Ancient DNA and the tropics: a rodent’s tale

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Most genetic studies of Holocene fauna have been performed with ancient samples from dry and cold regions, in which preservation of fossils is facilitated and molecular damage is reduced. Ancient DNA work from tropical regions has been precluded owing to factors that limit DNA preservation (e.g. temperature, hydrolytic damage). We analysed ancient DNA from rodent jawbones identified as Ototylomys phyllotis, found in Holocene and Late Pleistocene stratigraphic layers from Loltún, a humid tropical cave located in the Yucatan peninsula. We extracted DNA and amplified six short overlapping fragments of the cytochrome b gene, totalling 666 bp, which represents an unprecedented success considering tropical ancient DNA samples. We performed genetic, phylogenetic and divergence time analyses, combining sequences from ancient and modern O. phyllotis, in order to assess the ancestry of the Loltún samples. Results show that all ancient samples fall into a unique clade that diverged prior to the divergence of the modern O. phyllotis, supporting it as a distinct Pleistocene form of the Ototylomys genus. Hence, this rodent’s tale suggests that the sister group to modern O. phyllotis arose during the Miocene–Pliocene, diversified during the Pleistocene and went extinct in the Holocene.

1. Introduction

The Loltún cave is a geological formation located over a karst region on the Yucatan peninsula (figure 1a), which consists of a series of interconnected limestone chambers and tunnels and primarily karst terrains that allow the concentration of vertebrate remains (see the electronic supplementary material for a description). The quantity and quality of bone remains from Quaternary fauna preserved in this neotropical cave make it an extraordinary fossil reservoir. It includes approximately 4000 fossil and subfossils, predominantly mammalian, with neartic and neotropical affinities, and a faunal succession spanning from the Late Pleistocene to the Holocene [1,2]. Nearly 11% of the mammalian fossils from Loltún belong to the order Rodentia, including the monotypic species Ototylomys phyllotis. Identification was carried out by comparing the ancient molars and mandibles with those of extant rodents from the region [2]. A recent phylogeographic study shows that O. phyllotis has three divergent lineages throughout its geographical distribution, with haplotypes from Central America, Chiapas and Guatemala highlands and Yucatan peninsula (figure 1a), and that the origin of the genus was likely earlier than 3.35 Ma [3]. Although the historical affinity of the fossil samples preserved in Loltún is unknown, based on the extant O. phyllotis phylogeography, a plausible hypothesis is that they are related to the Yucatan peninsula lineage.
Figure 1. Results for cytochrome b sequence analyses of ancient and modern O. phyllotis samples. (a) Major lineages [3], where YP, Yucatan peninsula; CGH, Chiapas and Guatemala highlands; CAN, Central America nucleus and CR, Costa Rica. (b) Bayesian tree showing the haplotype groups recovered: three major lineages (as in a) and the LC. Numbers above the diagonal are posterior probabilities/aLRT-SH (values > 0.70); below are range of time of divergence in million years. (c) Haplotype network: circle size is proportional to haplotype frequency (numbers in parenthesis), short lines are mutational steps and diagram within circles corresponds to stratigraphic layers. (d) Stratigraphic schematic of El Túnel ([2]; figure modified from R. Velazquez), layers are organized from recent to oldest (1–8); m, metres. (Online version in colour.)

Comparing ancient and modern DNA sequences using molecular techniques is a framework that has been used to decipher the population origin of species or lineages [4,5]. However, the DNA of most ancient samples is heavily degraded and its preservation depends deeply on different processes that take place after cellular death, including availability of water and changes to the environmental conditions in which bone or soft tissue preservation occurs [6]. The high diversity and abundance of faunal remains from Loltún suggest that no drastic environmental changes have occurred since the Late Pleistocene. Historical humidity fluctuations have changed the microclimate around and inside the cave (e.g. turning tropical deciduous forest into grassland) sufficiently to allow for the presence of the noted diverse taxa [1]. Thus, while humidity has limited DNA preservation in tropical localities [7], the exceptional preservation of fossils inside Loltún was likely facilitated by the microclimate within the cave and by the nature of the karst terrains. Based on the preservation of our fossils and the value of ancient DNA to compare historical patterns of diversification [8], we aimed to address two objectives (i) to confirm the authenticity of ancient DNA in this tropical setting, and (ii) to assess their phylogenetic ancestry and relation with the known phylogeographic history of the species.

2. Material and methods

We selected 28 O. phyllotis hemimandibles and processed them following strict standards for ancient DNA recovery. These samples were found in several stratigraphic layers of two excavation units inside Loltún: El Túnel (layers 1–7) and El Toro (12 and 13). The jawbones were broken into three parts as described in the electronic supplementary material, figure S1, and approximately 30–70 mg were used for DNA extraction. DNA recovery was performed following Schwarz et al. [9]. We designed species-specific primers and included those used by [3] to amplify six overlapping fragments of ~100 bp each (electronic supplementary material, table S1). We included sequences of modern Ototylomys and two Tylomyini sister species (Nyctomys and Ototylomys) used as outgroups to reconstruct a phylogeny with Bayesian and Maximum-likelihood methods, using Beast v. 1.7.5 [10] and PhyML v. 3.1 [11], respectively. Time of divergence for major clades was estimated with Beast. We also estimated statistics of genetic diversity and demographic history, constructed a haplotype network and performed a Bayesian skyline analysis [12] to infer past population size changes. All details for ancient DNA extraction, primers, PCR amplification, methods and parameters for genetic, phylogenetic and demographic analyses are described in detail in the electronic supplementary material.

3. Results

Cytochrome b amplicons were successfully obtained for 16 jawbones (of the 28 selected samples from Loltún, an amplification success rate of 57%; electronic supplementary material, tables S4 and S5). A total of 666 bp was reconstructed for 12 samples, based on at least two of three independent amplicons for each 100 bp fragment (see the electronic supplementary material for details). This DNA fragment represents an unprecedented success for an ancient DNA sample from a tropical cave [7], considering comparable sequences come from temperate regions [13]. The Bayesian and ML trees (figure 1b and the electronic supplementary material, figure S2, respectively) showed a concordant topology, with high support for the O. phyllotis major clades and for a unique clade that includes all ancient samples (Loltún clade, LC) that differentiated from the other lineages (posterior probability = 1, aLRT-SH = 1). Surprisingly, LC diverged long before the modern O. phyllotis and after the Tylomyini sister species (16.46–9.54 Ma), whereas the divergence within the LC haplotypes occurred much later.
signal: negative Fu’sistics are in agreement with a recent population expansion indicates a recent diversification (figure 1a star-like shape and no more than three mutational steps, which layer. The latter is also evident in the haplotype network that has (0.1–0.5%; electronic supplementary material, table S3), indicatin-
other hand, genetic differentiation between layers was low (0.1–0.5%; electronic supplementary material, table S3), indicating a continuity between samples within the LC, irrespective of layer. The latter is also evident in the haplotype network that has a star-like shape and no more than three mutational steps, which indicates a recent diversification (figure 1c). Demographic statistics are in agreement with a recent population expansion signal: negative Fu’s $F_s$ index ($F_s = -0.620$) and low $R^2$ and raggedness indices ($R^2 = 0.12; \tau = 0.20$). The latter is supported by the Bayesian skyline plot, which shows a constant growth from 1.99 Ma and an effective population size ($N_e$) of less than 100 that decreases towards the present (electronic supplementary material, figure S3).

4. Discussion
Reed et al. [7] and studies cited therein suggest that tropical humid caves that are located within 30° of the Equator, at low elevations, or with high temperature and humidity, are unlikely to preserve DNA over millennia. The fact that we have successfully extracted and authenticated ancient DNA (amplicons of 100 bp fragments—totalling 666 bp—of the cytochrome $b$ gene) from jawbones preserved in Loltún cave, reiterates the fact that general rules on DNA preservation are limited in use. Understanding this preservation requires a more systematic review of the environmental context at this site. First, it has been recognized that dry and cold places, together with a rapid mineralization, allows DNA to persist longer and in fragments with adequate size to be amplified [14]. Faunal records from Loltún include species with nearctic and neotropical affinity in its older layers (Pleistocene, i.e. Canis dirus, Desmodus cf. D. draculæ) [11], which suggests that the Yucatan peninsula experienced a dry and cold period; subsequently, during the Holocene, the penin-
sula experienced several droughts that gradually gave way to the present-day humid conditions [15], all of which may have played a role in facilitating DNA preservation.

Taking into consideration factors regarding interpretation of results when working with ancient DNA (e.g. post-mortem damage and high substitution rate effects [16,17]), our study shows that the jawbones from Loltún, identified as $O$. phyllotis, are in fact a genetically distinctive clade that is basal to all $O$. phyllotis lineages. Despite molar similarities, Alvarez [2] reported a slight increase in size of these jaws, which became more pronounced in the deeper stratigraphic layers (12 and 13), and suggested that they could belong to a Pleistocene form of the Ototylomys genus. Our results support the latter: genetic distances estimated between the cave samples and modern $O$. phyllotis are extremely high, even falling in the range of genus-level distances. Ancient DNA studies have shown that the Holocene was characterized by cladogenesis, large-scale extinctions, migrations and changes in population size and con-
ectivity, resulting in the formation of new species and cryptic populations and also in the subsequent and historically more recent rapid loss of faunal diversity [18–20]. Our results are con-
gruent with these, where this rodent’s tale suggests that the sister group of modern $O$. phyllotis likely arose during the Miocene–Pliocene, diversified during the Pleistocene and went extinct in the Holocene; the demographic signal observed showing a con-
stant growth (2–0.5 Ma) followed by a population decrease near the present, highlights the extinction of this Pleistocene rodent, whose remains were fortunately preserved in the Loltún cave.

Acknowledgements. We gratefully acknowledge the Consejo de Arqueo-
logía, INAH for granting permission for destructive analysis, project support and fossil samples, and three anonymous reviewers.

Funding statement. T.A.G.G. acknowledges scholarships from CONA-
CyT (175434) and travel support from PAEP, UNAM, and thanks McMaster University’s support while conducting scientific visits. E.V.D. is grateful for financial support from CONACyT (101861).

References


Correction to ‘Ancient DNA and the tropics: a rodent’s tale’

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Biol. Lett. 10, 20140224. (2014; Published online 4 June 2014) (doi:10.1098/rsbl.2014.0224)

The present erratum is in regards to our article entitled ‘Ancient DNA and the tropics: a rodent’s tale’. We were made aware of problems with some of the ancient sequences submitted to GenBank and conducted a systematic review of all the files used in our study. We discovered that, unfortunately, an incorrect file was sent to GenBank and was also used in some of our downstream analyses. We immediately contacted GenBank, explained the situation and corrected the file. We have redone some analyses with the correct file and describe these changes below.

The following analyses were performed with the same parameters as described in the article. The new estimated best-fit substitution model under the Akaike Information Criterion (AIC) was HKY + I + G (A = 0.3298, C = 0.3080, G = 0.0981 and T = 0.2641; I = 0.2970 and γ = 0.7880). The ML (data not shown) and Bayesian trees (figure 1a) showed a concordant topology, with high support for the O. phyllotis major clades. All ancient samples were grouped within the Yucatan peninsula lineage (posterior probability = 0.82, aLRT-SH = 97). Divergence times remain the same for the complete phylogeny (figure 1a); the new results show that the divergence of the ancient haplotype basal to the Yucatan lineage occurred within the Pliocene (from 5 Ma onwards) and that of the rest of the ancient haplotypes occurred much later, coinciding with the diversification of modern haplotypes (1.9–0.33 Ma).

We obtained nine ancient haplotypes (figure 1b; accession numbers KJ751487–KJ751498). The entire ancient dataset did not deviate from neutrality (p > 0.10) (Tajima’s D = −1.388; Fu and Li’s D = −1.341; F = −1.542) and showed high haplotype (h = 0.939 ± 0.058) and low nucleotide diversity (π = 0.0093 ± 0.0018). Genetic differentiation between stratigraphic layers was low (0.1–0.5%), indicating continuity between samples within the Loltun ancient samples irrespective of layer. The haplotype network was constructed for the Yucatan peninsula lineage in accord with the estimated phylogenetic trees, given that the ancient set is no longer a unique clade and the ancient sequences are within this lineage. The resulting haplotype network shows that, with one exception (5AH), the ancient samples are not dispersed throughout the modern haplotypes. Instead, they are joined in a central star-like shape, with no more than nine mutational steps between them. The ancient set connects with other haplotypes from the Yucatan peninsula that are mostly located basal to subclades with recent diversifications (figure 1a,b).

To infer past population size changes, demographic indices and a Bayesian Skyline Plot were calculated for the Yucatan peninsula lineage. The skyline plot results remained consistent (relatively constant growth from 1.99 Ma and an Ne of less than 100 that decreases towards the present). Demographic statistics are in agreement with a recent population expansion signal: negative Fu’s Fs index (−30.083) and low R2 and raggedness indices (R2 = 0.0321; r = 0.0059). This historical demographic pattern was also confirmed by the neutrality test results, in which this lineage shows a signal of recent expansion indicated by significant (p < 0.02) and negative values of Tajima’s D and Fu’s D and F (D = −2.267, D = −5.466 and F = −4.949, respectively).

Importantly, despite the corrections to our sequences as well as the new analyses we performed, two of our three main results did not change: (i) the DNA signal is indeed real and tropical caves can be a good source of ancient DNA
regardless of theoretical predictions for these environmental conditions. We have successfully extracted DNA and amplified a fragment significantly longer than other reported from Quaternary rodents (666 bp for 12 samples). (ii) Genetic information obtained from unique fossil samples can reveal relationships that could not be assessed by morphology due to the limited availability of complete fossil samples. We were able to assess the phylogenetic ancestry of the ancient samples, in which our study shows that the jawbones from Loltun, identified as *O. phyllotis*, belong in fact to this species.

Our conclusion in the paper that the ancient samples formed a distinctive ancestral clade to modern *O. phyllotis* lineages is no longer valid. The corrected results show that the ancient samples fall within the lineage from the Yucatan peninsula. This result is consistent with our original hypothesis, which stated that the ancient samples were likely to be related to the Yucatan peninsula lineage. Our results remain congruent with the main processes of the Holocene, in that ancient and modern examples of *O. phyllotis* arose during the Miocene–Pliocene and diversified during the Pleistocene. To date we have not found any ancient haplotypes within extant populations, suggesting drift (extinction of ancient haplotypes) during the Holocene. This argues for remarkable genetic continuity within the Yucatan lineage over at least the last 500,000 years. Further genetic analyses on the well preserved remains from Loltun cave will allow for a more refined demographic analysis in the near future.

Figure 1. Results for cytochrome *b* sequence analyses of ancient and modern *O. phyllotis* samples. (a) BEAST tree showing the haplotype groups recovered: three major lineages (as in the manuscript), where blue (bottom), pink (middle) and green (top) bars are Central America Nucleus, Chiapas-Guatemala Highlands and Yucatan peninsula lineages, respectively; black lines indicate the nine ancient haplotypes. Numbers above the diagonal are aLRT-SH/posterior probabilities (values > 0.80); below are range of time of divergence in million years. (b) Haplotype network for the Yucatan peninsula lineage: circle size is proportional to haplotype frequency (numbers in circles), ancient haplotypes are indicated in red (shadowed areas). (c) Stratigraphic schematics of El Túnel; layers are organized from recent to oldest (1–6). (Online version in colour.)