil record then the fossil date of first appearance may be significantly younger than the actual date of origination. Biases that can cause the fossil record to underestimate times of origination include low preservation potential, or sampling intensity, and changes in clade diversity.

Our chances of sampling a clade are to a large extent dependent upon how many species it contains and whether those species are living in habitats conducive to fossilization. Thus, the probability of sampling a member of the clade Mammalia at any one time interval is very much greater than that of sampling any one mammalian genus, simply because the former usually encompasses a greater number of species that could be fossilized and discovered. Because clades initiate from a single taxon and diversify over time, it is less likely that a clade is sampled in the early part of its history than later on, when it has expanded to contain many species (Martin 1993).

Quality of preservation is another factor that changes over time for various reasons. Late Cretaceous mammals and birds are entirely small-bodied forms, and their record is in general significantly poorer in terms of quality of preservation than the corresponding Early Cretaceous or post-Paleocene record. Early Cretaceous Lagerstatten, such as at Liaoning in China and Las Hoyas in Spain, provide valuable windows on small terrestrial vertebrate faunas, but the only Late Cretaceous deposits yielding abundant articulated cranial and whole body material are in Mongolia (e.g., Norell & Clarke 2001). Elsewhere, Late Cretaceous mammals are known primarily from isolated bones, teeth, and jaws (Cifelli 2000). This contrasts with the relatively rich cranial and post-cranial skeletal record of the larger and more robust Paleogene mammals, and the occurrence of a few key Eocene Lagerstatten (e.g., Messel) preserving small terrestrial vertebrates. Although terrestrial deposits of latest Cretaceous age are widely developed in South America, North Africa, and Europe, such deposits have yielded few small mammal and

bird fossils, presumably for taphonomic reasons. It is harder to place fragmentary vertebrate material with any degree of confidence into a cladistic framework because of the large number of character states that remain unknown, and there is a surprising level of homoplasy in early mammalian dentition that confuses the issue (Cifelli 2000). Hence, an additional problem is that the poorer preservation of Late Cretaceous and Paleocene faunas makes it difficult to identify early stem-group members of extant orders.

Finally, even if we have sampled the rocks with great thoroughness, there is also a large-scale heterogeneity in the rock record that needs to be taken into account (Smith 2001). Some time intervals leave a relatively extensive rock record, whereas others are much more poorly represented due to large-scale transgression-regression cycles in Earth history. Furthermore, there can also be a shift from a terrestrial-dominated to a marine-dominated record for the same reason. The Late Cretaceous was a time of unusually elevated sea level, so that in Europe, for example, the majority of rocks surviving from that time interval are open marine deposits (Figure 2) in which terrestrial vertebrate remains are exceedingly scarce. In the Late Cretaceous of Europe, terrestrial deposits with vertebrate remains are absent between the Cenomanian and Campanian and thereafter are sparse and restricted in distribution (Le Loeuff & Buffetaut 1995).

Therefore, many pitfalls await those who wish to take the fossil record at face value.

Paleontological Dating of Bird and Mammal Originations

The fossil records of birds and mammals are quite similar, and in both cases there is a marked change between Cretaceous and Tertiary faunas. Cretaceous bird faunas are dominated by enantiornithines, hesperorniformes and ichthyorniformes, all universally acknowledged as stem-group birds (e.g., Chiappe 1995). By contrast bird faunas from the Paleocene onwards are composed predominantly of clades within the crown-group (Figure 3). Several modern orders (eagles, owls, cranes) appear in the Paleocene and by the Eocene most orders of living birds are represented (Chaterjee 1997, Blondel & Mourer-Chauviré 1998, Bleiweiss 1998a). Shorebirds, procellariformes, and waterfowl were all originally recorded from the Late Cretaceous of New Jersey, but these beds are now redated as early Paleocene (see Benton 1999). Thus, although Kurochkin (1995) has even argued that some late Early Cretaceous (ca. 100 million-year-old) fragmentary material might indicate the presence of Neognathae, convincing evidence for any Cretaceous crown-group bird is hard to come by. A possible Late Cretaceous parrot has been recorded (Stidham 1998) although this identification is disputed (Dyke & Mayr 1999). Only a possible presbyornithid recorded from the latest Cretaceous of Antarctica (Noriega & Tambussi 1995) seems to provide a firm record, since recent phylogenetic work has placed presbiornithids well within the extant order Anseriformes (Livezey 1997).

The paleontological record for mammals is very similar. There is no unambiguous evidence for any extant order of eutherian mammal occurring prior to the

Figure 2 Fossil bird and rock record of western Europe. Families of birds based on Blondel & Mourer-Chauviré (1998) with Cretaceous additions as follows: (1) undescribed material from the Maastrichtian of Maastricht (Paul Davies, personal communication 3/2001), (2) Buffetaut & Le Loueff (1998), (3) Enantiornithids from the Cambridge Greensand (see Chatterjee 1997). Rock record of terrestrial deposits based on map area for U.K. and France (scale = numbers of 1:50,000 or 1:63,360 series geological maps with rocks of that age and facies cropping out; see Smith 2001).

Paleocene (Alroy 1999, Archibald, 1999, Foote et al. 1999), yet almost all are present by the Middle Eocene (Figure 4). The oldest known monotremes are *Steropodon* and *Kollikodon* from the early Cretaceous (Albian) of Australia (Luo et al. 2001). The earliest stem group marsupials are similar in age (Cifelli 2000). The Late Cretaceous eutherians that are recorded are usually interpreted as stem group clades (e.g., Novacek et al. 1997) or representatives of supraordinal groups of Eutheria (e.g., zhelestids as stem group ungulates; Archibald 1996), although the latter is disputed (see Foote et al. 1999). Because early members of many groups differ only slightly in their dentition (Carroll 1985) and homoplasy is a major problem (Cifelli 2000), the dearth of articulated material makes phylogenetic placement of Cretaceous forms tentative at best. A direct reading of the fossil record leads to the conclusion that most, if not all, crown-group diversification of birds and mammals took place in a short time interval after the end of the Cretaceous (e.g., Feduccia 1995, 1996, Archibald 1999, Foote et al. 1999, Benton 1999).

Establishing Lower Bounds on Paleontological Dates

First occurrences in the fossil record establish only an upper bound on the times of origination. Although sister group relationships can be used to bracket divergence times, these are still based on upper bounds only (Figure 1). However, three methods have been proposed that place limits on the lower bound of age of origination. One adopts a maximum likelihood approach and calculates times of origin with error bars based on the number of extant species, the temporal distribution of taxa within the observed range, and a prespecified model of diversification (Marshall et al. 1998). Initial results show promise, but the method has yet to be published in detail. The other two methods, which are discussed below, involve the calculation of confidence intervals on fossil ranges and an analysis of sampling intensity.

CONFIDENCE INTERVALS Confidence intervals can be calculated for the end points of stratigraphical ranges based on the distribution of occurrences between first and last appearance of any taxon (Marshall 1998). These establish a lower bound at some predetermined level of confidence (e.g., 95%) within which it is possible that the clade could have gone unsampled by chance. Bleiweiss (1998a) used this approach to calculate the 95% confidence interval to the lower stratigraphical range of four orders of bird, and he showed that end points fell very close to the end Cretaceous. From this he deduced that the fossil record was providing a true picture and that modern bird orders could not have extended far into the Cretaceous.

Unfortunately, as Marshall (1999) was quick to point out, Bleiweiss assumed uniform sampling and preservation over time, something that is manifestly unrealistic. If there is large-scale heterogeneity of the rock record, with the Late Cretaceous having a much poorer terrestrial record than the Tertiary (Figure 2) or if in the early stages of a clade's history abundance and diversity is lower (Marshall 1999), then the confidence intervals are very much extended. What Bleiweiss demonstrated was that the Cretaceous-Tertiary marked a significant change in our record of bird diversity, but the causes for this change—whether sampling or biological—were not addressed. There are ways of calculating confidence intervals that are more realistic (Marshall 1998), but these require sampling and preservational biases to be specified a priori. As yet, such an approach remains untried, but is being developed (C. Marshall, personal communication, 5/2001). All we can be sure of is that the heterogeneity of the rock record and the strong bias against terrestrial records seen in some parts of the world in the Late Cretaceous (Smith 2001) will push back the lower bound for confidence intervals pertaining to early Tertiary originations deep into the Cretaceous.

SAMPLING INTENSITY Foote et al. (1999) took a different approach to setting lower bounds on the distribution of eutherian mammalian orders. They asked what was the likelihood that the earliest 35 million years of the eutherian crown-group fossil record (i.e., the Late Cretaceous) existed but was missing because of sampling. They took the basal divergence time indicated by molecular clocks (ca. 100 Ma)

and assumed a monotonic increase in taxa so that each of the nine modern orders of Eutheria predicted to have a Late Cretaceous record from molecular estimates had evolved by the end Cretaceous. They then used the stratigraphic distributions of stem group eutherian and non-eutherian mammals in the Late Cretaceous to derive an empirical estimate of mammalian preservation potential over that period. From this they concluded that, given the length of time, known sampling density, and estimated preservation potential, it was statistically highly unlikely that crown-group eutherian mammals had begun to diverge much before the end of the Cretaceous.

By using the fossil record of non-eutherian mammals as a control for estimating sampling parameters, this technique overcomes many of the problems associated with the fossil record described above. Foote et al. made a convincing case, but only within the very specific bounds of their data. Unfortunately the vast bulk of their primary data (more than 85% of the taxa known from two or more deposits that they used to calculate the preservation potential) comes from the Campanian-Maastrichtian of North America. Some Late Cretaceous formations in Mongolia, Uzbekistan, and Kazakhstan have recently yielded abundant and well-preserved terrestrial faunas, including mammals (see Cifelli 2000), which also imply an absence of modern faunas at this time, but elsewhere sampling is simply too sparse to say much. There are just four scrappy taxonomic records of mammals from Europe, and single mammal-bearing fossiliferous Late Cretaceous formations in South America, Madagascar, and India (Foote et al. 1999; supplementary data). Foote et al. demonstrated that fossil sampling of mammals in the Late Cretaceous of North America is adequate to allow realistic confidence that no extant orders of Eutheria were present, but their approach cannot say anything with statistical rigor about faunas from the rest of the world. This is an important caveat as new molecular phylogenies suggest a Gondwanan origin for many mammalian orders (see below).

Paleogeographic Constraints

Divergence times are sometimes estimated from the dating of major plate-tectonic events, such as the docking of two continental blocks or the opening of oceanic seaways between continental masses once joined. These events from the Cretaceous onwards are now really rather tightly constrained because of the geophysical evidence from deep-sea geomagnetic lineations and other sources.

The paleogeographical history of Gondwana is seen as especially critical to both bird and mammal evolution. The existence of distinct African (Afrotheria), South American (Xenarthra), and Laurasian (Laurasiatheria) clades identified from molecular studies (Murphy et al. 2001, Madsen et al. 2001, van Dijk et al. 2001) suggests not only that mammalian diversification commenced on southern landmasses, but that much of this diversification must have occurred during the Cretaceous (Springer et al. 1998, Madsen et al. 2001). Similarly, both Cooper & Penny (1996) and Cracraft (2001) have argued that much of the early diversification of

neornithines (crown-group birds) occurred in Gondwana in the Cretaceous, and Cracraft has used the breakup of Gondwana to place constraints on the time of origin of avian orders. Woodburne & Case (1996) provide an outstanding example of how plate tectonic events involved in the breakup of Gondwana can be integrated with phylogenetic hypotheses to make specific statements about the timing of events in marsupial evolution.

The fragmentation of Gondwana therefore provides a series of dates that specify the latest times at which there could have been free interchange between the faunas of Australia, South America, New Zealand, and Africa. Dating vicariance events by the initiation of mid-ocean ridge spreading is relatively straightforward, but the same problem arises here as with fossil dating, namely, that the separation of two continental landmasses marks the latest time at which effective interchange stopped, but it sets no lower bound. In some cases this upper bound is older than that set by a direct reading of the fossil record. However, chance transoceanic interchange after actual separation remains a possibility. Birds are less constrained than mammals in this respect, and Feduccia (1996) has consequently argued that the major flightless ratite clades, each confined to a separate continental block, evolved from a flying ancestor that dispersed well after the breakup of Gondwana. This of course requires that flightlessness evolved independently in each clade after dispersal, but that could be possible because many avian orders have their flightless clades.

Dating when continental blocks converged and docked, to create intercontinental land bridges for faunal interchange, is much more difficult from geological data. The formation of land bridges also depends to a large extent on global sea levels. High global sea levels in the mid to late Cretaceous partitioned Africa into two discrete land masses both isolated from Eurasia (Neraudeau & Mathey 2000) while Europe itself was an archipelago of small islands (Le Loeuff 1991). But by the Paleogene, with falling sea levels, Africa and Eurasia were firmly linked and sporadic interchange was occurring.

Biogeographic data thus unfortunately provide even less constraint on times of origination than does the fossil record.

MOLECULAR DATING

The idea behind molecular clock dating is deceptively simple. Point mutations on a gene sequence represent copy errors and such errors accumulate randomly over time. The amount of sequence differentiation observed between homologous genes of two taxa is a function of how long they have been separated. If the age of separation can be established from the fossil record for one pair of taxa, the rate at which genetic change has accrued since they last shared a latest common ancestor can be calculated. By extrapolation, this information can then be used to date the times of divergence of other pairs of taxa. In all cases where this method has been applied to the dating of bird and eutherian mammal divergences, many

orders have been found to have their origins deep in the Cretaceous (Hedges et al. 1996, Cooper & Penny 1996, Springer 1997, Kumar & Hedges, 1998, Penny et al. 1999, Bromham et al. 1999, Waddell et al. 1999b, Huelsenbeck et al. 2000, Nei et al. 2001).

However, molecular clocks are not error-free and come with their own suite of problems. Specifically, rates of molecular evolution may vary considerably, both across taxa and over time. Furthermore the accuracy of the technique depends upon having an accurate calibration point or points, and a reliable phylogeny with correct branching order and branch-length estimates.

Some Problems Associated with Molecular Dating

RATE VARIATION AMONG LINEAGES The idea that there is a universal molecular clock ticking away has long since been discredited (Vawter & Brown 1986, Saccone et al. 1993). Instead what we see is a great deal of rate variation; among loci on a gene (between site variation), between branches on a tree (species-specific rates), and probably within single lineages over time. Rate heterogeneity is a serious obstacle to accurate estimation of divergence times from molecular data. A rate calculated for one part of the tree may significantly underestimate divergence times on another part of the tree if molecular evolution there is slower or may overestimate if the rate is much faster (Norman & Ashley 2000). Early works that assumed a priori molecular rates of evolution were time homogenous over all taxa for any one gene have been heavily criticized (e.g., Ayala et al. 1998) and are now discredited. However, several methods have been developed recently to try to overcome the problem of rate heterogeneity.

Screening for rate homogeneity A set of taxa can be screened to test whether they show significant rate heterogeneity for particular gene sequences. If they do these data are rejected from further analysis and the molecular clock rate is estimated from the remaining time homogeneous sequence data. The most commonly used test for rate heterogeneity is that of Tajima (1993), although others have been suggested (e.g., Takezki et al. 1995, Yang et al. 1995, Norman & Ashley 2000). Kumar & Hedges (1998), for example, used Tajima's method to screen 658 relatively short gene and partial gene sequences for rate heterogeneity across a range of vertebrates. After rejecting those showing rate heterogeneity (22%), divergence times were calculated based on a single calibration point and assuming a uniform rate of evolution for each gene across all remaining taxa. From this they deduced that many of the orders of mammal and bird extended deep into the Cretaceous.

Unfortunately, these tests are not very strong and allow considerable rate variation to go undetected when sequence lengths are relatively small (i.e., 500–700 variable sites) (Bromham et al. 2000). They are also sensitive to taxonomic sampling (Robinson et al. 1998). Predicted times of origin may be wrong by as much as 50% if the undetected rate heterogeneity is as high as 1:3. As Hedges et al.'s gene sequences were mostly in the range of 500–700 bp length, a large degree

of uncertainty must be attached to their estimates of times of origination for bird and mammal orders. For this reason concatenating sequences (pooling data from multiple genes) with appropriate correction factors is now considered to provide more reliable estimates of divergence times (Nei et al. 2001).

Allowing for rate variation among taxa If divergence times can be estimated without requiring a rate calibrated from one part of the tree to be extrapolated to another part, then problems of rate heterogeneity can be largely avoided. Two approaches have been developed.

Springer (1997) used multiple calibration points across his tree of therian mammals and then adjusted each branch against a reference standard using regression analysis. This provided him with a set of correction factors that he could apply to control for rate acceleration or deceleration in different parts of the tree. Correction factors have also been used by Adkins et al. (2001) for dating rodent divergence times.

The quartet method (Rambaut & Bromham 1998) takes a different approach by breaking down a tree into sets of (nonexclusive) quartets composed of two pairs of taxa, each pair monophyletic with respect to the other. Each pair of taxa needs to have a time of divergence established from the fossil record. Standard rate tests are then used to identify (and remove) quartets with significant rate variation between the two taxa forming either of the pairs. This removes all quartets in which rate variation does not fit a two-state model. Each of the remaining quartets then provides an estimate of the time of origin for the common basal node. When large numbers of quartets are relevant to the same basal node, the error associated with this point estimate can be calculated.

Although the quartet method removes some of the problems of rate heterogeneity, it does assume uniformity of rate over time within each pair from basal node to tip in each half of the quartet. If molecular rates were systematically faster in the early part of a clade's history compared to its later history (e.g., Benton 1999), then this technique would still overestimate times of origin.

Building models of rate variation into estimates Instead of restricting analyses to just those taxa and genes that appear to show invariant rates of molecular evolution, an alternative approach is to try to incorporate a model of how rates of evolution are varying among taxa when calculating divergence times. A maximum likelihood approach can then be used to derive the best-supported date under the assumptions of the model. If each branch in a tree is assigned its own independent rate of evolution, then it becomes computationally impossible to derive divergence times. Consequently, some model is required that relates rates of evolution on one branch to that of its immediate neighbors. Four discrete methods of modeling rate variation have been proposed specifically for molecular dating.

Sanderson (1997) was the first to detail a method that allowed times of origination to be estimated from molecular data where rates were variable. He assumed that rates were autocorrelated across the tree and used a nonparametric model

to smooth rate changes between nodes. The preferred tree is then the one that maximizes covariance of rates over the tree. Thorne et al. (1998) took a similar approach by assuming autocorrelation of rates but using a parametric model for relaxing the clock. Huelsenbeck et al. (2000) used a double Poisson process to model rates of molecular evolution. First nucleotide substitutions along branches were assigned according to a Poisson process, then a second Poisson process was used to multiply the current rate by a gamma-distributed random variable. Maximum likelihood was then used to optimize the variables from the observed distribution of nucleotide substitutions assuming a given tree.

Cutler (2000) developed a very different approach. Point mutations were modeled not on a Poisson process, but on a more general stationary process assumption, which permits much wider variance in rates of evolution among taxa. When he applied his model to the question of when the metazoan phyla originated, Cutler still obtained a maximum likelihood date deep in the pre-Cambrian (1400 Ma). However, the error bars associated with this estimate were extremely large, and it was not possible to reject a Cambrian origin with statistical certainty. Relaxing the constraints on variance meant effectively that no goodness of fit test could reject the model.

Models such as those proposed allow relaxation of the assumption that rates of molecular evolution are uniform over time, but the models come with a price. The accuracy of the estimate depends upon the correctness of the model being assumed. If the model is more realistic than one assuming uniform rates, then the estimate of divergence times should be more accurate. But if the model is a poor fit to how rates really varied, then it can generate wildly inaccurate results (Zhang 1999). Choosing an appropriate substitution model of molecular evolution is important both for the accuracy of branching order and the estimation of branch length, as shown by Sullivan & Swofford (1997). Although actual patterns of amino acid or nucleotide substitution of a gene or protein are usually unknown, likelihood based models are now highly refined and offer very powerful and reliable means of constructing phylogenetic hypotheses from molecular data (Whelan et al. 2001). Models thus offer the potential of greater accuracy but may be less precise.

CALIBRATING THE MOLECULAR CLOCK All molecular clock approaches require one or more calibration points using dates derived either from the fossil record or from biogeographic constraints. There are two approaches—either calibration can rely on one or a small number of "well documented" dates where paleontological evidence seems highly reliable, or calibration can be achieved using a large number of independent dates so that a range of estimates is arrived at. The former approach has been criticized by both Lee (1999) and Alroy (1999) for placing too much reliance on a single paleontological date without considering its error. Error in setting a divergence time stems from the uncertainty associated with establishing the true phylogenetic placement of often rather difficult and incomplete fossil material. For example, uncertainty about which taxon represents the oldest member of the bird-mammal dichotomy could be responsible for as much as 10% error in

the dating of interordinal divergences of mammals and birds (Lee 1999), and a similar level of potential error arises over the dating of the marsupial-placental mammal split (Alroy 1999).

This problem is only compounded when one calibration date from the fossil record (e.g., the 300 Ma bird/mammal split) is used to estimate a second divergence date, and then both dates are used as "independent" calibration points. Tuinen & Hedges (2001), for example, used a 110 Ma primate/rodent divergence date in conjunction with the 300 Ma time point to calibrate the avian clock. However, the 110 Ma date is a molecular estimate derived from the 300 Ma estimate (Kumar & Hedges 1998) and is thus hardly an independent calibration point.

Multiple calibration points have the advantage that the variance that results from using different fossil dates can be used to establish confidence intervals on our estimates. However, with the latter approach it is important to remember that the distribution of error is not normal around the correct date (fossil dates are always underestimates), and so the more wrong dates that are included the further the mean will depart from the true date (Bromham et al. 2000).

In either case, calibration is likely to be more accurate if the divergence dates being estimated lie within the range of known calibration points rather than being extrapolated beyond them (e.g., Springer 1997) because extrapolation tends to lead to an overestimation of true divergence dates (Nei et al. 2001).

Finally, there are geochronological and correlation errors associated with all of these dates that are rarely, if ever, considered. In fact, the errors involved can be considerable, especially in less well-studied parts of the time scale, and consequently radiometric dates need to be treated with appropriate caution.

ACCURATE ESTIMATION OF PHYLOGENETIC RELATIONSHIPS Molecular distances between taxa can be calculated without recourse to a rooted phylogenetic hypothesis. However, in order to obtain information about rates of evolution, the network must be rooted correctly. One of the most common problems to beset molecular phylogenies is the use of too distant an outgroup for rooting (Smith 1994, Hillis & Wiens 2000). For genes such as the ribosomal RNAs, the variable sites within conserved regions can become saturated, effectively creating a predominantly random outgroup sequence. Transitions in certain mitochondrial genes can show evidence of saturation or partial saturation after as little as 10–20 million years (Springer 1997). Using predominantly random data to root an ingroup can result in midpoint rooting, often with high bootstrap support. With midpoint rooting the branch-length differences from root to tip among terminal taxa are minimized. Consequently rates of evolution calculated from a tree rooted on random outgroup data are more likely to pass a relative rates test.

Problems with rooting are apparent when using complete mitochondrial genome sequences to deduce tetrapod relationships (e.g., Cao et al. 1995, Takezaki & Gojobori 1999). They are also probably the cause for the mismatch between morphological and molecular rooting position for birds. Morphological data root the bird tree firmly with palaeognaths as sister group to the rest (Cracraft 2001).

Analysis of complete mitochondrial genome data using alligator as outgroup, however, roots the bird tree on passerines (Härlid et al. 1998, Mindell et al. 1999). Passerines appear to have a faster rate of molecular evolution when analyzed in other ways (e.g., Cooper & Penny 1996). Both Härlid et al. (1998) and Waddell et al. (1999b) used a bird tree rooted on passerines to calculate rates of evolution and times of ordinal origination. However, because their rooting almost certainly made short branches appear longer and long branches appear shorter, intertaxonomic rate variation was underestimated, and their estimated times of origin for bird orders are consequently invalid.

Rooting problems are probably quite widespread and have also been identified as the cause for the mismatch between ratite ingroup relationships (e.g., Lee et al. 1997). The precise root of the eutherian tree also remains ambiguous (Penny et al. 1999, Murphy et al. 2001, Liu et al. 2001).

Branching order is less crucial than root position for molecular clocks. There remains ambiguity of branching order for certain mammalian lineages (Springer & de Jong 2001, Liu et al. 2001, Eizirik et al. 2001) and bird lineages (Lee et al. 1997, van Tuinen et al. 1998), but whichever arrangement is taken makes little difference as these clades all extend into the Cretaceous, according to molecular clock estimates.

Molecular Dating of Bird and Mammal Divergences

The fact that rates of molecular evolution can vary both through time and across taxa is the single most fundamental obstacle to developing accurate estimates of times of origination from gene sequence data. Although various techniques have been proposed to circumvent the problem, none unambiguously allows the components of time and rate to be teased apart with any degree of precision (Cutler 2000). Many of the molecular clock analyses, therefore, remain problematic at best.

Of those dealing with birds, the analyses of Mindell et al. (1997, 1999), Härlid et al. (1998), and Waddell et al. (1999b), based on complete mitochondrial gene sequence data, place passerines at the base of the bird phylogeny and have deep divergence dates for most orders. However, these results must be treated with extreme caution because the rooting of their preferred tree is highly suspect (Mindell et al. 1999, Cracraft 2001) and its topology is inverted with regard to both stratigraphical order and the morphological tree. Morphological data strongly and unambiguously place ratites as the sister group to other birds, as do certain nonmitochondrial genes (Groth & Barrowclough 1999). Cooper & Penny (1996), using selected nuclear and mitochondrial genes, could not place the root of the bird tree with confidence (either ratites, galliformes, or psittaciformes came out as the basal clade according to the optimization procedure used and outgroup selected). Their tree was congruent with morphological data for the most part, and it identified passerines and strigiformes as having exceptionally long branches, whereas those of ratites were among the shortest. Bleiweiss (1998b) has shown that rates of molecular evolution in hummingbirds are most strongly linked to metabolic

rate and oxygen consumption, with the implication that clades of predominantly small-bodied birds such as passerines should be expected to have faster molecular clocks than the large-bodied ratites.

If the root of the bird tree based on complete mitochondrial gene sequence data is wrong, then rate heterogeneity among taxa is artificially reduced and molecular clock estimates are likely to be highly inaccurate and untrustworthy. The anomalously young age of divergence of the Ostrich and Rhea reported by Härlid et al. (1998) is easily explained if their rooting on passerines is incorrect. We therefore discount the evidence of Late Cretaceous divergence of bird orders presented by Mindell et al. (1997, 1999) and Waddell et al. (1999b).

Härlid et al. (1997) used amino acid and nucleotide data from complete mitochondrial gene sequences to date the neognath-palaeognath split, using a bird-mammal split at 300 Ma for calibration. This gave an estimated time of divergence of 80–90 million years (mid–Late Cretaceous). Using the same calibration point, plus an additional calibration point derived from the 300 Ma bird/mammal split, van Tuinen & Hedges (2001) estimated from multiple molecular data sets that the neognath-palaeognath split was 110–120 Ma ago. The extreme distance of the calibration point from the split of interest adds some uncertainty to the estimated divergence time, although, even if their calibration date is 10% in error (Lee 1999), the estimated origin of crown-group birds remains firmly in the mid-Cretaceous. However, they used large numbers of relatively short gene sequences rather than a concatinated sequence, so their relative rates test would not have removed problems of rate variance (e.g., Bromham et al. 2000, Nei et al. 2001).

Other estimates are less easy to discount. Cooper & Penny (1996) generated a molecular phylogeny based on the quartet method that is largely congruent with morphological phylogenies and thus potentially more accurately rooted. The quartet method they used is also far less sensitive to variation in rate, although, with just slightly fewer than one kilobase of sequence data, some rate variation is likely to have gone undetected. Nevertheless, the fact that estimated divergence times lie deep in the Cretaceous for at least 22 avian lineages, would require a massive change in rates of evolution between the internal branches and external branches of each quartet. It would imply that molecular evolutionary rates would have had to have been an order of magnitude faster in the early part of the history of bird diversification if orders were all to post-date the Cretaceous-Tertiary boundary.

The situation is very similar for therian mammals. Rate variation across the different clades is significant, and interordinal relationships until very recently remained poorly constrained with different data sets and different methods of analysis giving contradictory topologies (Liu & Miyamoto 1999, Waddell et al. 1999a, O'Leary & Geisler 1999, Murphy et al. 2001, Liu et al. 2001, Eizirik et al. 2001). The divergence dates obtained by Hedges et al. (1996) and Kumar & Hedges (1998) need to be treated with care because a single calibration point far removed in time from the nodes of interest was used and multiple sequences were analyzed independently rather than concatinated (Lee 1999, Bromham et al. 2000). Penny

et al. (1999) applied a LogDet approach to mitochondrial amino acid sequences of mammals and found that at least five orders and several supraordinal groups of eutherian mammal have a long Cretaceous history. However, the root position may be wrong, forcing an erroneously deep date for the basal divergence, and there were just two calibration points. Given the marked variation in molecular rates of evolution known to occur in mammals, their results must also be treated with caution.

The analysis of Waddell et al. (1999b) is very careful in terms of documenting possible sources of error, but it deals with relatively few lineages. They show that intraordinal divergences within Carnivora and Perissodactyla all postdate the Cretaceous-Tertiary boundary and that the crown group of eutherian mammals extends well into the Cretaceous. The conclusion is that some supraordinal stem groups extend far back into the Cretaceous, as does the order Carnivora, if this is indeed sister group to perissodactyls plus artiodactyls as recent trees suggest.

Springer (1997) carefully corrected for rate variation across the tree and examined diversification both within and between orders of mammal. He found that the orders Insectivora, Chiroptera, Rodentia, and possibly Primates all had origination dates within the Late Cretaceous (Figure 4). The majority of his interordinal comparisons also suggest stem-group lineages of two or more orders have deep Cretaceous roots. Furthermore, techniques that attempt to model rate variation (e.g., Huelsenbeck et al. 2000) or that model diversification taking sampling into account (e.g., Marshall et al. 1998) also point to Cretaceous divergences for at least some mammalian orders.

The most thorough analysis to date is that by Eizirik et al. (2001). They applied the quartet method to a concatinated database of almost 10,000 base pairs for 64 mammals. Interordinal divergences all fell within the Late Cretaceous (their 95% confidence intervals ranging from 64 to 109 Ma; Figure 4). The fact that diverse approaches all give similar Late Cretaceous divergence dates is compelling.

THE ORIGIN OF METAZOAN PHYLA

The second area where molecules and morphology are in serious disagreement concerns the origins of the metazoan phyla. Although the difference between the molecular and morphological estimates for bird and mammal origins may be as much as 50 million years, the discord between the two for the animal phyla may be as much as 500 million years, almost the entire length of the Phanerozoic. The debate has usually been centered on whether or not molecular data are consistent with a Cambrian explosion scenario. However, two points need to be emphasized. First, the debate, because of the nature of the available data, is focused not on the crown-group Animalia, but on crown-group Bilateria. It is the divergence of bilaterians from nonbilaterians and not the origins of Metazoa per se that is

generally being dated. The implication of this fact is that whatever date is accepted, much had already occurred in metazoan evolution prior to this time (e.g., *Hox* gene evolution—Ferrier & Holland 2001). Second, the fossil record itself indicates that crown-group bilaterians, possibly even stem-group molluscs (Fedonkin & Waggoner 1997), were present in the latest Precambrian at 555.3 $\pm$ 0.3 Ma ago (Martin et al. 2000). Hence, the key question is: Do crown-group bilaterians pre- or postdate the last Vananger (i.e., Marinoan) glaciation approximately 600 Ma ago? This has important implications for whether scenarios such as the hard-snowball Earth scenario of Hoffman and colleagues (Hoffman et al. 1998, Schrag & Hoffman 2001) are conducive for the survival of metazoans through this interval of time (Runnegar 2000, Hyde et al. 2001). Almost all molecular estimates, beginning with Runnegar's pioneering studies with hemoglobin (Runnegar 1982), strongly suggest that the origin of crown-group Bilateria predates 600 Ma, with estimates ranging from 630 (Lynch 1999) up to 1.2 Ga ago (Wray et al. 1996). However, paleontologists such as Budd & Jensen (2000) make a strong case that crown-group bilaterians cannot significantly predate the first appearances of trace fossils at about 555 Ma ago.

All of the problems discussed above, concerning both paleontological and molecular evidence, are exacerbated with the bilaterian origin question. The absence of any meaningful comparative fossil record throughout most of the Neoproterozoic means that there are no sampling controls and no way to calibrate and quantify the nature of the rock record.

The data for the molecular side of the question are also riddled with difficulties. First, calibrations are either based on one very distant estimate (the bird/mammal split at 300 Ma ago; e.g., Wang et al. 1999) or on several very suspect dates (e.g., Cutler 2000). As with the mammal/bird question, several authors have used secondary molecular dates as supposedly independent paleontological dates. For example, Gu (1998) used a fungal/metazoan split of 1100 Ma ago as an external calibration point for his study of metazoan divergences. However, this estimate is based on the molecular study of Doolittle et al. (1996) and again is not an independent calibration point. Unlike the mammal/bird question though, several papers have used literally imaginary divergence dates for the calibration of the clock under consideration. For example, Nikoh et al. (1997) used Dayhoff's (1978) 700 Ma divergence date for arthropods and vertebrates and claimed that this was based on the fossil record. Needless to say, aside from the "Twitya discs" (Hoffmann et al. 1990) there are no unequivocal metazoan remains that predate the Marinoan glaciation (Kaufman et al. 1997). Second, although better estimates of rate variation are now found in the literature (e.g., Ayala et al. 1998; Cutler 2000), all studies are still burdened by the fact that often the phylogeny derived from the gene(s) of interest is incorrect. Usually these studies can be boiled down to a three-taxon problem: nematodes (i.e., *Caenorhabditis elegans*), arthropods (i.e., *Drosophila*), and vertebrates. Several independent data sets all agree that nematodes and arthropods are each other's closest relatives with respect to vertebrates (Aguinaldo et al. 1997, de Rosa et al. 1999, Peterson & Eernisse 2001). However, most gene trees

involving *C. elegans* suggest that arthropods and vertebrates are sister taxa (Wang et al. 1999, Mushegian et al. 1998). Hence, as discussed by Mushegian et al. (1998), most *C. elegans* genes are evolving more rapidly than *Drosophila* genes, implying that any analysis that does not take this into account is potentially suspect. Finally, no analysis has systematically chosen taxa a priori to minimize rate heterogeneity and to use only molecules that give the correct phylogeny. The significant differences between animals such as *C. elegans* and vertebrates in terms of body size, physiology, and generation time (Martin & Palumbi 1993) probably lead not only to the tree being incorrect, but also to unreliable estimates of the divergence times between the two.

FAULTY RECORD OR FAULTY CLOCK?

The two lines of evidence that we can call upon for dating the origins of certain higher taxa, namely the fossil record and molecular clocks, come to different conclusions. But both also come with their own suite of problems. The fossil record suffers from relying on negative evidence while molecular clocks are plagued by problems of rate variation through time and across taxa. Are we any nearer to sorting out which (if either) is providing the more reliable picture?

The first thing to be clear about is what precisely morphological and molecular data disagree over (Alroy 1999). For example, although some have taken the bird/mammal fossil record more or less at face value (e.g., Feduccia 1996, Benton 1999), most accept that some diversification of crown group Eutheria and Aves occurred within the Cretaceous (Chaterjee 1997, Alroy 1999). Alroy (1999) agreed that maybe as many as 10 or 20 therian lineages extend back into the Cretaceous, even though fossil evidence is sparse, but held that a true explosive diversification in morphology still followed the end Cretaceous event. Molecular data tell us that diversification of crown-group eutherian supraordinal lineages was underway in the Late Cretaceous and that at least the stem group portion of a number of ordinal lineages had originated. So it is possible that, for both the bird/mammal and the bilaterian phyla controversies, molecular data are recording a deep history of stem-group taxa, while the fossil record accurately documents an explosive diversification of crown groups after their respective boundaries (Knoll & Carroll 1999, Alroy 1999). This would allow both sources of data to be congruent, with each telling us something important about the evolution of these groups. If this were so, then molecular data should date crown-group divergences after the respective boundary, and the fossil record should continue to reveal only stem-group forms before the boundary (see Figure 1).

There are, therefore, two critical questions that need to be addressed. First, is the fossil record good enough to be able to discriminate between a gradual increase in morphological diversity and a true explosive diversification? And second, do molecular estimates suggest significant intraordinal or intraphlyum diversification preceded the first appearance of clades in the fossil record?

That the Late Cretaceous terrestrial fossil record is much poorer than the Tertiary record in terms of sampling seems inescapable. Sites yielding faunas of small terrestrial vertebrates of Late Cretaceous age are uncommon outside the Late Turonian and Coniacian of Uzbekistan and Kazakhstan, and the Campanian and Maastrichtian of North America and Mongolia, but such sites are much more abundant and widespread after the Paleocene (e.g., Blondel & Mourer-Chauviré 1998). The rock record is strongly biased against terrestrial deposits in the Late Cretaceous due to the extremely high sea level at that time, and there is only limited environmental sampling. The Mongolian faunas are preserved in an aeolian setting and not necessarily representative of the continent as a whole, whereas the mid-Asian localities are from a low, moist coastal plain habitat (Cifelli 2000). Add to this the difficulty of determining the true relationships of what is often highly incomplete skeletal material that comprises the fossil record of this time interval, and it is clear that there is a significant improvement in the quality of the fossil record in the Eocene. So, although the fossil record could be providing a true picture, any realistic estimate of confidence intervals on the observed range of orders that take preservational biases into account would extend back to the mid-Cretaceous. Only in North America is the fossil record sufficient to apply rigorous statistical tests, and there we can be sure that the early Tertiary appearance of new taxa was at least partially due to major immigration into the region (Archibald 1993, Cifelli 2000). This presumably coincides with the appearance of land bridges, linking previously isolated landmasses as global sea level fell and Africa docked with Eurasia. So the fossil record remains effectively silent as to whether the burst of appearances in the early Paleogene is a result of biological diversification or improving preservational potential.

That molecular clock analyses are also beset with problems is undisputed. In particular, questionable rooting and inadequate treatment of rate variation across taxa invalidate a number of attempts to date times of origin. Nevertheless, these problems are now widely acknowledged and are being addressed in a variety of ways. The fact that all of these approaches still point to a Cretaceous history of at least some mammal and bird orders is, however, rather convincing, especially as such estimates usually come with confidence intervals. Furthermore, molecular data points to at least some intraordinal crown-group divergences within the Cretaceous (Rodentia, Insectivora, Chiroptera—see Springer 1997, Adkins et al. 2001; Ceratiodactyla, Perissodactyla—see Eizirik et al. 2001; Anseriformes, Apodiformes, Coracoformes, Passeriformes—see van Tuinen & Hedges 2001). The molecular data thus tends to support the view of a more gradual diversification at high taxonomic levels than is indicated by the fossil record.

One escape clause remains, however. Paleontologists such as Conway Morris (1999, 2000) point out that rates of morphological and molecular evolution could be correlated, citing the paper by Omland (1997). If, when morphological change was rapid, molecular rates of change also increased substantially, then estimates by all of the molecular clock methods discussed above would be invalid. However, the

correlation observed by Omland does not necessarily imply a causal link because both morphological and molecular changes could simply be accruing stochastically over time, as seems to be the case in echinoids (Smith et al. 1996). In any case, molecular clock estimates generally utilize genes with basic biochemical functions unconnected to phenotypic expression. Furthermore, it is difficult to explain how Paleozoic and Cenozoic dates agree between the two data sets, but only disagree within the Mesozoic (Easteal 1999). In other words, a marked acceleration of both morphological and molecular evolution within the latest Cretaceous–early Tertiary should have created a significant mismatch in all pre-Late Cretaceous divergence estimates, something that is simply not seen (Hedges et al. 1996, Kumar & Hedges 1998, Nei et al. 2001). Finally, even allowing for interphylum lineages to evolve at the fastest observed substitution rates seen amongst the recent taxa, Bromham & Hendy (2000) showed that this still does not bring the bilaterian divergences to the Cambrian.

For mammals and birds the evidence seems to be stacked in favor of there being a Late Cretaceous history of at least some orders outside North America and possibly Mongolia. However, when we turn to the early fossil record of metazoans and the reality of the Cambrian explosion, we find the data even less satisfactory. There is no equivalent to the non-eutherian mammals of the Cretaceous that can act as a control to test the sampling of pre-Cambrian fossils. Nor do we have any quantitative analysis of the nature and quality of the rock record over the critical time interval in the way we do for the Cretaceous-Tertiary, although Butterfield (1995) has hinted at a possible large-scale bias. Clearly many of the earlier molecular clock estimates can be challenged because they have not adequately taken the problem of rate variation into account (e.g., see Ayala et al. 1998, Lynch 1999) or because some of the calibration points are suspect (Lee 1999). Here molecular data are suggesting that the total group divergences of Bilateria occurred in the late Proterozoic, but they say nothing about whether there was an explosion of crown groups near the base of the Cambrian, as indicated by the fossil record. So in this case both molecular and paleontological data may in fact be providing a true picture of a deep history of stem groups and rapid diversification of crown groups (Knoll & Carroll 1999). Yet, in the absence of any rigorous way of establishing lower bounds on the ranges of metazoan phyla, and given our ignorance of the rock record biases, it seems to us that careful application of the molecular clock concept is the more secure way to establish the true pattern of evolution.

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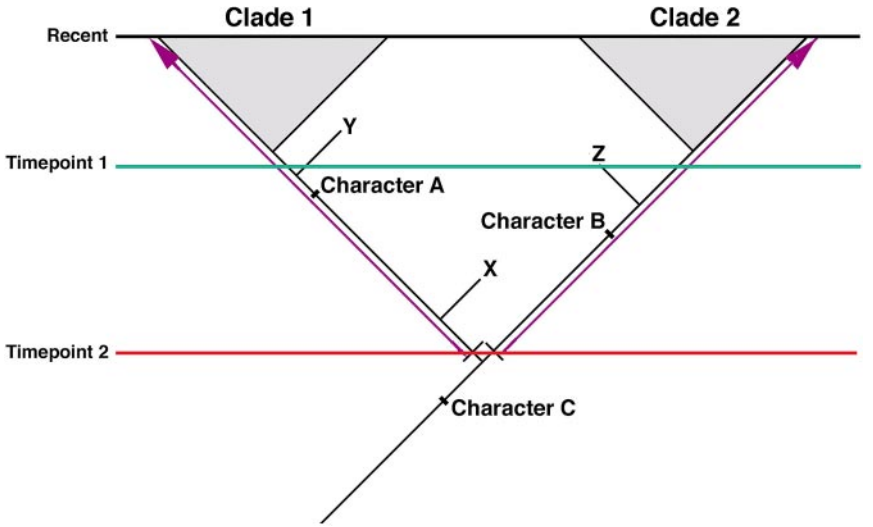

Figure 1 Cladistic and temporal framework for comparing and contrasting morphological versus molecular time points. The *hatched triangles* are the respective crown groups for clades 1 and 2; X, Y, and Z are stem-group taxa. Because taxon X can be placed only as part of clade $1 + 2$ recognized by Character C, the minimum divergence between clades 1 and 2, based on morphological characters (*black dashes*), would be established by taxon Z at Timepoint 1 (*green line*). Because the genomes of the two clades start to diverge from one another after separation (*purple arrows*) as point mutations accrue, molecular differences can be used to estimate time of divergence. (Note that this ignores a small number of intraspecific genetic differences that might exist prior to divergence.) Hence, molecular data estimate time of separation (Timepoint 2, *red line*), whereas palaeontological data estimate time of differentiation (Timepoint 1, *green line*). All else being equal, molecular estimates will normally be closer to the true time of divergence.

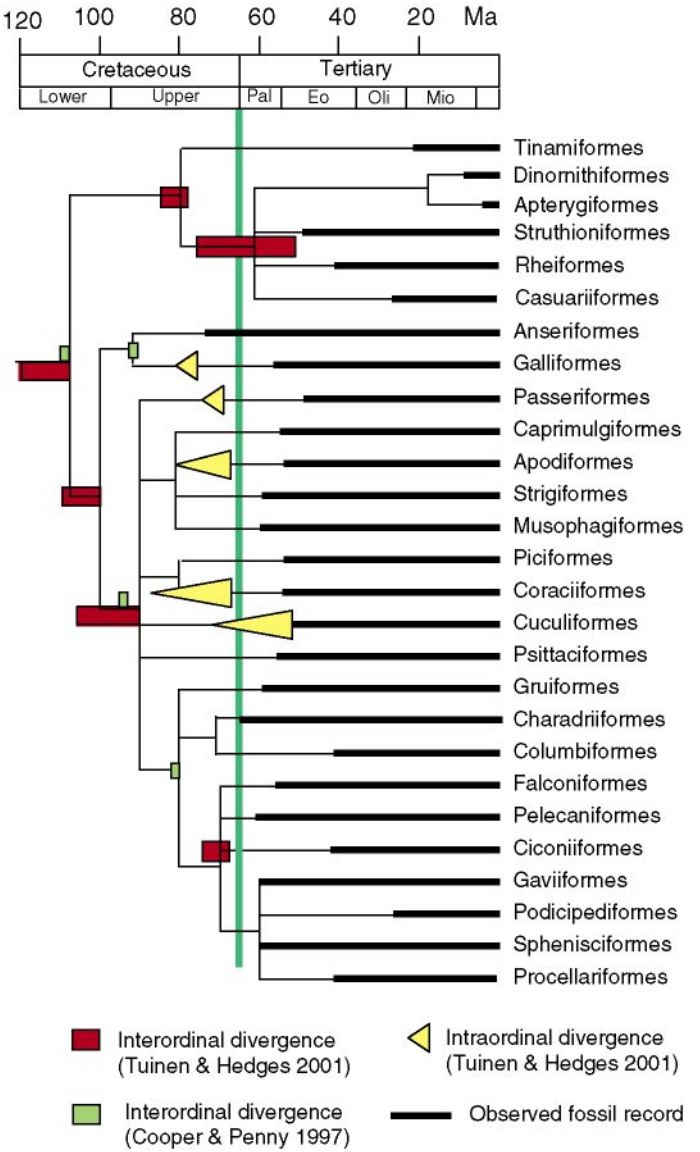

Figure 3 Molecular versus paleontological estimates of divergences within crown group birds. Phylogenetic relationships based on Cracraft (2001), with the exception of ratites where relationships are left unresolved. Molecular divergences taken from Tuinen & Hedges (2001) and Cooper & Penny (in Cooper & Fortey 1998, Figure 2).

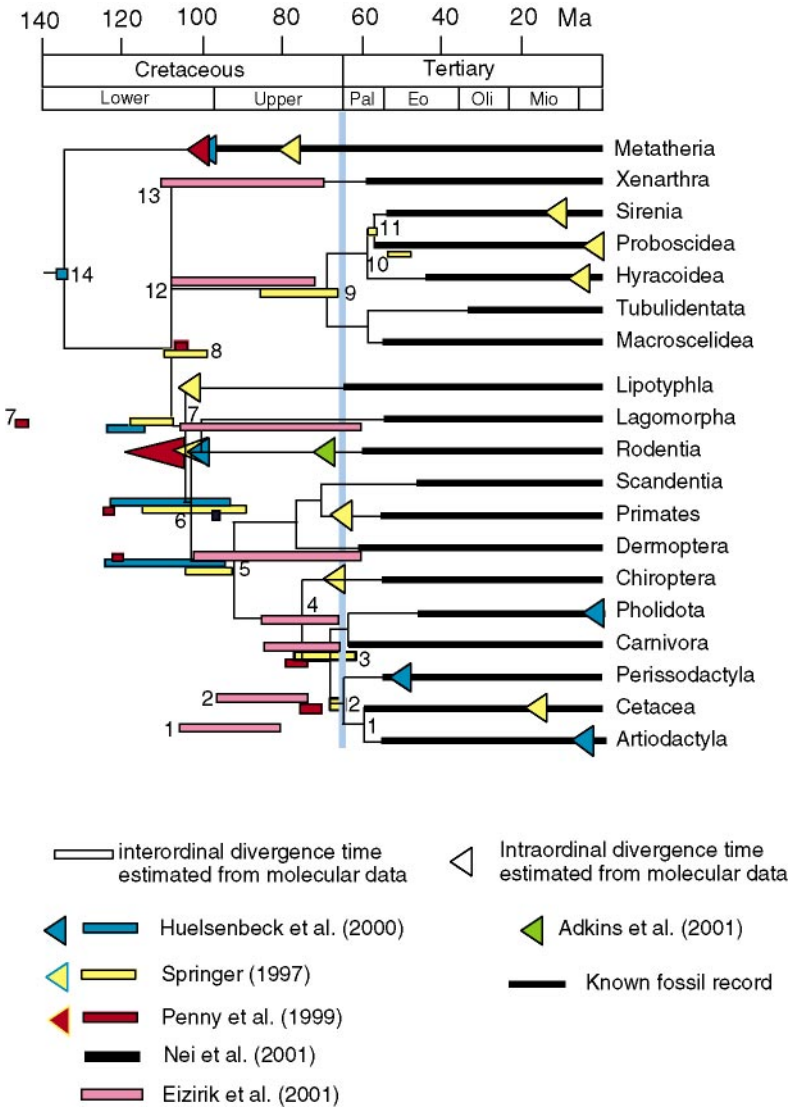

Figure 4 Some current molecular versus paleontological estimates of divergences within crown-group therian mammals. Phylogenetic relationships taken from Liu et al. (2001) except that the position of Xenarthra is left unresolved at the base. Molecular divergences taken from Huelsenbeck et al. (2000), Springer (1997), Penny et al. (1999), Eizirik et al. (2001), and Nei et al. (2001). Nodes 1–15 indicate the divergences being dated by molecular data.

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