Molecular Histology as a Novel Proxy for Ancient DNA and Protein Sequence Preservation

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Abstract

Researcher ability to accurately screen fossil and sub-fossil specimens for preservation of DNA and protein sequences remains limited. Thermal exposure and geologic age are usable proxies for sequence preservation on a broad scale but are of limited use for specimens of similar depositional environments and/or ages. Cell and tissue molecular histology is thus proposed as a proxy for determining sequence preservation potential of ancient specimens with improved accuracy. Molecular histology as a proxy is hypothesized to elucidate why fossil/sub-fossils of some depositional environments and or geologic ages preserve sequences while others do not and to facilitate selection of ancient specimens for use in molecular studies.

Keywords:

Molecular histology; ancient DNA; paleogenomics; paleoproteomics; fossil; sub-fossil

Introduction

Reports on the successful recovery of biomolecules from vertebrate fossils and sub-fossils ("subfossil" defined as being not fully fossilized) have increased exponentially over the prior 2 decades. Evidence supports the persistence of DNA sequences past 1Ma[1], and protein sequences have been reported to preserve into the Pliocene epoch (~3.5Ma) with minimal controversy[2-4]. Predictive and empirical studies have established that the potential of sequence data to persist into deep time depends substantially on thermal setting[3, 5-7] as well as the type of biological material examined; bone, dentine, enamel, or eggshell[3, 5, 8]. These advances in DNA and protein sequence recovery have expanded the opportunity for palaeoecological and paleoenvironmental studies to be conducted over a broader range of taxa and geological timepoints. Data reported from such studies are used to inform on and set conservation policy for extant wildlife populations that are of commercial interest, at risk of extinction, potentially invasive, at risk of low genetic diversity, etc.[9-12].

Despite these advances, the ability to predict which ancient specimens are likely to preserve molecular sequences still remains limited. A prevailing view within the primary literature is that specimens exposed to prolonged, elevated thermal conditions are less likely to preserve proteins and DNA[3, 5, 6, 13]. As an example, permafrost settings and late Pleistocene geologic timepoints have been shown favorable for the preservation of molecular sequences[5, 6, 8, 14, 15]. Such findings have supported the use of specimen thermal history and geologic age as proxies for molecular sequence preservation[3, 5, 6, 13]. However, this observation only holds in a general sense and to a relative degree. Other variables affecting protein and DNA preservation include sediment composition[16-20], moisture content[17-21], and oxygen content[17-23], among others. Consequently, multiple studies have reported differing degrees of sequence preservation even for specimens sharing similar thermal histories and/or geologic ages[8, 13, 15, 24, 25]. In such situations, thermal setting and geologic age are rendered marginally effective as proxies. Indeed, the complexity of variables influencing molecular sequence preservation in vertebrate remains means that any single variable selected for use as a proxy for sequence preservation will inevitably have substantial limitations.

A potential solution to these limitations is to directly examine the molecular histology of preserved subfossil/fossil tissues and cells. Molecular histology is defined by Campbell and Pignatelli (2002) as "an explanation of the morphological characteristics of a tissue in terms of the molecules present and the functional interactions between them"[26]. Molecular histology is how an organism's molecular makeup is manifested as cells and tissues, and variables including thermal history, geologic age, sediment composition, and others all directly affect how this molecular makeup preserves[17, 20, 27]. Thus, the preserved state of a fossil/sub-fossil's molecular histology (which includes molecular sequences), is representative of the combined effect of these diagenetic variables upon its molecular sequences. Substantial precedence exists in the scientific literature for the preservation of remnant cells and tissues within ancient vertebrate specimens[22, 28-40]. Data characterizing the molecular histology of such remains is herein hypothesized to be usable as a proxy for molecular sequence preservation. Correlating molecular histology with degree of sequence preservation across specimens spanning the fossil record would advance the use of such a proxy.

Testing this hypothesis can be accomplished using a suite of molecular techniques capable of characterizing both the morphological[29, 32, 33, 35, 40, 41] and chemical[22, 23, 29, 31, 36, 42, 43] aspects of molecular histology. Such data can then be used to identify connections between a fossil's observed molecular histology and its degree of molecular sequence preservation. Additionally, changes in fossil/sub-fossil molecular histology can be tracked across various geologic ages and depositional environments to reveal potential insights as to the role diagenetic variables play in sequence preservation. Ultimately, the study of fossil and sub-fossil molecular histology will introduce novel and practical methods for screening whether a given specimen should be selected for molecular sequencing, and it will increase understanding of how geologic age, thermal history, and other diagenetic variables influence sequence preservation.

Molecular Histology as a Potential Proxy for Ancient Sequence Preservation

The number of specimens reported to preserve molecular sequences decreases substantially beyond geologic ages of ~0.13-0.24Ma and ~0.8-1.0Ma for DNA and proteins respectively[5, 8, 44-48]. This is excluding specimens of permafrost settings and some cave deposits as these settings often confer exceptional preservation potential for molecular sequences[5, 14, 49-53]. The decrease in reported sequences from specimens exceeding these timepoints suggests substantial diagenetic alteration occurs to fossil/sub-fossil biomolecules over these timeframes. The extent of this diagenetic alteration is such that in many cases molecular sequences are degraded beyond the limit of detection of commonly used sequencing protocols. Still, molecular sequences, particularly protein sequences, have been reported from a few non-cave/permafrost specimens with geologic ages exceeding these thresholds[2-4, 34, 54-57]. A study on a Pliocene camel tibia from Ellesmere Island, Yukon, Canada, for example, managed to recover type-1 collagen peptides[2, 4]; in addition to two Mesozoic dinosaur specimens[34, 55-57], these are the only pre-Pleistocene bones currently reported to harbor sequence-able proteins. Two other camels from Miocene and Pliocene formations of Nebraska were analyzed in the Ellesmere Island tibia study yet failed to yield detectable peptide sequences[2, 4]. This begs the question of why some specimens like the exceptional Ellesmere Island tibia preserve protein and/or DNA sequence information while many other pre/early and even mid-Pleistocene specimens do not.

The prevailing view in the paleogenomic and paleoproteomic literature would be that the greater thermal exposure of the temperate Nebraska specimens facilitated protein degradation relative to the Ellesmere Island tibia[3, 5, 6, 13]. A warmer thermal setting accelerates the rate of diagenetic reactions affecting molecular histology, including molecular sequences[3, 58]. Advanced geologic age expands the temporal period over which these reactions have to progress and accumulate[18, 58]. Hence, a lower geologic age along with a cooler thermal setting is hypothesized to inhibit the extent of such diagenetic reactions and limit molecular sequence degradation. This and the degree materials such as bone, dentine, enamel, eggshell, and others resist degradation[3, 5, 8, 59] are often cited as key variables explaining examples of exceptional sequence preservation.

Indeed, a fossil or sub-fossil's thermal setting/history and geological age are generally used as proxies for predicting sequence preservation potential[3, 5, 6, 13]. However, even ancient specimens from similar timepoints and depositional environments are known to display great variation in sequence preservation[8, 13, 15, 24, 25, 60, 61]. In a study of 118 Xenarthrans from temperate to tropical locales, 6 speci-

mens from the Santa Clara formation (˜8.5-128Ka) of Camet Norte, Buenos Aires, Argentina, were analyzed. Of these, 2 specimens out of 6 demonstrated substantial evidence of protein preservation[61]. In this case, geologic age and thermal setting would be rendered relatively inaccurate as proxies since all specimens came from the same formation and would be expected to share a similar thermal history, yet not all preserve sequence information to a similar degree. Furthermore, a 2017 study by Mackie et al. examined the dental calculus of 21 Roman-era H. sapiens specimens from 3 European burial sites using LC-MS/MS sequencing. Reported sequence preservation varied widely between specimens and was unattributable to any specific variables[60]. These differences in preservation likely result from other variables including differences in composition[16-20], moisture content[17-21], and oxygen[17-23]content of burial sediments, among others. The complex range of variables potentially affecting sequence preservation supports that factors beyond geologic age and thermal history are responsible for specimens demonstrating exceptional sequence preservation. This limits the usefulness of any single diagenetic variable, such as geologic age or thermal history, as a proxy for DNA and protein sequence preservation.

A proposed solution to this limitation is to directly use fossil/sub-fossil molecular histology as a proxy for molecular sequence preservation. Molecular histology is the underlying basis for why diagenetic variables such as thermal history and geologic age can be used as proxies, in any capacity, for predicting sequence preservation. The cumulative effects of diagenetic variables are reflected in the preservational condition of a fossil or sub-fossil's molecular histology[17, 20, 27]. Directly studying molecular histology and correlating it with degree of sequence preservation bypasses the need to study any one of these variables individually. Thus molecular histology is hypothesized to be usable as an accurate proxy for molecular sequence preservation. Yet little empirical research exists to this point that has observed how molecular histology of fossil and sub-fossil specimens varies with degree of sequence preservation.

Literature Examples Highlighting the Potential of Molecular Histology as a Proxy

A few previous studies have examined fossil/sub-fossil molecular histology in a manner that can be linked to preservation potential for molecular sequences. A discussion of some findings relevant to the correlation of molecular histology with degree of sequence preservation follows.

A 2007 study by Schweitzer et al. used light and electron microscopy to survey the molecular histology of bone specimens ranging from modern day through to the Triassic[33]. The study reported that the molecular histology (especially the "collagenous" matrix) of specimens with dates exceeding 100-300Ka was substantially altered relative to specimens of younger timepoints. Light microscopy was herein used to replicate and reevaluate reported data for three of the 2007 study specimens (data not shown), the *M. columbi* femur (MOR 501 (formerly MOR 91.72), ˜12Ka), the *M. columbi*skull fragment (MOR 604, ˜100-300Ka), and the *M. americanum* rib (MOR 605, ˜100-300Ka). This was done according to the same demineralization protocol reported by the 2007 study[33].

Collagenous matrix of the mid-Pleistocene MOR 604 and MOR 605 specimens was highly fragmented and brittle, supporting substantial degradation. Histological structures resembling blood vessels readily broke free of the degraded matrix and were easily isolated. Both specimens exhibited evidence of exogenous, orange-brown mineralization across portions of structure surfaces even after hydroxyapatite was removed via acid demineralization. In contrast, the late Pleistocene MOR 501, also from the temperate region of Montana, U.S.A.[33], preserved a structured, relatively intact collagenous matrix. No evidence for exogenous mineralization of MOR 501 was detected with light microscopy.

Matrix from MOR 501 was more consistent in morphology with past reports on extant collagenous matrix[32, 41] than what was observed for matrix of MOR 604 and MOR 605. Collagenous matrix of MOR 604 and MOR 605 was closer in morphology to what has previously been reported for Mesozoic dinosaurs[32-34] and early-mid Cenozoic organisms[38, 39]. The stark difference in these observations supports a disparity in degree of type-1 collagen preservation between these specimens, which is predicted to affect potential sequencing analyses. Prior studies have reported type-1 collagen sequences from MOR 604[62] and MOR 605[57]. MOR 501 however has not previously been sequenced and a direct comparison regarding degree of type-1

collagen sequence preservation is not currently possible. Further, MOR 501, MOR 604, and MOR 605 were all recovered from the same geographic region, albeit different burial sites[33]. This supports the observed dichotomy in "collagenous" matrix preservation is thus likely less dependent on thermal setting.

Another study that analyzed molecular histology to a limited extent is that of the Pliocene Ellesmere Island camel tibia[2]. A cross-section of a vascular canal within the tibia was elementally mapped using energy dispersive X-ray spectroscopy (EDS). The analysis demonstrated that elements consistent with iron oxyhydroxides and barium sulfates co-localized to the vascular canal. The presence of such exogenous minerals supports that this tibia had undergone substantial chemical alteration. Both mineral precipitants are consistent with observations from older tertiary[37-39] and even Mesozoic specimens[16, 35, 36, 40, 41], and certainly precludes it from being considered a "sub-fossil". Despite the apparent chemical alteration to its molecular histology, the tibia still preserved collagen sequences identifiable via mass spectrometry[2].

Samples from the Pliocene tibia were not demineralized and examined with light microscopy within the study[2], however, thus precluding a direct comparison against observations for the MOR 501, MOR 604, and MOR 605 "collagenous" matrix morphology. The substantial mineralization detected by the EDS analysis is consistent with observations of mineralized histological structures within MOR 604 and MOR 605[33]. This supports a hypothesis that any "collagenous" matrix the Ellesmere Island tibia preserves is likely highly degraded morphologically, in a manner consistent with MOR 604 and MOR 605[33] as well as previous reports for Mesozoic dinosaurs[33] and pre-Pliocene Cenozoic[37-39] specimens.

Data from the two studies above already enables some predictions to be made regarding the relationship of the specimens' underlying molecular histology with degree of sequence preservation, and even some diagenetic variables. The dichotomy in molecular histology between extant specimens along with MOR 501 when compared against MOR 604, MOR 605, the Pliocene camel tibia, and Mesozoic dinosaurs is to this point a largely unexplored finding. Few, if any, studies have directly explored how these differences in "collagenous" matrix histology manifest in degree of recovered sequence data.

Based on the discussion above, bone specimens preserving ancient DNA are herein hypothesized to possess an intact, relatively robust collagenous matrix like that of MOR 501. If sequence-able DNA is present, collagenous matrix would also still be expected to be relatively intact. In contrast, bone specimens with a brittle, easily fragmented "collagenous" matrix like that of MOR 604 and MOR 605 are predicted to preserve, at most, remnant peptide sequences. If the collagenous matrix has degraded to the point it has lost structural integrity, the preservation of sequence-able DNA is not expected[18, 27, 59]. Further, this agrees with the trend of sequence-able DNA being rarely reported from specimens exceeding 0.13-0.24Ma in geologic age (excluding cave and permafrost deposits) as MOR 604 and MOR 605 are both assigned dates of ˜100-300Ka[33].

If such a hypothesis were supported, practical methods such as electron and even light microscopy may be capable of screening fossil/sub-fossil specimens for sequence preservation. However, the limited extent of the data that has been reported for ancient vertebrate molecular histology severely limits the conclusions that can be drawn regarding these relationships. This epitomizes the need emphasized by this review for extensive study of fossil vertebrate molecular histology.

Methods for Studying Fossil Molecular Histology

Examination of fossil/sub-fossil molecular histology is proposed for empirically studying how the cumulative effect of diagenetic variables upon a specimen's molecular histology correlates with degree of sequence preservation. Vertebrate elements with the highest potential for molecular sequence preservation include tooth enamel and dentine, bone, and eggshell[3, 5, 59]. Of these, bone by far is the most widely characterized within ancient specimens as to its non-mineral histological structures. Numerous studies have reported histological structures morphologically and chemically consistent with biological cells, vascular tissue, and "collagenous" matrix preserved within Cenozoic and Mesozoic bones[22, 28-40]. In particular, the organic portion of extant collagenous bone matrix is comprised of ˜90% type-1 bone collagen[40, 59]. This high proportion of a single, specific molecule is practical for comparison against purified collagen standards, extant controls, and across various ancient specimens.

The above histological structures are generally isolated via demineralization using a dilute acid[31, 33, 36]; this allows their molecular histology to be investigated using a suite of molecular methods. Characterization of morphology for these structures has historically been accomplished using a combination of light microscopy and both of transmission and scanning electron microscopy[31-33, 35, 36, 41]. Light microscopy is a practical method to rapidly screen specimens for the preservation of histological structures. The use of both transmission and scanning electron microscopy together is particularly advantageous. While both offer nano-scale optical resolution, the former images sample cross-sections while the latter sample surface[63, 64]. Both methods are also readily capable of detecting a distinct ˜67nm banding pattern unique to collagen protein helices[40, 65-67]. Observation of this banding pattern indicates either the presence of a collagen helix or compounds replicating its structure.

Studying the chemical aspect of molecular histology generally requires localizing chemical signal to a specific histological structure. Two methods with precedence for use within molecular paleontology are time-of-flight secondary ionization mass spectrometry (ToF-SIMS) and Raman spectroscopy:

ToF-SIMS rasters a microscale-diameter ion beam in a square, grid-like pattern across a specimen's surface. At each point in the square analysis "grid", the chemical content of the specimen at that specific point is detected and recorded as a spectrum of molecular and fragment ions. A specific ion can then be plotted according to its recorded intensity at each point in the grid to form a molecular map that mirrors the area analyzed across the specimen's surface. The specific types of ions detected via this process vary depending upon specimen chemical makeup; this allows the unique histological structures of a specimen to be targeted so that chemical makeup can be connected to morphology[68-70]. A few studies have employed ToF-SIMS to analyze ancient specimens[29-31, 36, 52, 71-73]. One recent publication used the method to analyze the molecular histology of demineralized epidermis from an exceptionally preserved Jurassic ichthyosaur[31]. Ionic fragments consistent with peptides or related compounds, along with polyaromatic hydrocarbons, were successfully localized to the ichthyosaur epidermis. Recorded intensities for polyaromatic hydrocarbon and peptide-related ion fragments (such as those detected in the Jurassic ichthyosaur[31]) can be compared across extant and ancient histological structures. For example, elevated levels of polyaromatic related ions in one specimen relative to another would be predicted to indicate a higher degree of chemical degradation[68, 74-77]. This is one potential method for evaluating changes in fossil/sub-fossil molecular histology by geologic timepoint and depositional environment.

Raman spectroscopy utilizes a monochromatic laser to irradiate a single point a few microns in diameter on a specimen surface[78-80]. As the laser's photons contact the specimen surface, a small number of them are inelastically scattered by the specimen surface; that is, they either gain or lose energy after contacting the specimen surface[81, 82]. The degree to which these photons change energy depends on the type of molecular bond vibration the photon contacted within the specimen. Detecting the change in these photons' energies forms a spectrum revealing the types of molecular bond vibrations present where the laser contacted. This allows specific histological structures to be analyzed for the types of molecular bonds present in their chemical makeup[78-80, 83]. A recent study attempted to analyze the molecular histology of fossil tissues using Raman spectroscopy[22]. However, perusal of their published findings raised questions as to whether some of their data represented true Raman signal or was an artifact of autofluorescence[84]. Raman spectroscopy with a laser wavelength below 250nm is a well-established solution to eliminate autofluorescence[42, 85] but has seen little use within molecular paleontology historically[42]. However, similar to the ion intensities with ToF-SIMS, Raman signal intensity for specific bond vibrations can be compared across extant and ancient specimen molecular histology. Indeed, this method has seen substantial use historically in correlating thermal history with molecular makeup for a wide range of humics and kerogen macromolecules in petroleum and soil science[76, 86, 87].

Data collected using these described techniques can be correlated with the degree to which molecular sequences are recoverable from fossil and sub-fossil specimens. Both the intensity of Raman signal for specific bond vibrations and the relative ion abundances from ToF-SIMS can readily be compared against the degree to which a specimen preserves molecular sequence information. In the case of collagen peptides, both forms

of electron microscopy can be used to evaluate the relative abundance of ˜67nm banding present within bone matrix. This too can be compared against the degree of type-1 collagen sequence information recoverable from a given specimen.

Conclusion

Thermal setting and geologic age have been commonly used as proxies for predicting molecular sequence preservation potential[3, 5, 6, 13]. Late Pleistocene and Holocene specimens from cooler regions, especially permafrost deposits, have been shown to generally possess the highest preservation potential for molecular sequence information[5, 6, 8, 14, 15]. However, depositional environments are influenced by other variables including moisture[17-21] and oxygen content[17-20, 22, 23], ion species present, and sediment composition[16-20]. These confounding variables limit the usefulness of thermal setting and geologic age as proxies outside of a broad scale.

Direct analysis of fossil and sub-fossil molecular histology is a potential answer to this limitation. The molecular histology of a specimen's preserved cells and tissues reflects the cumulative effects of environmental variables upon its constituent biomolecules, including DNA and protein sequences[17, 20, 27]. Observed degradation of cell and tissue molecular histology is hypothesized to correlate with constituent biomolecules having undergone degradation. This agrees with the limited data in the primary literature on the correlation of molecular histology with sequence preservation potential[2, 33, 57, 62]. Thus, the preserved state of fossil/sub-fossil molecular histology is predicted to be an accurate proxy for molecular sequence preservation. A potential limitation to this approach is that some aspects of molecular histology may be beyond resolution or limit of detection for current molecular methods. However, modern molecular instrumentation regularly functions on the micro- and nanoscale in terms of resolution and limit of detection[63, 64, 68, 69, 78-80, 88], thus minimizing this limitation as a potential obstacle. The use of fossil/sub-fossil molecular histology as a proxy for sequence preservation has potential to elucidate why ancient specimens of some formations and timepoints preserve sequences while others do not; such understanding would facilitate the selection of ancient specimens for use in future ancient DNA and paleoproteomic studies.

Competing Interests

There are no competing interests to declare.

References

1. Valk, T.v.d. et al. Million-year-old DNA sheds light on the genomic history of mammoths. Nature 591, 265-269 (2021).

2. Rybczynski, N. et al. Mid-Pliocene warm-period deposits in the High Arctic yield insight into camel evolution. Nat. Commun. 4, 1550 (2013).

3. Demarchi, B. et al. Protein sequences bound to mineral surfaces persist into deep time. eLife 5, e17092 (2016).

4. Buckley, M., Lawless, C. & Rybczynski, N. Collagen sequence analysis of fossil camels, Camelops and c.f. Paracamelus, from the Arctic and sub-Arctic of Plio-Pleistocene North America. Journal of Proteomics 194, 218-225 (2019).

5. Welker, F. et al. Enamel proteome shows that Gigantopithecus was an early diverging pongine. Nature 576 (2019).

6. Hofreiter, M. et al. The future of ancient DNA: Technical advances and conceptual shifts. BioEssays 37, 284-293 (2015).

7. Kehlmaier, C. et al. Tropical ancient DNA reveals relationships of the extinct Bahamian giant tortoise Chelonoidis alburyorum. Proc. R. Soc. B 284, 20162235 (2017).

8. Wadsworth, C. & Buckley, M. Proteome degradation in fossils: investigating the longevity of protein survival in ancient bone. Rapid Communications in Mass Spectrometry 28, 605-615 (2014).

9. Jensen, E.L. et al. Ancient and historical DNA in conservation policy. Trends in Ecology & Evolution (2022).

10. Der Sarkissian, C. et al. Unveiling the Ecological Applications of Ancient DNA From Mollusk Shells. Frontiers in Ecology and Evolution 8 (2020).

11. Alter, S.E., Newsome, S.D. & Palumbi, S.R. Pre-Whaling Genetic Diversity and Population Ecology in Eastern Pacific Gray Whales: Insights from Ancient DNA and Stable Isotopes. PLoS ONE 7, e35039 (2012).

12. Leonard, J.A. Ancient DNA applications for wildlife conservation. Molecular Ecology 17, 4186-4196 (2008).

13. Wadsworth, C. et al. Comparing ancient DNA survival and proteome content in 69 archaeological cattle tooth and bone samples from multiple European sites. Journal of Proteomics 158, 1-8 (2017).

14. Ngatia, J.N. et al. Distinguishing extant elephants ivory from mammoth ivory using a short sequence of cytochrome b gene. Sci. Rep. 9, 18863 (2019).

15. Ancient DNA: methods and protocols, Vol. 840. (Humana Press, 2012).

16. Schweitzer, M.H. et al. A role for iron and oxygen chemistry in preserving soft tissues, cells and molecules from deep time. Proc. R. Soc. B 281, 20132741 (2014).

17. Briggs, D.E.G. The Role of Decay and Mineralization in the Preservation of Soft-Bodied Fossils. Annual Review of Earth and Planetary Sciences 31, 275-301 (2003).

18. Lindahl, T. Instability and decay of the primary structure of DNA. Nature 362, 709-715 (1993).

19. Schweitzer, M.H., Schroeter, E.R., Cleland, T.P. & Zheng, W. Paleoproteomics of Mesozoic Dinosaurs and Other Mesozoic Fossils. Proteomics 19, 1800251 (2019).

20. Gupta, N.S. Biopolymers: A molecular paleontology approach. (Spring Science+Business Media Dordrecht, 2014).

21. Lennartz, A., Hamilton, M.D. & Weaver, R. Moisture Content in Decomposing, Desiccated, and Mummified Human Tissue. Forensic Anthropology 3, 1-16 (2020).

22. Wiemann, J. et al. Fossilization transforms vertebrate hard tissue proteins into N-heterocyclic polymers. Nat. Commun. 9, 4741 (2018).

23. Wiemann, J., Crawford, J.M. & Briggs, D.E.G. Phylogenetic and physiological signals in metazoan fossil biomolecules. Science Advances 6, eaba6883 (2020).

24. Hill, C.L. & Schweitzer, M. Mammoth (Mammuthus) from the Doeden Gravels, Eastern Montana: Biometric and Molecular Analyses. Current Research in the Pleistocene 16, 120-123 (1999).

25. Fortes, G.G. et al. Ancient DNA reveals differences in behaviour and sociality between brown bears and extinct cave bears. Molecular Ecology 25, 4907-4918 (2016).

26. Campbell, R.J. & Pignatelli, M. Molecular histology in the study of solid tumours. Mol. Pathol. 55, 80-82 (2002).

27. Briggs, D.E.G., Evershed, R.P. & Lockheart, M.J. The Biomolecular Paleontology of Continental Fossils. Paleobiology 26, 169-193 (2000).

28. Lindgren, J. et al. Microspectroscopic Evidence of Cretaceous Bone Proteins. PLoS ONE 6, e19445 (2011).

29. Lindgren, J. et al. Molecular composition and ultrastructure of Jurassic paravian feathers. Sci. Rep. 5, 13520 (2015).

30. Lindgren, J. et al. Biochemistry and adaptive colouration of an exceptionally preserved juvenile fossil sea turtle. Sci. Rep. 7, 13324 (2017).

31. Lindgren, J. et al. Soft-tissue evidence for homeothermy and crypsis in a Jurassic ichthyosaur. Nature 564, 359-365 (2018).

32. Schweitzer, M.H., Wittmeyer, J.L., Horner, J.R. & Toporski, J.K. Soft-Tissue Vessels and Cellular Preservation in Tyrannosaurus rex. Science 307, 1952-1955 (2005).

33. Schweitzer, M.H., Wittmeyer, J.L. & Horner, J.R. Soft tissue and cellular preservation in vertebrate skeletal elements from the Cretaceous to the present. Proc. R. Soc. B 274, 183-197 (2007).

34. Schweitzer, M.H. et al. Biomolecular Characterization and Protein Sequences of the Campanian Hadrosaur B. canadensis. Science 324, 626-631 (2009).

35. Armitage, M.H. & Anderson, K.L. Soft sheets of fibrillar bone from a fossil of the supraorbital horn of the dinosaur Triceratops horridus. Acta Histochemica 115, 603-608 (2013).

36. Surmik, D. et al. Spectroscopic Studies on Organic Matter from Triassic Reptile Bones, Upper Silesia, Poland. PLoS ONE 11, e0151143 (2016).

37. Boskovic, D.S. et al. Structural and Protein Preservation in Fossil Whale Bones from the Pisco Formation (Middle-Upper Miocene), Peru. Palaios 36, 155-164 (2021).

38. Cadena, E. Microscopical and elemental FESEM and Phenom ProX-SEM-EDS analysis of osteocyte- and blood vessel-like structures obtained from fossil vertebrates of the Eocene Messel Pit, Germany. PeerJ 4, e1618 (2016).

39. Cadena, E. In situ SEM/EDS compositional characterization of osteocytes and blood vessels in fossil and extant turtles on untreated bone surfaces; different preservational pathways microns away. PeerJ 8, e9833 (2020).

40. Boatman, E.M. et al. Mechanisms of soft tissue and protein preservation in Tyrannosaurus rex. Sci. Rep. 9, 15678 (2019).

41. Schweitzer, M.H., Zheng, W., Cleland, T.P. & Bern, M. Molecular analyses of dinosaur osteocytes support the presence of endogenous molecules. Bone 52, 414-423 (2013).

42. Long, B., Zheng, W., Schweitzer, M.H. & Hallen, H. Resonance Raman imagery of semi-fossilized soft tissues. Proc. SPIE 10753 (2018).

43. Thiel, V. et al. Occurence and fate of fatty acyl biomarkers in an ancient whale bone (Oligocene, El Cien Formation, Mexico). Organic Geochemistry 68, 71-81 (2014).

44. Mitchell, K.J. & Rawlence, N.J. Examining Natural History through the Lens of Palaeogenomics. Trends in Ecology & Evolution 36, 258-267 (2021).

45. Meyer, M. et al. Palaeogenomes of Eurasian straight-tusked elephants challenge the current view of elephant evolution. eLife 6, e25413 (2017).

46. Buckley, M., Larkin, N. & Collins, M. Mammoth and Mastodon collagen sequences; survival and utility. Geochimica et Cosmochimica Acta 75, 2007-2016 (2011).

47. Froese, D. et al. Fossil and genomic evidence constrains the timing of bison arrival in North America. Proceedings of the National Academy of Sciences 114, 3457-3462 (2017).

48. Lindqvist, C. et al. Complete mitochondrial genome of a Pleistocene jawbone unveils the origin of polar bear. Proceedings of the National Academy of Sciences 107, 5053-5057 (2010).

49. Dabney, J. et al. Complete mitochondrial genome sequence of a Middle Pleistocene cave bear reconstructed from ultrashort DNA fragments. Proceedings of the National Academy of Sciences 110, 15758-15763 (2013).

50. Meyer, M. et al. A mitochondrial genome sequence of a hominin from Sima de los Huesos. Nature 505, 403-406 (2014).

51. Meyer, M., Arsuaga, J.L. & Filippo, C.d. Nuclear DNA sequences from the Middle Pleistocene Sima de los Huesos hominins. Nature 531, 504-507 (2016).

52. Orlando, L. et al. Recalibrating Equus evolution using the genome sequence of an early Middle Pleistocene horse. Nature 499, 74-78 (2013).

53. Valk, T.v.d. et al. Million-year-old DNA sheds light on the genomic history of mammoths. Nature 591, 265-269 (2021).

54. Cappellini, E. et al. Early Pleistocene enamel proteome from Dmanisi resolves Stephanorhinus phylogeny. Nature 574, 103-107 (2019).

55. Cleland, T.P. et al. Mass Spectrometry and Antibody-Based Characterization of Blood Vessels from Brachylophosaurus canadensis. J. Proteome Res. 14, 5252-5262 (2015).

56. Schroeter, E.R. et al. Expansion for the Brachylophosaurus canadensis Collagen I Sequence and Additional Evidence of the Preservation of Cretaceous Protein. J. Proteome Res. 16, 920-932 (2017).

57. Asara, J.M., Schweitzer, M.H., Freimark, L.M., Phillips, M. & Cantley, L.C. Protein Sequences from Mastodon and Tyrannosaurus Rex Revealed by Mass Spectrometry. Science 316, 280-285 (2007).

58. Ramsøe, A. et al. Assessing the degradation of ancient milk proteins through site-specific deamidation patterns. Scientific Reports 11, 7795 (2021).

59. Wang, S.-Y., Cappellini, E. & Zhang, H.-Y. Why collagens best survived in fossils? Clues from amino acid thermal stability. Biochemical and Biophysical Research Communications 422, 5-7 (2012).

60. Mackie, M. et al. Preservation of the metaproteome: variability of protein preservation in ancient dental calculus. Sci. Technol. Archaeol. Res. 3, 74-86 (2017).

61. Presslee, S. et al. Palaeoproteomics resolves sloth relationships. Nat. Ecol. Evol. 3, 1121-1130 (2019).

62. Schweitzer, M., Hill, C.L., Asara, J.M., Lane, W.S. & Pincus, S.H. Identification of Immunoreactive Material in Mammoth Fossils. Journal of Molecular Evolution 55, 696-705 (2002).

63. Bozzola, J.J. & Russell, L.D. Electron microscopy: Principles and Techniques for Biologists. (Jones and Bartlett, Sudbury, Mass.; 1999).

64. Handbook of Microscopy. (Springer Nature Switzerland AG, Gewerbestrasse, Switzerland; 2019).

65. Lin, C.P., Douglas, W.H. & Erlandsen, S.L. Scanning electron microscopy of type I collagen at the dentin-enamel junction of human teeth. Journal of Histochemistry & Cytochemistry 41, 381-388 (1993).

66. Tzaphlidou, M. The role of collagen in bone structure: An image processing approach. Micron 36, 593-601 (2005).

67. Gottardi, R. et al. Supramolecular Organization of Collagen Fibrils in Healthy and Osteoarthritic Human Knee and Hip Joint Cartilage. PLoS ONE 11, e0163552 (2016).

68. Thiel, V. & Sjovall, P. Using Time-of-Flight Secondary Ion Mass Spectrometry to Study Biomarkers. Annual Review of Earth and Planetary Sciences 39, 125-156 (2011).

69. Sodhi, R.N.S. Time-of-flight secondary ion mass spectrometry (TOF-SIMS):-versatility in chemical and imaging surface analysis. Analyst 129, 483-487 (2004).

70. Touboul, D. & Brunelle, A. What more can TOF-SIMS bring than other MS imaging methods. Bioanalysis 8 (2016).

71. Lindgren, J. et al. Molecular preservation of the pigment melanin in fossil melanosomes. Nat. Commun. 3, 824 (2012).

72. Schweitzer, M.H. et al. Analyses of Soft Tissue from Tyrannosaurus rex Suggest the Presence of Protein. Science 316, 277-280 (2007).

73. McNamara, M.E., Dongen, B.E.v., Lockyer, N.P., Bull, I.D. & Orr, P.J. Fossilization of melanosomes via sulfurization. Palaeontology 59, 337-350 (2016).

74. Sjövall, P. et al. Analysis of kerogens and model compounds by time-of-flight secondary ion mass spectrometry (TOF-SIMS). Fuel 286, 119373 (2021).

75. Oberlin, A. Carbonization and Graphitization. Carbon 22, 521-541 (1984).

76. Delarue, F. et al. The Raman-Derived Carbonization Continuum: A Tool to Select the Best Preserved Molecular Structures in Archean Kerogens. Astrobiology 16, 407-417 (2016).

77. Buseck, P.R. & Beyssac, O. From Organic Matter to Graphite: Graphitization. Elements 10, 421-426 (2014).

78. Smith, E. & Dent, G. Modern Raman Spectroscopy: A Practical Approach. (John Wiley & Sons Ltd., Hoboken, NJ; 2019).

79. Pan, Y. & Hu, L. Applications of chemical imaging techniques in paleontology. National Science Review 6, 1040-1053 (2019).

80. Ferraro, J.R., Nakamoto, K. & Brown, C.W. Introductory Raman Spectroscopy. (Academic Press, 2003).

81. Smekal, A. Zur Quantentheorie der Dispersion. Naturwissenschaften 11, 873-875 (1923).

82. Raman, C.V. & Krishnan, K.S. A new type of secondary radiation. Nature 121, 501-502 (1928).

83. Hill, A.H., Manifold, B. & Fu, D. Tissue imaging depth limit of stimulated Raman scattering microscopy. Biomed. Opt. Express 11, 762-774 (2020).

84. Alleon, J. et al. Pushing Raman spectroscopy over the edge: purported signatures of organic molecules in fossil animals are instrumental artefacts. BioEssays 00, e2000295 (2012).

85. Abbey, W.J. et al. Deep UV Raman spectroscopy for planetary exploration: The search for in situ organics. Icarus 290, 201-214 (2017).

86. Ferralis, N., Matys, E.D., Knoll, A.H., Hallmann, C. & Summons, R.E. Rapid, direct and non-destructive assessment of fossil organic matter via microRaman spectroscopy. Carbon 108, 440-449 (2016).

87. Schito, A., Romano, C., Corrado, S., Grigo, D. & Poe, B. Diagenetic thermal evolution of organic matter by Raman spectroscopy. Organic Geochemistry 106, 57-67 (2017).

88. Marini, M. et al. The structure of DNA by direct imaging. Science Advances 1 (2015).