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Treasa Creavin

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Please find attached the response your suggested changes. They were valuable and we have included virtually all of them. We have also, as you suggest, modified the figure. We have also removed the reference to correspondence with Alexei Drummond and thanked him in the acknowledgements. Alexei has indicated to us his agreement to do this.

The authors hope this ms is now suitable for publication in *Trends in Genetics*.

Yours (on their behalf)

Prof David Lambert

Molecular and morphological evolution in tuatara are decoupled

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In the 1970s Marie-Claire King and Allan Wilson [1] suggested that macromolecules and anatomical and behavioral features of organisms evolve at independent rates. Before then, the common view was that rates of molecular and morphological evolution are positively correlated. In accordance with this traditional view, Miller *et al.* [2] question our finding that tuatara (*Sphenodon*, Reptilia) exhibit a rapid rate of molecular evolution [3] since *Sphenodon* are a relict taxon with little skeletal change from Cretaceous relatives. Miller *et al.* [2] raise a number of points that we address here.

First, they [2] argue that our conclusion is solely based on the point estimate of the tuatara molecular rate and ignores the associated 95% Highest Probability Density (HPD). The point estimate we obtained for tuatara (1.56 substitutions/site/million years (s/s/My), HPD 0.83-2.34) is the highest recorded for vertebrate animals. However, we did not ignore the HPD; we explicitly stated that the tuatara rate is not significantly different from the high rates recorded for penguins, aurochs and moa [3].

Miller *et al.* [2] also suggested that our estimate of the evolutionary rate of the hypervariable regions (HVRs) of the tuatara mitochondrial (mt) genome is “implausibly high”, compared with estimates from pedigree analyses and mutation accumulation lines. In fact these methods result in rates as high as 2.5 and 8.9 s/s/My respectively [4-5] and these are ~60% and six fold greater than what we estimated for

tuatara using time serial samples. Furthermore Miller *et al.* [2] calculated a tuatara “mutation rate” by simply dividing our evolutionary rate by a generation time estimate. Such a modified evolutionary rate is not comparable to an actual mutation rate obtained using pedigree methods or mutation accumulation lines, because they estimate different parameters in different ways.

They also suggest [2] that the high molecular rate recorded in our study [3] might be due to what they argue is the low genetic variability (~2%) of our tuatara dataset. This claim is also incorrect. The nucleotide diversity of the nine other species compared in our study ranged from 0.2% to 6.2% (Figure 1a). Obviously, the diversity of our tuatara dataset is well within this range and the nucleotide diversity of only three species (horse, bison and brown bear) is significantly greater than that of tuatara ($P > 0.05$) (Figure 1a). Moreover, contrary to Miller *et al.*’s [2] hypothesis, the evolutionary rates for these three species with high diversity are significantly slower (< 0.4 s/s/My) than that estimated for tuatara (1.56 s/s/My) [3]. Therefore the claim that low nucleotide diversity will upwardly bias our Bayesian analysis of molecular rates is unfounded.

Although Miller *et al.* [2] suggest that there is no evolutionary signal in the dataset we presented, they nevertheless analyzed a subset of our original data and then claimed that their rate estimate is informative. By analyzing just the ancient samples, Miller *et al.* [2] estimated a much lower molecular rate (0.076 s/s/My, HPD 0.0016 – 0.32) for tuatara. This rate has very large confidence intervals close to zero and the mean is 47 times that of the lower HPD that actually suggests a lack of signal due to small sample size. Furthermore, they performed three randomization tests by interchanging the ages within the ancient samples. These tests resulted in rate estimates that are slightly less than, but not significantly different from ours, which led them to conclude that the tuatara dataset does not contain sufficient information to estimate a valid rate of evolution. However, Miller *et al.* [2] did not include the modern samples in their randomization process. The latter constitute 55% (41/74 sequences) of the dataset. Because their approach retained 55% of the signal, it is not surprising that the resulting rate estimates are not different from that estimated using the true ages. We repeated their analysis with a complete randomization protocol that randomized the ages of all sequences, (ancient and modern), and we then estimated the evolutionary

1 rate using BEAST [6]. The evolutionary rates for each of our 20 randomized datasets
2 were much lower than the rate of 1.56 s/s/My reported by us (Figure 1b). The results
3 of our reanalysis are precisely what would be expected from randomizing the ages of
4 all samples, when there is signal in the data.

5
6 We also conducted a simulation study to test if the results presented by Miller *et al.*
7 [2] are derived from a methodological artifact due to ignoring the modern sequences
8 (see supplementary material for further details). When we randomized the dates of
9 ancient simulated sequences only, we obtained similar results to those reported by
10 Miller *et al.* (Figure S1a) [2]. In contrast, randomizing the ages of modern and
11 ancient simulated sequences (Figure S1b) gave similar results to those shown in
12 Figure 1b, again indicating strong support for our original estimate [3].

13
14 Miller *et al.* [2] also suggested that tuatara populations are highly structured
15 genetically and that this structure will have contributed to the rapid evolutionary rate
16 we recorded. They claim that mainland (ancient) and offshore island (modern)
17 populations of tuatara are genetically differentiated. However, their claim was based
18 on patterns of geographic structure of tuatara from microsatellite DNA [7, 8]. This
19 inference is invalid because our study is about rates of evolution of mitochondrial
20 DNA. Microsatellites typically show much more variation and reflect a different time
21 and geographic scale to that of mitochondrial DNA [9]. Moreover, median joining
22 network analyses using NETWORK 4.5 (<http://www.fluxus-engineering.com>) show
23 that mtDNA sequences of tuatara from different island populations intermingle.
24 Furthermore, our modern and ancient samples although geographically distinct, are
25 not genetically discrete and their mtDNA sequences also intermingle in a
26 phylogenetic network (Figure 1c).

27
28 Finally, Miller *et al.* [2] noted that our Bayesian methods assume either a constant
29 population size or exponential growth, whereas tuatara populations have declined
30 substantially since humans arrived in New Zealand approximately 730 years ago [10].
31 However, all but three of the bones used in our original study [3] predate this decline.
32 Therefore, the recent demographic history of tuatara cannot explain the high
33 molecular rate we estimated.

1 It should be noted however that currently available methods for evolutionary rate
2 estimation, including BEAST [6], are based on simple models of evolution.
3 Therefore future rate estimates that take into account parameters such as DNA
4 damage, migration, bottlenecks and population subdivision will influence the point
5 rate estimates of all the vertebrates. However the high rate estimate we reported for
6 tuatara [3] is unlikely to change. Hence, despite its morphological stability, it is clear
7 that tuatara evolve quickly at the level of neutral genetic variation, when compared to
8 other vertebrates. This is not surprising because the biological processes underlying
9 DNA sequence evolution, and those that govern changes in morphology are very
10 different. When it comes to tuatara, Allan Wilson and his coworkers would no doubt
11 be pleased.

12 **Acknowledgements**

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15 University and the Allan Wilson Centre for Molecular Ecology and Evolution for financial
16 support. We are grateful to Simon Ho for providing the alignments of HVR sequences and to
17 Alexei Drummond for helpful discussion.

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Figure 1 Nucleotide diversity in tuatara. **1a.** Nucleotide diversities of bison, brown bear and horse are significantly higher than that of tuatara (* $P < 0.05$ and ** $P < 0.01$). **1b.** Evolutionary rates of the mitochondrial HVR regions of tuatara estimated using serial samples. The original rate estimated by Hay *et al.* [3] is shown. White dot represents the point estimate and the 95% HPD intervals are shown. The results from 20 replicates in which the ages of all samples were randomized, are indicated. Black dots represent the point estimates and the 95% HPD are also given. The geographic distributions of modern and ancient samples used in this study are shown. **1c.** Median joining network of tuatara haplotypes is shown. Ancient sequences are indicated in red, modern in yellow and hypothetical haplotypes in black. Branch lengths approximate the number of nucleotide differences between haplotypes and the sizes of circles are proportional to the number of samples.

Figure 1a,b,c
[Click here to download high resolution image](#)

(a)

(b)

(c)

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