Reply to "The linking of plate tectonics and evolutionary divergences" by Phillips et al.

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Phillips et al. [1] reply to our finding that genetic divergence between subterranean metacrangonyctid amphipods from opposite shores of the Atlantic is congruent with vicariance by plate tectonics [2]. They highlight three presumed shortcomings in our analyses: 1) 3rd codon positions (3rd CPs) of the mitochondrial genes used to reconstruct the metacrangonyctid phylogeny are saturated, and consequently should be excluded; 2) substitution rates across the tree do not fit an uncorrelated lognormally distributed (UCLD) clock, and implementation of a random local clock (RLC) model would be more appropriate; and 3) the two dates that we used to calibrate the tree are fairly recent compared to the overall tree length, while the inclusion of a deep fossil calibrator could have improved dating. However, much of the criticism of Phillips et al. applies more to their modification of our data set than to the original data itself. Specifically, their addition of several highly divergent taxa - driven by the necessity to include taxa encompassing the new deep calibration node they propose (see point 3) -, largely alters the properties of our original data matrix (see points 1 and 2) such that their criticisms become relevant. We maintain that 3rd CPs saturation and deviation from lognormal rates largely apply to the new and not to the original data set. We also have some concerns about the fossil calibration used by Phillips et al.

Both the stemminess metric calculated by Phillips et al. [1] and our Xia and Lemey test indicate that 3rd CPs are indeed more saturated than 1st and 2nd positions. However, values for each of the three CPs in our original dataset are lower than critical values [2], suggesting that there is still phylogenetic signal at 3rd CPs despite partial saturation. That 3rd mitochondrial CPs are
partially saturated comes as no surprise, as this feature has been extensively shown to occur at various taxonomic levels [3]. However, the occurrence of partial saturation does not necessarily imply lack of phylogenetic signal and implementation of partitioning over CPs and relaxed-clock models has been shown to improve molecular phylogenetic and dating analyses in such conditions/circumstances [4]. Notwithstanding, in order to evaluate the relevance of this argument analytically, we reanalyzed the data after exclusion of 3\textsuperscript{rd} CPs and we show that this modification has a limited impact on age estimates (Fig. 1 and Supplemental Information).

Phillips et al. second concern relates to a presumed violation of the assumption that rates across the tree are not lognormally distributed although this seems to be mainly caused by the addition of distant outgroups (see Phillips et al.’s Fig. S1 [1]). In order to explicitly compare the two clock models (UCLD and RLC) in a formal phylogenetic Bayesian framework, we used the posterior simulation-based analog of Akaike’s information criterion recently developed by Baele et al. [5]. The test indicates that UCLD clock, implemented in the original analysis, outperforms RLC (see Supplemental Information for details). For the sake of comparison, we nevertheless reanalyzed the original dataset, with and without 3\textsuperscript{rd} CPs, applying a RLC model as suggested by [1]. New age estimates, although generally younger, still fall within the confidence age interval estimated using UCLD clocks (see Fig. 1), indicating that the original results are robust with respect to the use of different clock models and the effect of 3\textsuperscript{rd} CPs.

Phillips et al. also refer to clock calibration issues [1]. We fully agree that, ideally, molecular clock calibrations are best implemented by deploying several well-dated fossils robustly assigned to particular nodes positioned at different timescales in a given phylogeny [3]. However, fossils calibrations in molecular phylogenies are far from being a silver bullet, for several reasons. Fossils may be incorrectly assigned to the crown and not to the stem of a clade; age of fossils may be considerably younger than the origin of their respective clade; and data limitations may compromise both fossil taxonomic placement and dating [3, 6]. Furthermore, the fossil record is notoriously incomplete and patchy, and in many instances appropriate fossil calibrators are simply not available. In fact, the fossil record of the Amphipoda is extremely poor, with the oldest fossils known corresponding to casts preserved in Eocene Baltic amber not older than 54-40 million years (my) [7] (Supplemental Information). Facing the absence of an appropriate fossil calibrator for the
taxa under investigation, Phillips et al. add a number of non-metacrangonyctid amphipod, isopod, decapod and hoplocarid outgroups to our original alignment [1] in order to encompass an inferred date for the separation of the Subclasses Eumalacostraca and Hoplocarida derived from fossil information, and in doing so they introduce two possible problems in the analysis. This is an extremely deep node in relation to our ingroup, and raises significant concern over the overall rate stability (see above). Furthermore, they used the age of Hesslerella to date the split of the Subclasses Eumalacostraca and Hoplocarida, seemingly overlooking that Hesslerella is an undeniable crown-group phreatoicidean [8, 9], a member of the Peracarid order Isopoda, and as such its age (325 Mya) should be considered a minimum constraint age for the Peracarida. Thus, it should be assigned to the node Isopoda/Amphipoda in Phillips et al. tree instead of to the Hoplocarida/Eumalacostraca node (Fig. S1). It is important to mention here that the Eumalacostraca includes three Superorders: Syncarida, Eucarida (to which the decapods belong) and Peracarida, the latter including the amphipods and Isopods, among other groups (Fig. S1).

The calibration of molecular clocks and deduction of subsequent evolutionary timescales have always been open to debate and discussion [3, 6]. We acknowledge that there are a number of assumptions and parameters that can be applied to both phylogeny estimation and molecular clock calibration that can have an impact on the resulting estimates, and that a full understanding of the relative importance of vicariance and dispersal to explain the distribution of metacrangonyctid amphipods would require taxonomically well-sampled, robust multi-loci phylogenies of the lineages forming the superorder Peracarida, with reliable and appropriately distributed palaeographic and fossil calibrations.

The criticisms of Philips et al. demonstrate the power of parameter choice to drive biogeographic inference, but to paraphrase the great Stratford Bard (or was it the Bible?) "the devil can cite Scripture for his purpose". The differing results of Philips et al. are largely driven by their modification of the original dataset and a possibly inappropriate deployment of fossil calibration. Potentially powerful parameters do indeed carry great responsibility.

References
1. Phillips, M.J., Page, T.J. de Bruyn, M., Huey, J.A., Humphreys, W.F., Hughes, J.M., Santos,


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Figure 1. Estimation of node ages under relaxed lognormal and random local clocks.
Comparison of node age estimates obtained using different analyses. Common to all are the palaeogeographical events used for calibration, the phylogenetic tree and corresponding node numbers reported in [2] and the use of independent substitution models and clocks for each codon site. Original analysis (uncorrelated lognormal relaxed clock, 3rd CPs included): blue dots represent mean estimates, gray lines represent 95% HPD intervals. Data reanalysis: orange squares represent mean estimates applying an uncorrelated lognormal clock model with 3rd CPs excluded; pink triangles with random local clocks and 3rd CPs included, black dots with random local clocks and 3rd CPs excluded. The key node linking both sides of the Atlantic in the phylogeny reported in [2] is highlighted with a grey box.
1. Exclusion of Third Codon Positions and Estimation of Divergence Times

Phillips et al. [S1] criticize that the 3rd codon positions (CPs) of the mitochondrial protein-coding genes are highly saturated in our phylogeny, and that this resulted in an underestimation of deep divergences relative to shallow divergences. To further explore this we have performed a reanalysis run in BEAST v1.7.2 [S2] using our original dataset and assumptions [S3] but excluding 3rd CPs, to test if their exclusion influences the estimation of divergence times. Following reanalysis, estimates do not differ substantially (Fig. 1), with the exclusion of 3rd CPs generating a divergence time of 72.3 mya (50.5-97.3 95% highest posterior distribution, HPD) for the node of importance. On the other hand, saturation of 3rd CPs does become a more serious issue when deeply divergent amphipod outgroups are included, as in the case for the analyses of Phillips et al. [S1].
3. Calibration Issues and Outgroups

In addition to the sequences of the two amphipods we obtained (*Pseudoniphargus daviui* and *Bahadzia jaraguensis*), Phillips et al. add other non-metacrangonyctid amphipod outgroups to our original alignment, namely Genbank mitochondrial sequences from *Caprella mutica* and *C. scaura* [S1]. They also include sequences of two isopods (*Armadillidium vulgare* and *Ligia oceanica*), two decapods (*Penaeus monodon* and *Scylla tranquebarica*) and a hoplocarid (*Squilla mantis*). They then apply an inferred date of separation of the Subclasses Eumalacostraca (to which the Amphipoda, Isopoda and Decapoda, among others, belong) and Hoplocarida derived from fossil information to calibrate their tree.

We argue that the use of the divergence between these two crustacean Subclasses as a calibration point, based on fossil dating, largely drives the discrepancy between their divergence date estimate and ours. We consider that such calibrations may be biased due to the inappropriate placing of the Phreatoicidean fossil (see main text and Fig. S1). We have commented already in the main section on the condition of *Hesslerella*, a Palaeozoic fossil isopod assigned to the suborder Phreatoicidea [S5]. The fact that it is not included in any living family does not invalidate its phreatoicidean condition, and we do not find any basis in the literature to consider it as a "stem isopod" as Phillips et al. do in their reply (see [S6]). In addition, recent mitogenomic data certainly supports Phreatoicidea at a basal position within the Isopoda [S7]. Surprisingly, Phillips et al. did not include in their analyses the mitogenome of *Eophreatoicus*, a member of the Phreatoicidea and thus of the lineage of isopods with the oldest fossil record [S6-S8]. We have perfomed a preliminary phylogenetic analysis including *Eophreatoicus* plus all isopodan and amphipodan mitogenomes available in nucleotide databases. In this analysis, we have used the node leading to *Eophreatoicus* (assigning it the age of *Hesslerella*) to calibrate the tree. This analysis produced age estimates for the metacrangonyctids that were compatible with our initial calculations based on palaeogeographical considerations.

Both hoplocaridans and eucarids would represent in principle useful taxa to provide deep calibrations given their old fossil record and high number of mitogenomes available. We think, however, that the inclusion of more distant outgroups other than isopods (the only peracarids aside
amphipods for which mitogenomes are currently available), could produce considerable overestimations in deep-age calibrations and these are by no means "directly scalable" as Phillips et al. assume in their reply. Peracarids display rather high mitochondrial nucleotide substitution rates compared to both hoplocaridans and eucarids (Decapoda+Euphausiacea) [S9], and consequently long tree branches. This has been related to the high number of gene rearrangements affecting the mitogenome of peracarids such as changes in strand bias, replication origins and inversion of genes [S7]. This makes mitogenome-based deep phylogenies of Crustacea unreliable, and particularly for estimation of divergence times.

Most of the available amphipod fossils preserved in Eocene Baltic amber dated at 54-40 million years (my) can be assigned to modern genera within the freshwater families Niphargidae (Niphargus) and Crangonyctidae (Synurella) [S10], a feature that lends credence to the extreme persistence (i.e., at geological time scales) of Metacrangonyx lineages. Nevertheless, since the age of the known amphipod fossils does not extend to deeper calibration times, we used two relatively recent palaeogeographic events that affected the Moroccan High-Atlas (37.2–25.0 mya) and the Balearic Islands in the Mediterranean (16–5.5 mya) to calibrate our tree [S3]. Using these calibrators we obtained an average long-term pairwise sequence divergence of 10.9% per million years for Metacrangonyx [S3], a rate that would increase by at least 4-fold assuming the fossil calibration proposed by Phillips et al. [S1].

References


**Figure S1. Pancrustacean phylogenetic tree.**

Simplified tree depicting the major Crustacean relationships and the relevant clades mentioned in the text. Also shown are the phylogenetic position of Hesslerella according to Phillips et al. and according to more accurate taxonomic interpretations.
Hesslerella fossil new placement

Hesslerella fossil by Phillips et al.
Hesslerella fossil new placement

Hesslerella fossil by Phillips et al