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Flying rocks and flying clocks: disparity in fossil and molecular dates for birds

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Major disparities are recognized between molecular divergence dates and fossil ages for critical nodes in the Tree of Life, but broad patterns and underlying drivers remain elusive. We harvested 458 molecular age estimates for the stem and crown divergences of 67 avian clades to explore empirical patterns between these alternate sources of temporal information. These divergence estimates were, on average, over twice the age of the oldest fossil in these clades. Mitochondrial studies yielded older ages than nuclear studies for the vast majority of clades. Unexpectedly, disparity between molecular estimates and the fossil record was higher for divergences within major clades (crown divergences) than divergences between major clades (stem divergences). Comparisons of dates from studies classed by analytical methods revealed few significant differences. Because true divergence ages can never be known with certainty, our study does not answer the question of whether fossil gaps or molecular dating error account for a greater proportion of observed disparity. However, empirical patterns observed here suggest systemic overestimates for shallow nodes in existing molecular divergence dates for birds. We discuss underlying biases that may drive these patterns.

1. Introduction

For many major radiations, molecular divergence dating analyses have yielded age estimates far predating the oldest fossil evidence [1–4]. This phenomenon has spurred debates over timing of divergences in individual clades [5–7]. However, no large-scale empirical comparisons of molecular divergence estimates versus fossil ages have been undertaken, hindering identification of potential biases. Birds present a prime target for such investigations. The avian radiation has received intense attention from molecular biologists and palaeontologists [3,4,7–16], because the timing of this radiation bears on interpretations of survivorship patterns across the Cretaceous–Palaeogene mass extinction. Longstanding controversy exists over whether the modern bird radiation extends deep into the Cretaceous, implying mass survival, or is restricted primarily to the Cenozoic, implying an explosive radiation [3,4,7–16].

In essence, this debate has centred on how much of the disparity between the fossil and molecular data can be attributed to molecular dating error, which may be due to discordance between gene trees and species trees, methodological error (inappropriate models or calibration error), or stochastic error, and how much can be attributed to the fossil record, which may be due to lack of specimen preservation and discovery, the temporal gap between species divergence and the evolution of diagnostic morphological characters, and the ability of palaeontologists to correctly identify fossils to clades [e.g. 5,11,17,18]. While molecular error may lead to either overestimation or underestimation of divergence ages, the fossil record can only provide minimum estimates of the age of species divergences, assuming that fossils are correctly identified and dated. Thus, debate often reduces to the question of how incomplete the fossil record might be. The avian fossil record has numerous large gaps, as indicated by ghost lineages or minimum implied gaps required by the shape of the avian tree and the stratigraphic

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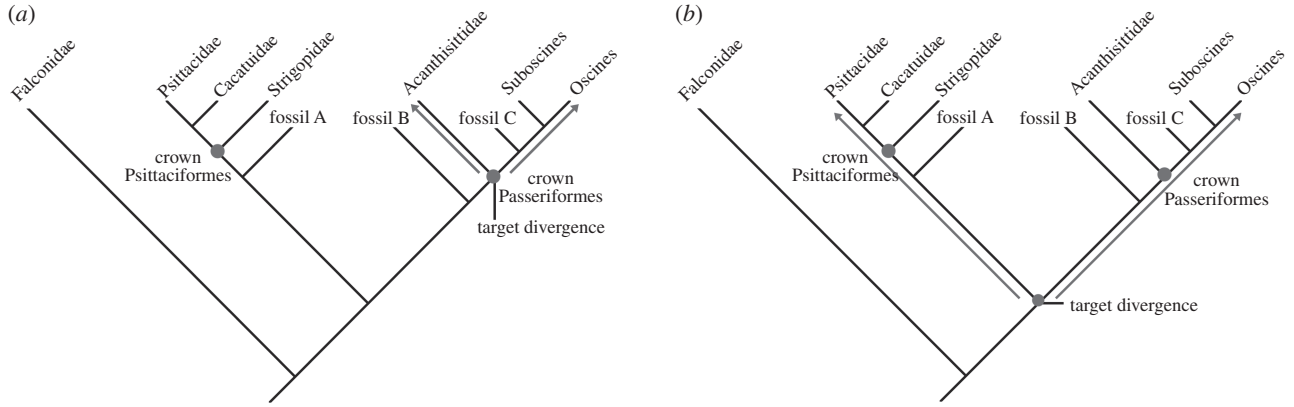

Figure 1. Data collection strategy for the fossil age datasets. In this example, the taxon of interest is Passeriformes. (*a*) In the crown fossil dataset, the divergence of interest is the deepest divergence within the crown clade. Thus, the oldest fossil that falls *within* the crown clade provides the fossil age for 'crown Passeriformes'. Fossil C is the only appropriate fossil. (*b*) In the stem fossil dataset, the divergence of interest is that between Passeriformes and its sister taxon Psittaciformes. Thus, the oldest fossil that falls along the lineage leading to crown Passeriformes (or within crown Passeriformes) or anywhere along the Psittaciformes lineage provides the fossil age for 'stem Passeriformes'. In this case fossil A, fossil B or fossil C can all be considered. Fossil A is relevant because if a group is present in the fossil record at a given time, this presence indicates its sister group must also have been present [19]. Note that the crown and stem datasets each consider both sides of the divergence emerging from the target divergence, and therefore use the same amount of information relative to the node representing the target divergence.

distribution of fossils [e.g. 14]. Our goal in this study is not to resolve how deep the radiation of crown birds extends into the Mesozoic. Instead, we undertake a broad survey of molecular divergence time estimates and fossil ages in order to elucidate consistent patterns of disparity between the two data types.

2. Material and methods

We collected two datasets. Our molecular age dataset comprises published estimates for the divergence of 67 avian clades from their sister clades (stem ages), as well as estimates for the basal divergence within each of these clades (crown ages). The targeted clades correspond roughly to extant avian orders, though we broke non-monophyletic orders into finer units to avoid problems associated with polyphyletic groups. Our fossil dataset comprises the ages of the oldest stem fossil and oldest crown fossil for each of these 67 clades (figure 1). Collecting both stem and crown ages is desirable because (i) these nodes are clearly defined and have received substantial attention from divergence studies and (ii) the basal divergence of each clade (crown age) will, by definition, be younger than the divergence of that clade from its sister clade (stem age), providing some temporal structure for comparisons.

A large-scale phylogenomic study [20] was used as a framework to identify sister clades. Results from mitochondrial, nuclear and mixed gene studies were surveyed using the bioinformatics resource TimeTree [21], which curates published divergence estimates. TimeTree results were confirmed against the original papers, and we conducted additional literature searches to incorporate dates from analyses not available through TimeTree. Fossils were not included as dated tips in any of the surveyed analyses, although this practice is becoming more common in studies of other taxa [e.g. 22,23].

A total of 332 stem divergence dates and 126 crown divergence dates were collected. In cases where two targeted clades are sister taxa (e.g. Galliformes and Anseriformes), stem divergence estimates for both clades will be identical in a given study. There are 19 such pairings in our dataset, and we excluded dates from one side of each such pairing when calculating disparity to avoid duplication. Some crown clades yielded no divergence results because they are monotypic or have not been subjected to a divergence dating analysis.

Ages for the oldest known fossil stem and crown representative of each of the targeted clades were collected from the literature (electronic supplementary material, tables S5 and S6). We chose to err on the side of accepting older fossil records in cases where material was incomplete or where crown status has been proposed based on morphology but has not been resolved via phylogenetic analysis. This practice is conservative with regard to the magnitude of disparity between fossil ages and molecular divergence estimates, in that any erroneous inclusions will tend to reduce disparity. In cases where a fossil could not be dated using numerical methods, we selected the midpoint of the finest geological time division to which it could be assigned.

In the fossil record, a clade with a limited range can still be inferred to be at least as old as the oldest record of its sister taxon, because the presence of the sister taxon at a given time horizon indicates the divergence between the two had already occurred [19]. In cases where the fossil record of a targeted clade's sister taxon predates the oldest fossil within the targeted clade, we used the sister age in our comparisons. As in the TimeTree comparisons, the global phylogeny [20] was used to determine sister clade. For 36 targeted clades, considering sister taxa provided an older date for stem divergences. It is possible for more than half of the 67 clades to have an older sister taxon because the avian tree is not perfectly balanced (e.g. the oldest record of Psittaciformes provides an age for the divergence between Psittaciformes and its sister clade Passeriformes, and also for the stem age of the more deeply branching Falconidae).

Paired sample Wilcoxon signed-rank tests were used to evaluate whether clade ages inferred from different data sources were obtained from identical population distributions, without assuming an underlying normal distribution. Fossil age and molecular age were treated as repeated observations of the age of each clade.

Statistical tests were also performed in R to test for correlations between molecular date : fossil age ratio (relative disparity) and analytical methods employed by the 47 analyses that yielded our divergence dataset. We applied one-way ANOVA to test for statistical differences between mean relative disparity (averaged over all dated nodes) in studies using different methods of inference

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Table 1. Summary data from comparisons of 371 molecular divergence dates and oldest fossil records within 67 avian clades. Average ratio of mean clade divergence estimate to oldest fossil age is provided, along with disparity and standard deviation (s.d.) in millions of years (Myr). Nodes with no fossil more than 1.0 Myr in age were excluded from calculations of ratios but included in calculations of disparity (see the electronic supplementary material).

	all divergence dates			mitochondrial dates			nuclear dates		
	ratio	disparity	s.d.	ratio	disparity	s.d.	ratio	disparity	s.d.
divergence from sister clade (stem divergence)	1.56	21.41	± 13.1	1.71	29.43	± 14.1	1.46	16.55	± 14.4
basal crown divergence	2.85	25.43	± 18.4	3.64	33.05	± 20.3	1.59	13.13	± 18.0

(penalized likelihood, non-parametric, Bayesian and strict clock) and clock models (strict, uncorrelated, autocorrelated and rate smoothing). The latter category pools non-parametric and penalized likelihood approaches, which are considered separately under methods of inference. In cases where significant differences were detected, we performed pairwise comparisons applying the Bonferroni correction for multiple comparisons. We also conducted Welch two sample *t*-tests for significant differences between studies using unpartitioned versus partitioned sequence data, and between studies using fossil calibration points versus secondary calibration points. Finally, we tested whether relative disparity depended on study publication date via linear regression. Further details are provided in the electronic supplementary material.

3. Results

Substantial disparity between molecular estimates and the fossil record was noted for most splits (table 1 and figure 2): molecular estimates were on average 1.56 times as old as the earliest stem fossils of each clade (or its sister taxon), with an average disparity of 21.38 Ma. Disparity was higher for crown divergences than for stem divergences. Molecular estimates were on average 2.85 times as old as the earliest crown fossil in birds, with an average disparity of 25.43 Myr. Mitochondrial genes yielded older ages than nuclear genes in almost all cases (53 of 58 divergences for which both were available). Wilcoxon signed-rank tests indicate that ages derived from mitochondrial studies were significantly older than those derived from nuclear studies, and that relative disparity was significantly higher for crown comparisons than for stem comparisons ($p \leq 0.01$, see the electronic supplementary material). A Breush–Pagan test for heteroskedasticity carried out using the R package 'car' [24] failed to reject the hypothesis that discrepancy between fossil and molecular age estimates is independent of clade age ($\chi^2 = 0.1889984$, d.f. = 1, $p = 0.6637516$). Moreover, visual inspection reveals a 'funnel-in' pattern consistent with the interpretation that fossil and molecular age estimates are more congruent for older clades than for younger ones (figure 2*e*).

Significant differences between dates yielded by different methods were detected only in comparisons between Bayesian and strict clock inference and between strict clock and uncorrelated clock models. In both cases, this mismatch was driven by a single study that used a strict clock approach. No significant differences in relative disparity were found between inference methods or clock models when this point was excluded, between studies that used partitioned versus unpartitioned sequence data, or between studies that used fossil calibrations versus secondary calibrations. Linear regression of disparity on publication date does not support a significant relationship between these variables, indicating that the magnitude of disparity is not simply greater in older studies.

4. Discussion

Our results suggest that, for Aves, discord between molecular divergence estimates and the fossil record is pervasive across clades and of consistently higher magnitude for younger clades. The old ages inferred via mtDNA appear to be a primary driver of disparity between fossil dates and molecular age estimates, although nucDNA results also contribute to this effect. Moreover, this disparity pushes divergences across biologically meaningful temporal boundaries. Stem divergences of 42 clades with earliest fossil records restricted to the Cenozoic are pushed into the Mesozoic by molecular estimates, implying a wave of survival across the Cretaceous–Palaeogene mass extinction boundary that is uncorroborated by the fossil record. This observation has, of course, previously been stated [e.g. 3,16]. Owing to the nature of the fossil record, which in our study is expected to provide only minimum estimates of clade ages, it should be clear that a large portion of this disparity must be due to gaps in the records of individual clades. Determining the actual amount of disparity that is due to fossil gaps versus molecular divergence estimation errors would require knowing the true divergence ages. Because these ages can never be known with certainty, we assert that it is more productive to explore several consistent patterns of disparity.

Unexpectedly, relative disparity is substantially higher for crown than for stem divergences. This observation is difficult to attribute to fossil preservation biases. The quality of the fossil record is expected to improve from the past towards the present, because more fossil bearing rocks are preserved from younger deposits [25]. If disparity were primarily driven by gaps in the fossil record, one would expect the gap between the divergence of a lineage and its oldest known fossil to be smaller on average for the basal crown divergence in each clade, which by definition occurred more recently than the stem divergence. Another potential explanation is identification bias. If palaeontologists fail to properly identify the oldest fossil of a clade, that clade's stratigraphic range will be artificially shortened. This is an unlikely driver of the patterns observed here, because crown fossils are relatively straightforward to identify: all diagnostic characters shared by the common ancestor of extant representatives should be present in the oldest crown fossil. For stem fossils, however, it is not possible to predict *a priori* when in the history of the lineage each diagnostic trait evolved. We thus argue that

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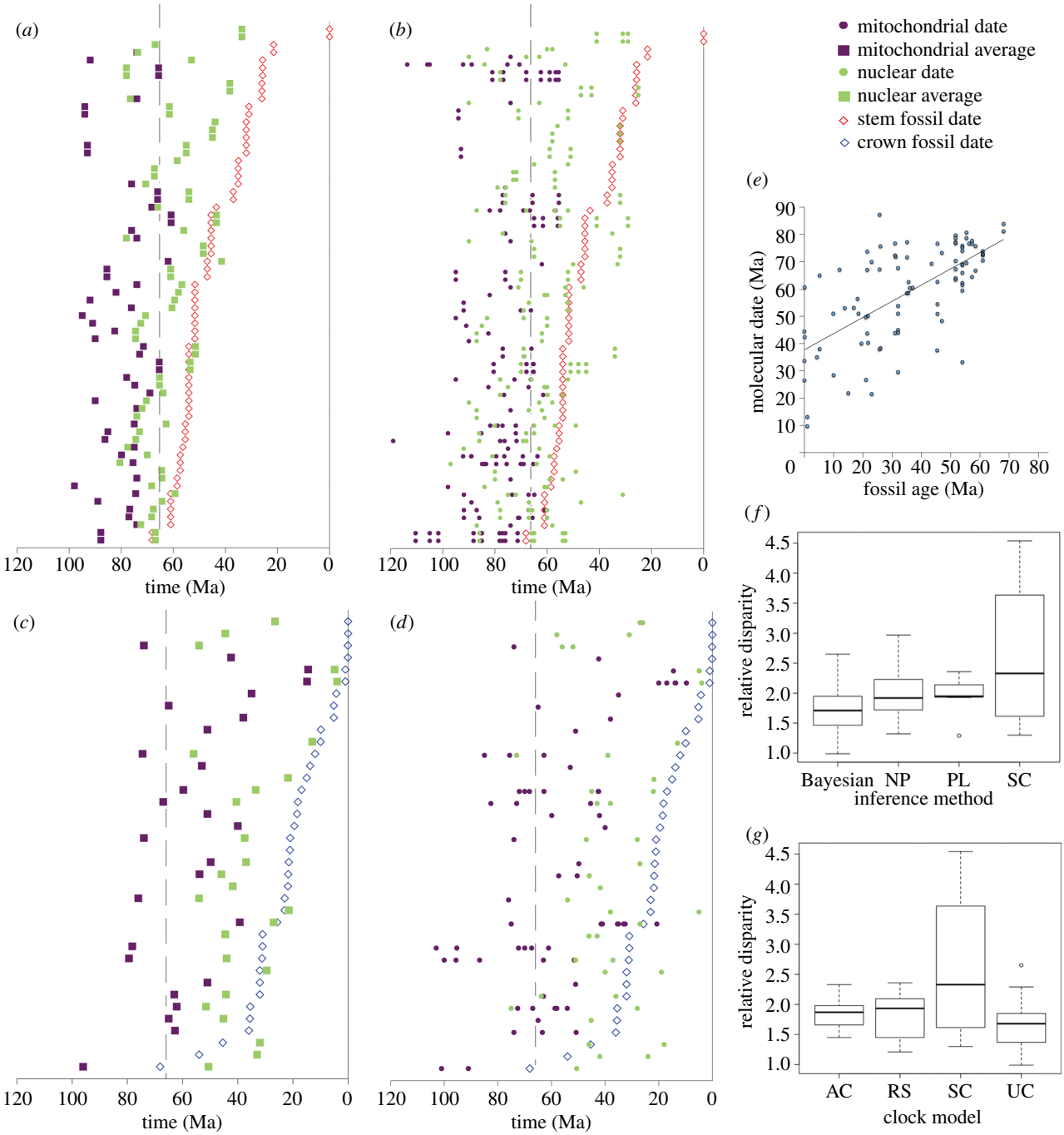

Figure 2. Disparity between fossil ages and molecular divergence dates in Aves: (*a*) average molecular dates and fossil ages for the divergence of each of 67 clades from its sister clade (stem divergences); (*b*) individual molecular dates and fossil ages for stem divergences; (*c*) average molecular dates and fossil ages for the basal crown divergence in each clade; (*d*) individual molecular dates and fossil ages for basal crown divergences; (*e*) plot of average molecular dates to fossil ages for all crown and stem divergences; (*f*) comparison of ratios for average molecular divergence dates to fossil ages inferred by Bayesian, non-parametric (NP), penalized likelihood (PL) and strict clock (SC) methods; (*g*) comparison of ratios for average molecular divergence dates to fossil ages under autocorrelated (AC), rate smoothing (RS), strict clock (SC) and uncorrelated (UC) clock models. (Online version in colour.)

there are likely to be more stem fossils than crown fossils lingering with 'incertae sedis' identifications. In sum, biases in the fossil record predict larger gaps between genetic divergences and fossil occurrences for stem divergences than for crown divergences, yet the opposite pattern is observed.

An explanation for the greater disparity in the crown dataset should thus be sought among issues related to molecular branch length estimation. Simulation studies reveal that 'tree compression' resulting from underestimating hidden substitutions on deep-diverging branches may lead to error in divergence analyses [26,27]. Critically, simulations suggest that if a deeper node is calibrated, divergence error effectively shifts to shallower nodes [6,26]. Owing to model misspecification, standard nucleotide (NT) coding strategies will tend to overestimate shallow divergence ages, and purine/pyrimidine (RY) coding strategies will tend to underestimate shallow divergence ages under this type of calibration regime at least in mtDNA studies [26]. Given the prevalence of NT coding strategies, tree compression effects may partially explain observed patterns of disparity between molecular divergence estimates and the fossil record. For our sample, dated divergences were frequently shallow relative to nodes calibrated in the analysis generating the estimate. Calibration depth is difficult to quantify given the frequent use of multiple calibrations

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and because it is impossible to rank the depth of two nodes on opposite sides of a balanced tree *a priori*. However, it can be observed that crown nodes will occupy more shallow positions relative to stem nodes overall.

Disagreement between gene trees and species trees is another potential source of bias. Unless there is gene flow, gene divergence will typically predate speciation, because genetic polymorphisms present at species divergence must have arisen prior to that event [17]. Therefore, simply equating gene trees with species trees is expected to lead to overestimated divergence dates. If there is a large discrepancy between gene tree and species tree divergence, this phenomenon could account for a substantial portion of overall disparity. In such a case, the ratio of molecular age to fossil age is expected to be more biased for younger divergences than for older ones, leading to greater overestimates for younger nodes. The magnitude of this effect is related, in part, to the depth of the targeted nodes: it is predicted that such error will become insignificant as true species divergence age increases [17].

Examination of the nodes targeted in this study and relevant avian life-history data suggests that mismatch in divergence time between gene trees and the species tree is not a satisfying explanation for the patterns of disparity elucidated here. Coalescence time is scaled in time units of $2N_e$ generations, where N_e is effective population size. For birds, most estimates of N_e are at least an order of magnitude smaller than 10^6, and average age at reproduction is less than 10 years, commonly close to 1 year [e.g. 28–30]. Hence, the interval between genetic divergence and species divergence is expected to be less than 1 Myr. In general agreement with this hypothesis, applying a multispecies coalescent to marsupial mammals resulted in an average decrease in inferred ages of shallow nodes of approximately 3 Myr compared with previous results [31]. The increase predicted for birds should be lower, owing to shorter average generation times compared with marsupials.

Given that the crown nodes examined here had an average fossil age of 20.9 Myr (a minimum estimate for clade age), it is unlikely that discordance between gene trees and the species tree is the primary driver of the differences between the crown and stem datasets. Still, we note that divergence estimates surveyed in our study relied on the concatenation of multiple loci, rather than incorporating an explicit model of gene-lineage coalescence into the phylogenetic inference procedure. Methods that enable multilocus species tree inference and apply the multispecies coalescent [32–34] offer a promising way to evaluate the effects of gene-lineage coalescence on disparity between molecular divergence time estimates and the fossil record.

Greater disparity from the fossil record for mitochondrial versus nuclear results is a second consistent pattern. Higher substitution rates and greater rate heterogeneity may be driving factors, although rate heterogeneity among nuclear and mitochondrial genes has rarely been evaluated [35]. Mitochondrial data suffer from greater NT composition bias and site saturation [27]. Nuclear genes generally exhibit lower levels of site saturation [36], and studies using only nuclear genes may therefore yield younger estimates compared with those incorporating mitochondrial sequences if model misspecification is a factor [37] and the calibrated node is deeper than the node of interest (as is generally the case). It has been anticipated that increased focus on nuclear data will reinvigorate avian divergence studies [8]. Future studies should reveal whether wider sampling of nuclear loci results in directional shifts in age estimates.

5. Conclusion

As it is unlikely that the oldest fossils of all surveyed clades have been discovered, some disparity must always be attributed to gaps in the fossil record. However, the patterns elucidated here motivate careful consideration of other sources of error. Though often mischaracterized as scrappy, the fossil record of modern birds is now sampled from hundreds of thousands of specimens from throughout the Cenozoic [13]. As increasing efforts have yielded vast numbers of new specimens but failed to reconcile the gap between molecular and fossil evidence, it becomes less plausible to attribute disparity solely to gaps in the fossil record. Patterns observed in stem/crown comparisons and mitochondrial/nuclear comparisons are not expected to be affected by limits of the fossil record, and thus deserve particular attention. We suggest that the magnitude and pattern of disparity observed here should encourage concern for potential systemic biases in molecular dates as well as proper calibration strategy [37–45].

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