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Rocks and clocks: calibrating the Tree of Life using fossils and molecules

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A great tradition in macroevolution and systematics has been the ritual squabbling between palaeontologists and molecular biologists. But, because both sides were talking past each other, they could never agree. Practitioners in both fields should play to their strengths and work together: palaeontologists can provide minimum constraints on branching points in the Tree of Life with considerable precision, and estimate the extent of unrecorded prehistory. Molecular tree analysts have remarkable modelling tools in their armoury to convert multiple minimum age constraints into meaningful dated trees. As we discuss here, work should now focus on establishing reasonable, dated trees that satisfy rigorous assessment of the available fossils and careful consideration of molecular tree methods: rocks and clocks together are an unbeatable combination. Reliably dated trees provide, for the first time, the opportunity to explore wider questions in macroevolution.

The evolving relationship between rocks and clocks

Classically, the fossil record has provided the timescale for evolutionary history. This role was codified as the unique contribution of palaeontology to the neodarwinian synthesis by Simpson in his seminal *Tempo and Mode in Evolution* [1]. This is, however, no longer the case. There have been four decades of evolving molecular clock theory [2] and, since the 1990s, this has become the tool of choice in attempts to calibrate evolutionary time. One might be seduced into thinking that fossils are no longer of any use in a world of molecular biology. After all, the fossil record has been berated countless times for its failure to match molecular clock estimates for the timing of evolutionary events. Recently, however, there has been an almost cultural change in the manner in which these mismatches are interpreted. As molecular clock methods have diversified with ever-increasing complexity, attempting to capture the reality of molecular evolution, their inherent assumptions have become not only weaker, but also more numerous [3]. The fossil record is being examined anew to inform these assumptions, and to provide more, and increasingly reliable, estimates to calibrate the clock, and to provide an independent test of different molecular clock methodologies.

From their conception, the performance of molecular clocks was measured against the fossil record. Zuckerkandl and Pauling's seminal study of haemoglobin evolution [4] demonstrated close agreement between molecular clock and palaeontological estimates for the timing of the origin of major groups of vertebrates. Subsequently, such precise concordance has rarely been achieved [5] and, invariably, discrepancies have been attributed to the vagaries of the fossil record.

However, fossil and molecular date estimates are, more often than not, in general accord [6] and this trend has increased [7] as molecular clock analyses have become more sophisticated [3]. A few infamous examples of gross discrepancy remain, such as estimates for the dates of origin of complex animals, birds and flowering plants [8], but others have been resolved through a better understanding of palaeontological and molecular data, through discussion and collaboration between palaeontologists and molecular biologists, and through the development of new molecular clock methods.

This is encouraging because, directly or indirectly, all molecular clock analyses rely on palaeontological data for calibration. Collaborations between palaeontologists and molecular biologists are increasingly commonplace as, together, they attempt together to uncover an accurate temporal scale for the one true Tree of Life. As we discuss here, this has been exemplified in recent years in two main areas: (i) the development of reliable calibration points and methods; and (ii) the testing of molecular clock analytical methods.

Calibrating the molecular clock

The fossil record is an imperfect record of evolutionary history, but precisely how imperfect? During the early 1980s, the palaeontologist Bruce Runnegar championed the molecular clock as an independent means of testing the palaeontological timescale of evolutionary history [9]. However, molecular clocks require fossil calibration and various approaches have been taken, including the extrapolation of a previously inferred rate of molecular evolution [10] and use of multiple calibration points [11], although some have attempted to eschew palaeontological data altogether [12].

However, the most commonly adopted approach to calibrating the molecular clock has been to find a single palaeontological age estimate that is perceived to be reliable. Invariably, this is assessed by comparing the fit of the stratigraphic ranges of fossils to evolutionary trees in a variety of ways. Obviously, lineages that diverge one after the other should exhibit the same order of fossil representatives in the rocks [13]; and, because lineages split at one point in time, the oldest fossil records of both lineages should be the same age [14]. Deviations from these

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ideals provide a measure of confidence in the fossil evidence for lineage-splitting events, and the stratigraphic spacing of individual fossil finds can even be used to define a confidence interval enveloping an inferred true time of first occurrence [15].

Of these measures, the bird–mammal lineage splitting event has become the most widely adopted calibration point in molecular clock studies, either directly, or indirectly through the use of inferred rates of molecular evolution. However, a debate has erupted over the appropriateness of this calibration [16] that has highlighted a tension between palaeontologists, who believe that a calibration should be a close approximation of an evolutionary event, and molecular biologists, who are often more concerned with the quantity of molecular data available that can give a statistically meaningful rate of evolution. Predictably, the alternative calibration points that have been proposed [16,17] cannot be applied to most of the sequence data available in public databases such as GenBank and Ensembl [18].

Unfortunately, both of these positions are justified. If a fossil calibration underestimates the date of an evolutionary event, the inferred rate of molecular evolution will be too low and all clock estimates derived from that rate and date will also be underestimates [18]. This fact has been accepted by molecular biologists for years and matters little if it is understood that clock estimates generally exceed fossil-based estimates. If fossil calibrations are accepted instead as minimum constraints, as appears universally to be the case [8], the only bad calibrations are those that are erroneously older than the age of the splitting event that they purport to constrain [19]. However, fossil calibrations are frequently used as actual dates out of computational expediency [19], rather than as minimum bounds in rate estimates, for lack of maximum bounds, but this is changing [20,21].

The availability of molecular data is also a serious consideration, because the scientific questions that molecular clock analyses can address are constrained by the availability of sequence data. Furthermore, because of overdispersion, where the substitution rate variance exceeds that expected under Kimura's theory of neutral evolution, a large data set is inevitably required for the derivation of a statistically significant average rate [22]. It is understandable then why the fossil calibration for the bird–mammal split has been so widely adopted because it encompasses 30 of the 41 animal genomes for which broad coverage is available.

Nonetheless, debate over the fossil-based date for the bird–mammal split has highlighted general problems with the way in which palaeontological data are presented and used in molecular clock analyses. For instance, the security with which the fossils (on which the date is based) are placed within the phylogeny has been questioned [23], and the age of the sequence of rocks in which the fossils were discovered has proved hard to constrain [16,24]. There are many errors inherent in identifying the oldest member of a clade and even more errors that might influence the date that is ultimately presented for calibrating the rate of molecular evolution (Box 1). These errors can be large and yet they are rarely, if ever, considered in the application of fossil calibrations.

Minimum constraints and maximum date estimates

To overcome some of the problems associated with molecular clock calibrations, we have recently presented a synthesis of palaeontological, phylogenetic, stratigraphic and geochronological data pertaining to fossil age constraints for the lineage splits between each of the animal genomes available from Ensembl [19]. Palaeontological data can only provide reliable minimum constraints on lineage splitting events. However, palaeontological databases such as Fossil Record 2 [25] are littered with oldest-possible, rather than the oldest-secure records for lineages, and the age estimates for these individual records also tend to err towards the oldest-possible interpretations. Thus, to comply with the expectation that fossil calibrations are minimum constraints, we have filtered, as best we can, the available data to find the oldest-secure fossil record for each lineage split, and returned to the primary geological literature to determine the range of possible age estimates, from which we propose the youngest possible date for use.

Minimum constraints alone are difficult to incorporate into molecular clock analyses because of the lack of maximum constraints. These might be assessed by considering the probability of how far back the evolutionary history of a clade extends below the minimum constraint [18,20,21,26,27]. There are several ways to use fossil occurrences to predict a confidence interval or age-range extension within which a lineage arose (Box 2), and these can be used to inform maximum constraints on substitution rates or molecular clock estimates. A maximum date can be used as a simple constraint or in modelling the probability density of the true time of lineage inception between the minimum and maximum constraints, and beyond [18,19,21,26], such as might be required for oldest-possible ages for calibrating fossils and, indeed, oldest-possible records. In Figure 1, we augment our existing database of calibrations with fossil-based estimates for the additional lineage splits required by the addition of sequenced genomes added to Ensembl since the completion of our review [19] and revise some of the dates presented therein. Justification for all calibration dates is provided at http://www.fossilrecord.net.

Testing molecular clock methods

Although much emphasis has been placed on the mismatch between molecular clock and palaeontological estimates of lineage-splitting events, comparisons between molecular clock estimates find no better accord. The development of multifarious methods of molecular clock analysis [28] has not improved the situation and, indeed, the gradual convergence of molecular clock and palaeontological estimates has been interpreted by some as evidence that clock analyses can now be manipulated to achieve just about any desired outcome [7,27], rather than as evidence that they can model rate heterogeneity more realistically.

Possibly so, but among these methods and their inherent assumptions, one or more of the methods must be performing more efficiently than others, but how can we tell? This question has been investigated in several recent studies that have taken the position that congruence between independent data sets provides the best insight into the performance of molecular clock methods [29–31]. The

Box 1. Why fossils cannot indicate actual branching dates

The fossil record cannot be read literally: there are many gaps, and many organisms, even whole groups, have never been preserved [53]. There have been suggestions [54] that the fossil record depends on the rock record. However, the widespread congruence between the order of fossils in the rocks and the order of nodes in cladograms [55] cannot be explained away so easily. Rather, it provides confirmation that the fossil record is more real than artefact. This does not deny the fact that there are many gaps. At low levels of resolution (e.g. large clades over coarse timescales), the significance of the gaps might be negligible, but in finer-scale studies (e.g. species over thousands of years), the gaps can overwhelm any biological signal [56]. Nevertheless, an identifiable fossil demonstrates the divergence of the lineage to which it belongs and, thus, provides cast-iron constraints on the temporal dimension of the Tree of Life that cannot be obtained by any other means.

There are many sources of error in estimating the actual date of origin of a clade, and these can be divided into five broad categories.

- Phylogenetic topology: is the cladogram correct, with robust support values for the node in question, and for the node above and below?
- Fossil record sampling: the oldest known fossil will not be the earliest member of a lineage and the oldest actual fossil is unlikely to ever be sampled.
- Identification: are all the fossils correctly identified and correctly assigned to their lineages? Identifying the oldest fossil representative of a clade is difficult because: (i) they emerge within a single point in time and space (Figure I); (ii) the earliest representatives will invariably lack fossilizable apomorphies of the living members of the clade; and (iii) fossils are usually incomplete and so it can be difficult to determine whether the absence of clade-specific diagnostic character reflects the nature of the organism or of its fossilization history.
- Exact age-date assignment (absolute dating): is there a good radiometric date that can be assigned to the fossiliferous horizon? What is the observational error on the radiometric date in question? Is this date in stratigraphic proximity to the fossil occurrence (Figure I)?
- Correlation (relative dating): dating of the fossils used in calibration is rarely direct and, usually, dates are assigned through correlation from the rock section in which the fossil was recovered to another in which absolute age dates are available. This process can be simple or tortuous, with concomitant consequences for dating error (Figure I).

Our conclusion [19] is that fossils cannot provide accurate estimates of evolutionary splitting events, but they can provide firm minimum age constraint on such events, the error on which is the uncertainty over the date of a named geological formation from which the integral fossils have been recovered. The errors associated with the age of geological formations can be remarkably small and there is a strong international programme working continuously to improve the exact age dating of fossiliferous rock successions and the age correlations between rock sections [57].

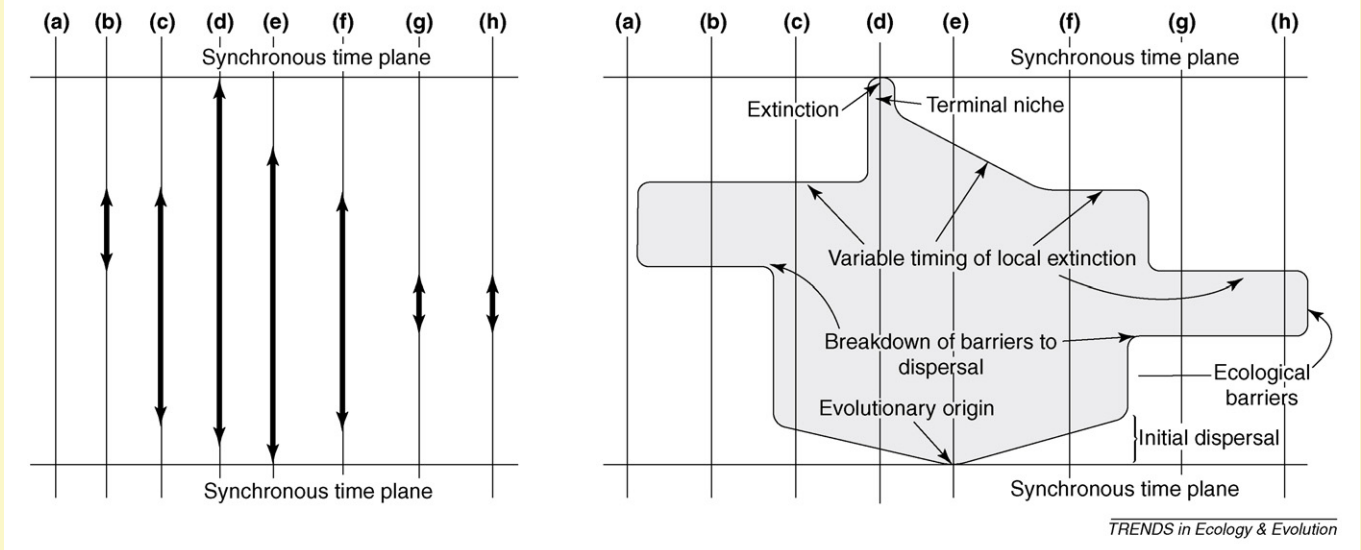

Figure I. Errors inherent in dating rock successions and their biological causes. The chance of detecting a fossil depends on a range of biological, geological and human factors. **(a–h)** are a series of time-equivalent rock sections in different geographical regions, most of which (but not all) record the time range of a single taxon. The bold arrows in the left panel represent the age range of the taxon in each of the rock sections with a vertical time axis from old (bottom) to young (top). The record is different for each, because the clade in question had a complex biogeographical history (interpreted in the right panel); no single section provides the true time range of the clade, and some sections, for example (a), preserve no record of it at all. Evidently, the oldest record is in (e). Invariably, however, this section will not have been dated by geochronology and so it is necessary to correlate to another which has, say (h), but where the first appearance of the taxon will be younger, perhaps much younger. To identify and obtain a date for the earliest record, it is necessary to either date all sections geochronologically (which is too time consuming and expensive) or integrate the data from many sections using the method of graphic correlation [58]. Where this kind of analysis has been undertaken, such as across the K–T extinction event (65 Mya), the differences in date of first appearance from one section to another are hundreds of thousands of years [59]. For older sections and groups with poorer fossil records, dating inaccuracies arising from correlation must be in the order of millions of years. Despite the fact that graphic correlation has been universally adopted in the oil industry, the academic community has been slow in the uptake.

most sophisticated of these is a team effort by Andrew Smith and colleagues [30] on sea urchins, an ideal group for comparing the fossil record and molecular clock-based estimates because they have a diverse representation both among living biota and the fossil record. Furthermore, because much of their taxonomy is based upon readily fossilized skeletal characters, it is possible to integrate both living and fossil sea urchins into one morphological analysis of evolutionary relationships and to calibrate the component lineage splitting events using their abundant fossil record.

Smith and colleagues compared several methods for best fit to the data: the Langley-Fitch strict clock method [32], and a variety of relaxed clock methods that allow for the rate heterogeneity observed in the data, including parametric Bayesian [33], nonparametric rate smoothing

Box 2. Probability densities of clade origins

If it is accepted that the oldest securely identified fossil in a clade gives a minimum age constraint on the date of the branching point at the base of a clade (Box 1), how is the maximum constraint to be estimated?

One approach is phylogenetic bracketing, which obtains not only minimum, but also maximum constraints on the timing of a branching event using the date of the preceding and subsequent branching episodes [16,17]. Broader constraints can be derived using the earliest stem-member of the overall clade to provide a maximum constraint, and the earliest member of the crown group to provide a minimum constraint [24,60]; propagated errors can then be placed on both of these dates to provide the overall extent of the bounds. Another approach is to estimate the timing of the branching event itself by modelling the pre-fossil history of diversification and/or its attendant diminishing preservation probability (lower abundance, fewer species and perhaps smaller body size) [61,62].

Both approaches are problematic. Phylogenetic bracketing assumes that the branching events above and below a calibration more reliably capture the timing of the branching event in question than does the estimated date of the calibration itself. Meanwhile, modelling approaches are particularly well suited to testing molecular clock estimates but are perhaps too assumption laden to use in molecular clock calibration. Nevertheless, both approaches rely on assumptions about the shape of a diversifying clade. Empirical observations suggest that clades generically diversify following a logistic curve [61,63,64], in line with expectations from standard birth–death models in macroevolution [65] and ecological models such as the Lotka–Volterra models of competition and the island biogeography model [66,67].

The logistic model might then be an appropriate description of the probability distribution of fossil finds from the genealogical origin of a clade to the first fossil find. If a 95% confidence interval is marked near the base of the logistic probability distribution (Figure Ib), a very deep tail might still be subtended that allows for the remote chance of one day finding a truly ancient fossil, while retaining a reasonable assumption that this is unlikely. Such a distribution could be qualified on evidence of older, systematically uncertain fossils (Figure Ic). For example, in dating the origin of Chondrichthyes, the oldest secure fossils are partial skeletons from the Early Devonian [68] (~395 million years ago) but there are isolated scales reported from the Ordovician [69] (460 million years ago) that share one or two apomorphies with living Chondrichthyes. The evidence is insufficient to regard them as secure records of Chondrichthyes: they could lie on the stem to Chondrichthyes plus Osteichthyes, or even more basally in the tree [70]. Nonetheless, such early, but uncertain fossils should influence the probability distribution to include an expansion of the logistic curve.

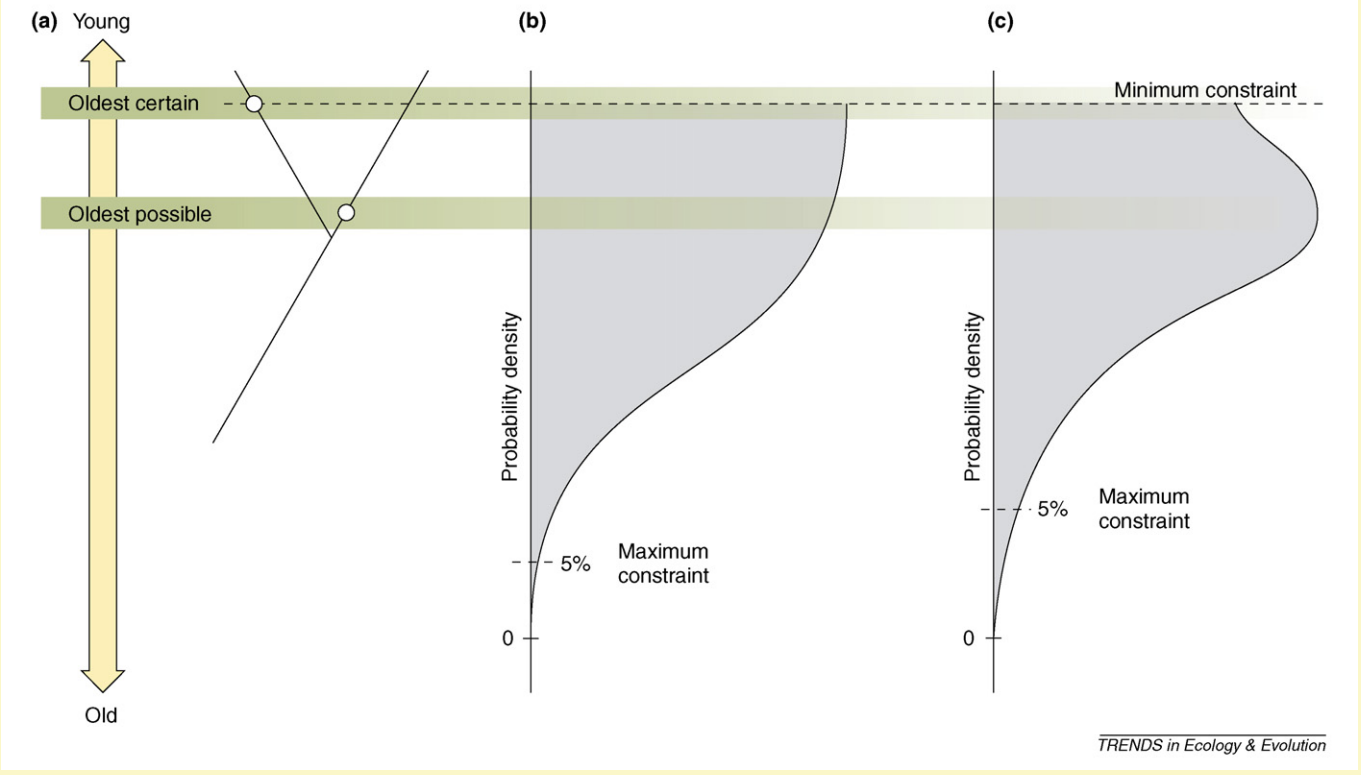

Figure I. Two hypothesized patterns for the distribution of probabilities between the maximum and minimum constraints on the date of origin of a clade **(a)**. In **(b)**, the curve is a logistic, corresponding to a standard birth–death model of diversification and an equilibrium at 'normal' diversity, when fossils become abundant. In **(c)**, an assumption is added that there might be some older fossils whose affinity is less than certain, corresponding to an expansion of the probability distribution.

[11] and semiparametric penalized likelihood [34]. Relative performance was considered by comparing clock estimates of nodes to palaeontological estimates and, on the whole, there was good concordance, with only a 10% difference between known record and missing record inferred from clock estimates. However, this general concordance masks the variance in clock estimates that arose through varying the phylogenetic and methodological assumptions.

In particular, the number of calibrations used in an analysis affected the performance of tree-finding methods. With only one basal calibration, the strict clock method performed poorly, underestimating divergence times, whereas the relaxed clock methods generally performed well. Adding further calibrations improved the performance of the clock methods, particularly the strict clock method, but some of the relaxed clock methods performed more poorly. In fact, the strict clock method performed best

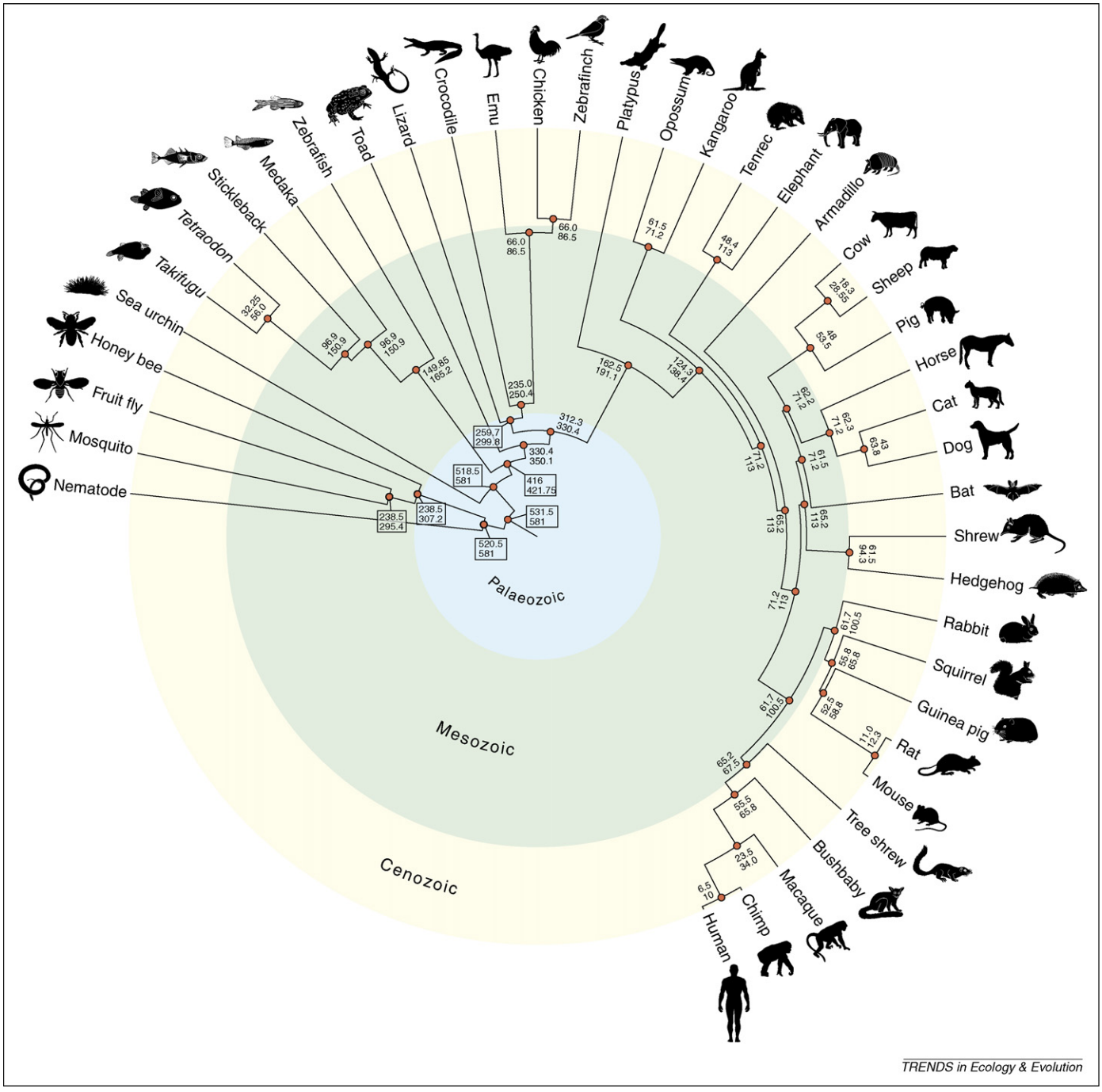

Figure 1. Tree of relationships of the key genome model organisms showing minimum (bold) and maximum (roman) fossil-based dates for each branching point. The pattern of relationships is based on a consensus of current views. The minimum age constraints are based on the oldest fossil confidently assigned to either of the two sister groups that arise from each branching point. The maximum age constraint is based on bracketing (maximum ages of sister groups) and bounding (age of an underlying suitable fossiliferous formation that lacks a fossil of the clade). Full justification for each minimum and maximum fossil-based age constraint is available at http://www.fossilrecord.net.

of all when multiple calibrations were used, suggesting that even when rate heterogeneity is apparent, relaxed clock methods may not perform best.

Although this comparative study [30] showed that, in most instances, molecular clock methods approximated well the palaeontological estimates in a group with a good fossil record, palaeontological estimates were generally younger than their molecular counterparts [6], although not universally so. There was a significant mismatch between palaeontological and molecular dates in three parts of the tree regardless of the clock methods used, and these reflected a known poor fossil record, or fields where further research is needed.

So it appears that we have turned full circle. The fossil record is now being marshalled to provide a guide to the performance of molecular clock estimates. Clock methods are now more diverse than they were during the 1960s, and initial studies suggest that relaxed clock methods return sensible date estimates that complement the fossil record as our guide to evolutionary history.

What is the point?

Ultimately, it might be possible to determine which clock method and model of rate heterogeneity is most appropriate within a given circumstance, but, we must ask ourselves, what is the point? Although some molecular clock studies have focussed on attempts to test established hypotheses of tempo and mode of evolution [35–38], many verge on the vainglorious, providing a timescale for the gamut of evolutionary history [39,40], with no obvious purpose except perhaps to provide a means of classification [41]. Indeed, the errors on molecular clock estimates are often so broad that, although they provide excellent tests of evolutionary hypotheses contingent on timing [35], they are insufficiently precise on their own to provide a basis for correlating organismal evolution with Earth history. Hedges and colleagues, for instance, have argued for a causal relationship between Neoproterozoic Snowball Earth events and the diversification of animals and plants based on molecular clock age estimates [42,43]. However, the errors on the molecular clock estimates are so broad that, setting aside methodological problems [44] and a complete incongruence with fossil data [45], these evolutionary events cannot definitively be correlated with the cryogenic phase of Earth history, let alone with any of the component Snowball Earth events.

Molecular clocks are not yet up to the job [7], but neither is the fossil record. Congruence between palaeontological and molecular clock estimates, combined with the geological context of palaeontological data, currently provides the best approach to holistic attempts to uncover the interplay between evolving organisms and their environment. This was the approach taken by Kevin Peterson and colleagues in an integrative molecular clock study that attempted to unravel the emergence of animal phyla [46–49]. As usual, the estimates derived from the study, using distance and maximum likelihood methods, were hampered by broad standard errors, but the results were compared for accuracy against not only palaeontological data, but also the gamut of geological evidence and macroecological expectations of the unfolding scenario of metazoan diversification (Figure 2). For example, the ecological consequences of early metazoan evolution would be negligible because early metazoans were sponge-like organisms. The origin of eumetazoans would be a different matter, however, with the newly evolved gut facilitating novel feeding strategies such as macrophagy. These expectations are met with evidence for a revolution in

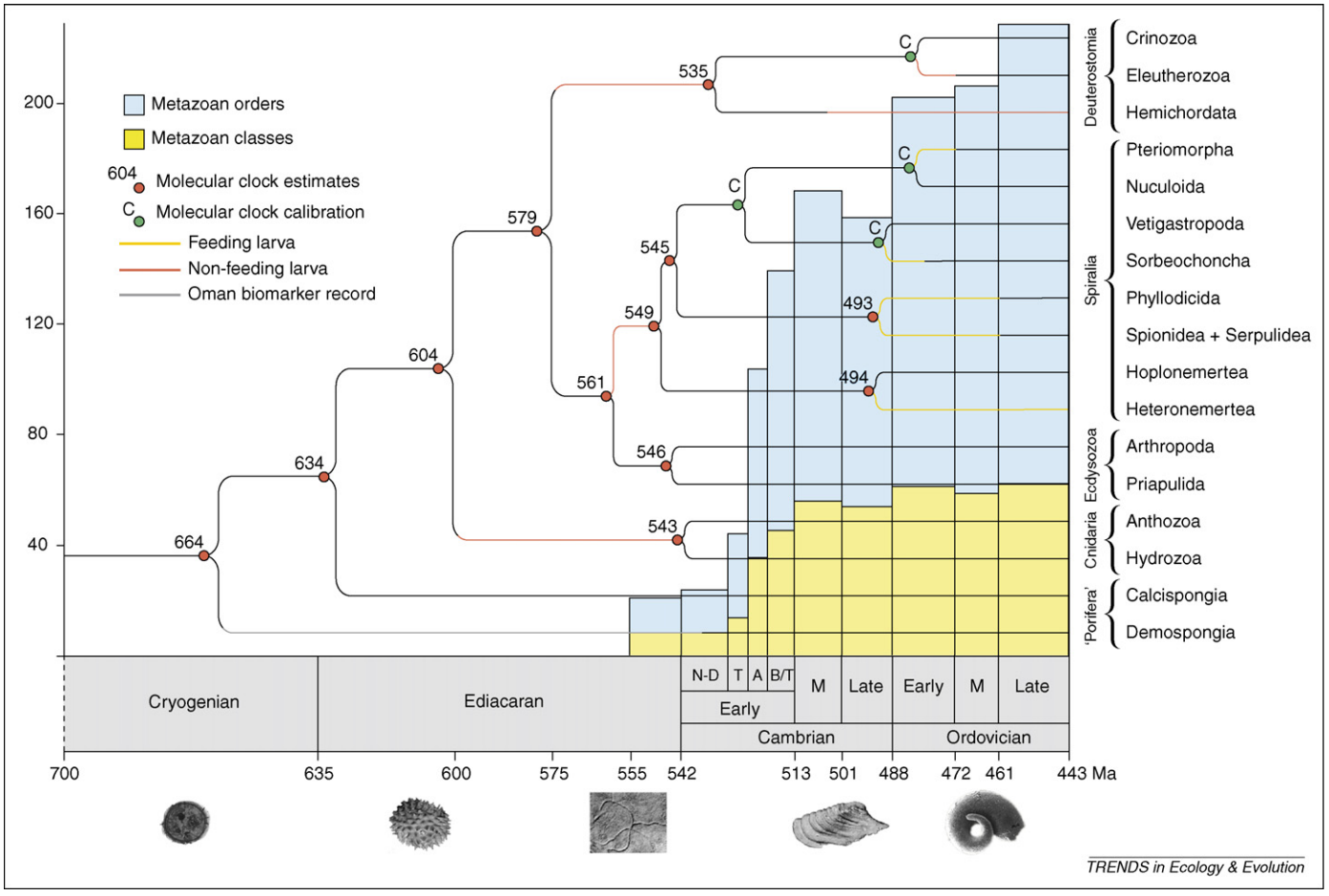

Figure 2. Concordance of palaeontological data, phylogenetic hypotheses, macroevolutionary events and molecular clock estimates from the work of Peterson and colleagues [46–49]. The fossil record of marine invertebrates from the Cambrian through the Ordovician (calibrated to the Y axis) is compared with the divergence estimates of the molecular clock of Peterson and Butterfield [48]. Some of the calibration points are shown ('C') and the divergence estimates are given in red boxes. Also shown is the evolutionary history of feeding larvae as determined by both the molecular clock and the fossil record [49]. Shown at the bottom (from left to right) are the change in acritarch morphology from pre-Marinoan to post-Marinoan, the first appearance of large macroscopic trace fossils and the change in morphology of gastropods with non-feeding larvae to gastopods with feeding morphology. Abbreviations: A, Atdabanian; B/T, Botoman/Toyonian; N-D, Nemakit-Daldynian; T, Tommotian. Reproduced with the permission of the authors and the Palaeontological Association [52].

the plankton: acritarchs that had survived for eons became extinct and the rate of plankton evolution increased by an order of magnitude, and all of this in close temporal concordance with the clock estimate for the origin of eumetazoans based on distance (but not likelihood) methods [48]. The later origin of planktonic larval stages among a diverse and polyphyletic grouping of eumetazoan phyla, long hypothesized as a life-history strategy to evade predation [50], also coincides with a dramatic rise in the diversity of epifaunal suspension feeders [49].

This study has been criticised, because the methods underestimated branch length and the fossil calibrations were used in the analysis to date divergence events precisely, rather than as minimum constraints. Both of these factors are guaranteed to return dates in closer accord with palaeontological evidence and remedial analyses of the original data sets have yielded substantially older dates [27,51]. However, it is worrying that the macroecological scenario, substantiated on a breadth of molecular phylogenetic, comparative anatomical and ecological data, and corroborated by geological and palaeontological evidence (with their attendant relative and absolute time constraints), is so strongly divorced from an inordinately deeper and ultimately cryptic metazoan evolutionary history. It suggests that something is awry and the discrepancy cannot be explained away by the old chestnut of fossil record vagary. Rather, it requires that we revisit a host of assumptions concerning phylogenetic topology, comparative anatomy and the nature of long-extinct ancestors, upon which all of our evolutionary and ecological scenarios are based. We also need to question the nature of the geological, as well as the palaeontological, record. And, if we are to accept a cryptic pre-fossil history of metazoans, this in turn requires organisms sufficiently small that even evidence of their activity would not enter the fossil record, which changes assumptions concerning generation time and mutation rate on which molecular clock analyses are based. However, allowing for shorter generation time and higher attendant mutation rates would lead ultimately to shallower molecular clock estimates that are in closer accord with palaeontological and geological evidence.

If nothing else, by integrating diverse sources of data, Peterson and colleagues have raised the bar, requiring that debate moves on from disciplinary partisanship, to participants' consideration of the totality of evidence, and revealing how reciprocal illumination can result.

Conclusion

Rock- and clock-based perspectives on the timescale of evolutionary history have long been adversarial, but we are witnessing a return to a more complementary approach to calibrating evolutionary events in Deep Time. The fossil record is imperfect but, on their own, the performance of molecular clock methods is unknowable. Together, however, rocks and clocks could provide an integrative approach to uncovering not only the timing and tempo, which was the original aim of dating evolutionary trees, but also the ecological and environmental context of evolutionary events in Earth history. Surely, this is the goal of establishing an evolutionary timescale.

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