

Caveats on the Use of Fossil Calibrations for Molecular Dating: A Comment on Near et al.

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The use of genetic data to estimate divergence times for evolutionary lineages is a controversial subject (Lee 1999; Smith and Peterson 2002; Graur and Martin 2004; Pulquério and Nichols 2007). There are many obstacles to developing accurate molecular clocks, some of which are inherent to molecular data (e.g., rate heterogeneity; Hillis et al. 1996; Graur and Martin 2004). One of the more salient problems facing molecular systematists is how to use the paleontological record as a source for calibration dates (Graur and Martin 2004; Reisz and Müller 2004). Specific problems include the selection of inappropriate fossils (Lee 1999; Benton and Ayala 2003) and the fact that fossils will almost always underestimate lineage origins (Marshall 1990).

Near et al. (2005; abbreviated NEA hereafter) propose a new method for identifying the most erroneous fossils so that they can be excluded in order to develop internally consistent divergence dates. In doing so, NEA provide very clear-cut examples of the serious pitfalls facing molecular systematists using fossil calibration dates. Although we focus our discussion on the article by NEA, it is important to note that many (if not most) studies using fossil calibrations suffer from a less than rigorous handling of the paleontological record. We hope that by highlighting the problems exemplified by NEA, our comment will serve as

a cautionary signpost for future workers. Ideally, their studies will treat paleontological data more rigorously.

Reported Ages of Fossil Calibration Points

The most important criterion for a valid fossil calibration point is that it should be based on accurate information that is used appropriately. Fossil age estimates are the initial data from which all calibrated molecular clock analyses are based; yet, despite their importance, many studies do not adequately substantiate these crucial data. This approach is exemplified by NEA's study, in which 15 of 17 of the absolute ages they use are not supported by the references they provide (table 1). In the two cases where NEA's supporting references do include absolute age estimates, a careful reading of the primary sources shows that these dates are accompanied with qualifying remarks such as "about" (Hirayama 1998) or "approximately" (Meylan et al. 2000). NEA chose to ignore these qualifying remarks, strip these dates of any error, and report them as overly precise estimates of "110.0" and "210.0" million years ago. Such estimates imply that the ages of these fossils are datable to the nearest 100,000 years. However, the actual age estimates for these fossils may not be constrained to within ~1 to 10 million years of the reported value (table 1).

An important point for all researchers to realize is that all geologic dates possess error that is a function of uncertainty derived from the limits of stratigraphic and geochronologic precision. For any study performing calculations on geologic dates, this unavoidable error needs to be recognized and, depending on the use of the age estimate, potentially carried through every stage of the analysis. Unfortunately, most molecular dating studies ignore this source of error, a trend that was exposed and strongly condemned by Graur and Martin (2004). Despite citing Graur and Martin, NEA ignored stratigraphic error in their own study. Therefore, the precise calibrations reported by NEA are not substantiated by the available evidence (table 1).

Assuming that precise and accurate dates could ever be substantiated, we also question NEA's use of multiple

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Table 1: Comparison of ages of fossil calibration points reported by Near et al. (2005), with their supporting references

Node	Reference given	Absolute age given in reference	Relative age given in reference	Absolute age (Ma) estimated from reference	Absolute age (Ma) in Near et al. 2005
1	Gaffney 1986	None	Mid Keuper and late Triassic (Norian)	218.5–202.1	210.0
2	Gaffney 1990	None	In the Keuper, of Norian age		110.0
3	Meylan et al. 2000	Approximately 110 Ma	Early Aptian	Approximate age given	110.0
4	Meylan and Gaffney 1991	None	Early Cretaceous	149.5–98.8	110.0
5	Gaffney et al. 2001	None	Uncertain of age/locality	Impossible	110.0
6	Hirayama 1998	About 110 million years before the present	Early Cretaceous: Late Aptian or Early Albian	Approximate age given	110.0
7	Yeh 1965	None	Lower Cretaceous	149.5–98.8	100.0
8	“Hutchison 1981” (should be Hutchison and Bramble 1981)	None	Paleocene	65.8–55.6	65.0
9	Hutchison 1980	None	Graybullian	54.8–53.1	52.0
10	de Lapparent de Broin 2001	Taxon not mentioned	Taxon not mentioned	Impossible	50.0
11	Hutchison 1991	None	Lysitian	53.3–52.7	50.0
12	de la Fuente et al. 2001	None	Upper Campanian–Lower Maastrichtian	76.4–69.3	71.0
13	Hutchison 1996	None	Chadronian	37.3–33.8	34.0
14	Sukhanov 2000	None	Aptian-Albian to Santonian	126–82.8	90.0
15	Williams 1954	None	Lower Miocene	23.1–15.9	18.0
16	Gaffney et al. 1989	None	Early to middle Miocene	23.1–11.6	15.0
17	Wood 1976	None	Late Miocene	11.6–5.3	11.6
18	Jackson 1988	None	Hemphillian	9.0–4.6	5.0

Note: Ma = millions of years ago. Stratigraphic error is included in each age range. Absolute age estimates are based on Clyde (2001), Gradstein et al. (2004), and Woodburne (2004).

“fixed” calibration points. NEA made seven of the 10 retained calibration points (after deletion of seven other “inconsistent fossils”) fixed estimates, meaning that they are treated as indicators of a fossil lineage’s precise age of origination. Yet, the possibility that any given fossil represents the exact time that a lineage appears is extremely remote. Taxa must appear in the fossil record some time after their initial divergence, so many have argued that fossil calibrations should be considered minimum estimates of the ages of lineages and splitting events (Marshall 1990; Benton and Ayala 2003; Benton and Donoghue 2007). The computer program that NEA used for their penalized likelihood analysis, r8s (Sanderson 2002, 2003), requires at least one fixed calibration point. Conceding that one fixed point may be necessary, we believe that the choice of which point to use will be difficult to justify and should be done with great care. Using a fossil calibration point that is both well dated and well placed in a phylogeny would be an important first step, but this fixed point should also conform to all of the criteria for useful calibrations provided by Müller and Reisz (2005). They recommend that all calibration dates be based on taxa that have fossil records with small temporal gaps and a phylogenetic framework displaying minimal ghost lineages. Fossil lineages that fit these criteria have fairly complete fossil records, so fossil calibration points derived from these groups are less likely to grossly underestimate the age of the nodes they are calibrating. None of the fixed points used by NEA meets these criteria.

Phylogenetic Placement of Fossil Calibration Points

The phylogenetic placement of extinct taxa is a major challenge for molecular dating studies based on fossil calibrations. It seems likely that calibration points based on incorrect phylogenetic hypotheses can yield grossly incorrect dates for many nodes, and so the justification for choosing taxa for each node should be rigorous and explicit. NEA provide a method for identifying such misleading fossils a posteriori, but we submit that the choice of fossil calibrations should be justified a priori. For example, NEA use the early turtle *Proterochersis robusta* to date the crown group of turtles (Testudines). We will set aside the fact that the absolute age of *P. robusta* is not given in any of their supporting references (table 1); the phylogenetic placement of this taxon is not well established. Rougier et al. (1995) conclude that *P. robusta* is not a crown member of Testudines, as NEA claim, a hypothesis that is supported by recent studies (Sukhanov 2006; Joyce 2007). NEA do not address the controversy surrounding this taxon, but removing this calibration point could change the estimated age of the crown group by 100 million years (table 1). In at least one other case, NEA’s phylogenetic hypothesis is

entirely novel (e.g., their placement of the “Lindholme-myddidae” as basal to *Platysternon*) and has not been reported anywhere else in the literature. These unjustified and potentially inappropriate placements could have a major impact on their divergence estimates and raise questions about how fossils were assigned to certain nodes.

Müller and Reisz (2005) recommend that the phylogenetic position of a potential fossil calibration should be well established, but this is not the case for the fossils chosen by NEA. NEA (p. 139) state, “Placement of fossils on the inferred molecular phylogeny was guided by cladistic analysis of 115 morphological characters presented in earlier studies of turtle phylogenetic relationships (Shaffer et al. 1997).” It is unclear how these characters “guided” the placement of taxa because the morphology-based trees of Shaffer et al. (1997) differ substantially from the molecule-based tree used in the calibration analysis of NEA. Because character codings for the fossils used by NEA are not reported in either study, it seems that NEA did not assign fossils at nodes based on explicit, repeatable methods (e.g., a cladistic analysis). The placement of fossils at nodes is a key assumption for using fossil calibrations, so it should be done as explicitly as possible. Justifying appropriate turtle fossils for calibration may prove challenging because morphological and molecular analyses give conflicting pictures of basal relationships (Krenz et al. 2005; Danilov and Parham 2006; Parham et al. 2006; Joyce 2007), with neither data set showing strong support for key nodes.

Conclusions

The conclusions of molecular clock studies using fossil calibrations are only as valid as the initial assumptions regarding the paleontological data (and in some cases may be entirely based on those assumptions; Lee 1999). Identifying whether paleontological data are suitable for a fossil calibration study may be difficult, but it is no less important than choosing an appropriate genetic data set. In the present example, the fossil taxa do not meet the criteria outlined for effective calibration points (Müller and Reisz 2005). The expressed goal of NEA (p. 138) was to develop a method to “identify misleading fossils” through cross-validation as way to identify errors in the phylogenetic placement and dating of fossils. However, if fossil taxa are placed ad hoc or calibration points are fixed on unsubstantiated dates, it is impossible to know whether inconsistent fossils are misleading or simply misinterpreted. Clearly, explicit estimates of fossil ages and phylogeny need to be established before a study such as that of NEA can be expected to give realistic and informative results. This is a daunting challenge and will require substantially more attention to the paleontological record. Molecular dating

studies based on unsubstantiated paleontological data can be useful heuristic examples of potential methods but should not be confused with rigorous depictions of actual data or patterns.

Important caveats for future workers involve the substantiation and accurate reporting of geologic dates from the literature, the inappropriate use of fixed calibration points, the objective phylogenetic placement of fossil taxa, and the novelty of estimated ages of origination. Given that these issues have been explicitly raised here, we submit that it is more important than ever for practitioners of fossil-calibrated molecular dating to treat paleontological data with the same rigor as molecular data. We strongly encourage the community as a whole to require that the ages and phylogenetic placements of fossils be substantiated by explicit evidence.

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