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Weighing the mass spectrometric evidence for authentic *Tyrannosaurus rex* collagen

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Abstract

We use authentication tests developed for ancient DNA to evaluate claims by Asara *et al.* of collagen peptide sequences recovered from mastodon and *Tyrannosaurus rex* fossils. Although the mastodon passes, absence of amino acid composition data, lack of evidence for peptide deamidation, and association of the α1(I) peptide sequences with amphibians not birds, suggests that *T. rex* does not.

Comment

Early reports of DNA preservation in multi-million year old bones (i.e. dinosaurs) have been largely dismissed (see 1 and SOM T1, 2) but reports of protein recovery are persistent (see 3 for review). Most of these studies used secondary methods of detection, but protein sequence, arguably the gold standard for molecular palaeontology, has now been claimed for the first time (2). Following initial optimism generated by reports of dinosaur DNA, there arose a gradual awareness of the problems and pitfalls which bedevil analysis of ancient samples (1), leading to a series of recommendations for future analysis (1, 4). As yet, there are no equivalent standards for fossil protein, so here we apply the recommended tests for DNA (4) to the authentication of the reported protein sequences (2) (Table 1).

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Likelihood of collagen survival

The extremely hierarchical structure of collagen results in unusual, catastrophic degradation (5) as a consequence of fibril collapse. The rate of collagen degradation in bone is slow because the mineral 'locks' the components of the matrix together, preventing helical expansion which is a pre-requisite of fibril collapse (6). The packing which stabilises collagen fibrils (6) also increases the temperature sensitivity of degradation (E_a 173 kJ mol^{-1}; Fig. 1). Collagen decomposition would be much faster in the *T. rex* buried in the then megathermal (>20 °C) (7) environment of the Hell Creek formation (collagen $t_{½}$ ~ 2 ka) than it would have been in the mastodon lying within the Doeden Gravel Beds (present day mean temperature 7.5 °C; collagen $t_{½}$ 130 ka; Fig. 1).

Risk of contamination

The molecular target (collagen) is ideal for this investigation; the protein has a highly characteristic motif and yet also is sufficiently variable to enable meaningful comparison between distant taxa if sufficient sequence is obtained (Fig. 2). In comparison with ancient DNA amplification, contamination by collagen is inherently less likely. Furthermore because the bones were excavated by the authors, obvious contamination sources such as animal glue (used in conservation) can be excluded. Concentrating protein from the large amounts of bone used (2.5 g) may have heightened the risk of extraneous proteins entering the sample during extraction, but there have been no systematic studies of this phenomenon. Independent extraction and analyses would have strengthened claims for the authenticity of the origin of the peptides (and potentially ameliorated the original problems of data interpretation) (4).

The remarkable soft-tissue preservation of the investigated *T. rex* specimen (MOR 1125) has been documented (8); however microscopic preservation does not equal molecular preservation (9). Immunohistochemistry provides support for collagen preservation, although no data regarding inhibition assays with collagen from different species or cross-reactivity with likely contaminants (e.g. fungi 10) are presented. Curiously no amino acid compositional analysis was conducted (c.f. 11) although immonium ions were identified (by TOF-SIMS). In our experience, collagen-like amino acid profiles have been obtained in all bones from which we could obtain collagen sequence (Fig. 1, inset).

Proof of sequence authenticity

The spectra (12) (SOM of 12) are inconsistent with many of the original sequence assignments (13, 14) (SOM Table 1 and 13). A common diagenetic modification, deamidation, not considered in the original publication, may shed light on authenticity. The facile succinimide mediated deamidation of $N_{1156}G$ (14) occurred in peptide Ost 5 (see Table 1 of 13 for nomenclature) from ostrich, presumably during sample preparation. Direct hydrolytic deamidation is slower (14) and an expectation of elevated levels of such products is reasonable for old samples. We agree with the new interpretation (13) of the spectrum illustrated in Fig. 2b of 3 as α_1(I) G_{362}SEGPEGVR$_{370}$, the deamidated (Q→E_{367}) form of the sequence found in most mammals (12). By way of contrast, none of the three glutamine residues in "*T. rex*" peptides are deamidated (Table S1 SOM). Only time will tell if Q→E is a useful marker for authentically old collagen, but from the evidence presented the mastodon sequence looks *more* diagenetically altered than *T. rex*.

The unusual, fragmented nature of the reported *T. rex* sequence does not make it amenable to standard, model-based phylogenetic analysis. Instead, we examined the phylogenetic signal of the α1(I) fragments of mastodon and *T. rex* using Neighbor-Net analysis and uncorrected genetic distances. Using the originally reported sequences (2), both the *T. rex* and mastodon signal displayed an affinity with amphibians (Fig. 2a) (12). Our re-

interpretation of the spectra changes the affinity of mastodon but not of *T. rex* (Fig. 2b). Two other peptides are reported from *T. rex* (3); we question the interpretation of the (frog) α1(II) spectrum, but not the α2(I) assignment which *is* identical to chicken.

We require more data to be convinced of the authenticity of the *T. rex* collagen. Nevertheless, the handful of spectra reported for the temperate Pleistocene mastodon fail neither phylogenetic nor diagenetic tests, highlighting the potential of protein mass spectrometry to bridge the present gulf in our understanding between the fate of archaeological and fossil proteins (Fig 1.). In order to avoid past mistakes of ancient DNA research(1), we would recommend that future fossil protein claims are considered in the light of tests for authenticity such as those we have used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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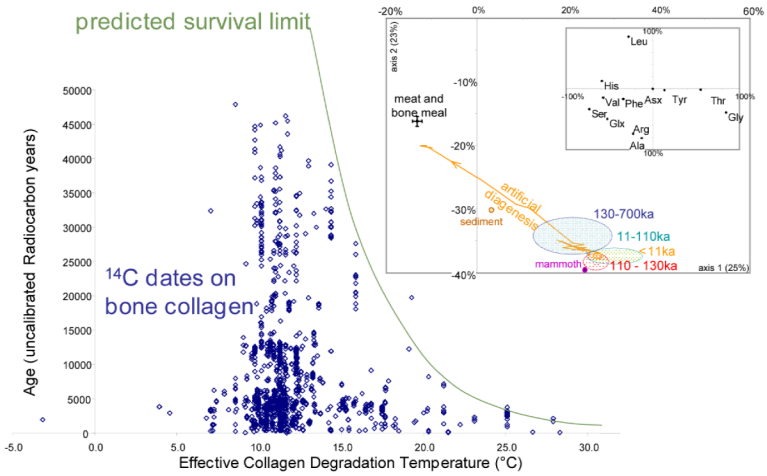

Figure 1.

Plot of radiocarbon age versus estimated effective collagen degradation temperature for radiocarbon dated bones from laboratory databases (principally Oxford and Groningen). The line represents the expected calendar age at which 1% of the original collagen remains following a zero-order reaction; almost no bone collagen survives beyond this predicted limit. **Inset**. 99% confidence intervals of amino acid compositions by first two principal component analyses (48% of total variance) for < 11 ka (n = 324), 11-110 ka (n = 210), 110-130 ka (n = 26) and 130-700 ka (n = 31) bones from NW Europe. Pliocene samples are not plotted, as their composition (n = 8) is highly variable and yields of amino acids are low. The orange line indicates a compositional trend observed when compact bone is heated for 32 days at 95 °C, which reduces collagen to 1% of initial concentration, each inflection representing a separate analysis (n = 32). The composition becomes more similar to mixed tissues samples (meat and bone meal, n = 32) principally due to the depletion of Gly. An amino acid profile for mammoth (m) is consistent with collagen, unlike the associated sediment sample (s) (data from 11).

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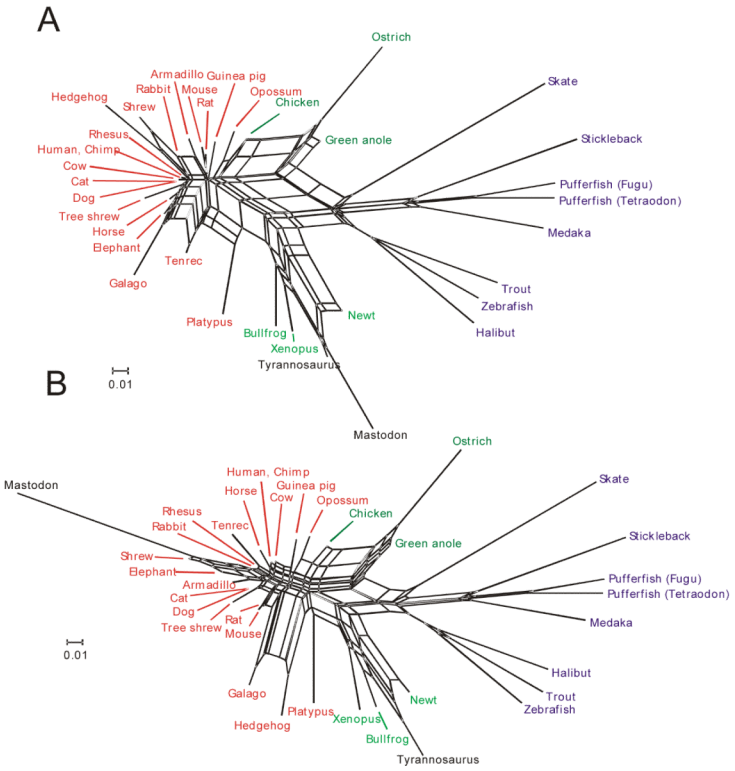

Figure. 2.

Phylogenetic networks of α1(I) sequences using Neighbor-Net analysis (**A**) with the original assignments (2) and (**B**) following reinterpretation of the mass spectrometric data (12). *T. rex* does not group with bird/reptile using either set of sequence alignments. More sequence is required for a full, model-based phylogenetic analysis

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Table 1

Key questions to ask about ancient biomolecular investigations (taken from 4)

Test	Sample	Pass	Observation
Does the age, environmental history and preservation of the sample suggest collagen survival?	Mastodon 300-600 ka	✓	Collagen $t_{½}$ @ 7.5 °C = 130 ka
	T. rex 65 Ma	✗	Collagen $t_{½}$ @ 20 °C = 2 ka
Does the biomolecular and/or macromolecular preservation of the sample, the molecular target, the innate nature of the sample and its handling history suggest that contamination is a risk?	Biomolecular preservation	?	Range of evidence presented (8) but no amino acid compositional data.
	Macromolecular preservation	✓	...but macromolecular ≠ biomolecular preservation (9).
	Molecular target	✓	
	Handling history	✓	...but large (2.5 g) samples processed
Do the data suggest that the sequence is authentic, rather than the result of damage, and contamination?		✗	Errors in interpretation of spectra (see SOM Table 1 and 13)? Damage induced errors in sequence.
Do the results make sense, and are there enough data to make the study useful and/or support the conclusions?	Mastodon	✓	Weak affinity to mammals
	T. rex	✗	Affinity of α1(I) peptides amphibians, not birds or reptiles