




RAPID COMMUNICATION

CHEMICAL EVIDENCE OF PRESERVED COLLAGEN
IN 54-MILLION-YEAR-OLD FISH VERTEBRAE

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Typescript received 9 August 2019; accepted in revised form 21 November 2019

Abstract: Collagens are the most abundant proteins in the animal kingdom. They form the structural framework of connective tissues such as bones, tendons and skin, and play an important biomechanical role in supporting tissue functions. The preservation of collagen in deep time is a topic of intense debate. Here we provide indisputable evidence for the presence of collagen in early Eocene fish vertebrae using online pyrolysis comprehensive two dimensional gas chromatography time-of-flight mass spectrometry (py-GC×GC-TOFMS) and immunofluorescence analysis. The presence of cyclic dipeptides such as diketodipyrrole, 2,5-diketopiperazine of proline-proline and 2,5-diketopiperazine of proline-glycine along with other nitrogen-bearing

molecules in the pyrolysis products of the studied fossils unequivocally demonstrate that collagen can withstand degradation and diagenetic alteration. Immunofluorescence study also confirms the presence of collagen-I in the fossilized fish vertebrae. Contrary to common opinion, the present findings suggest that the preservation of collagen in fossilized soft tissues is not rare. We propose that one of the essential factors controlling the preservation of collagen is the establishment of a suitable microenvironment within the fossil, inhibiting diagenetic alteration including microbial decay.

Key words: collagen, fish vertebrae, Eocene.

COLLAGENS are the most abundant protein in the extracellular matrix and in connective tissue of vertebrates (Shoulders & Raines 2009). In vertebrates, collagens are found in skin, bones, cartilage, tendons and corneas (Wess 2005). This structural protein family provides tensile strength, torsional stiffness and prevents mechanical failure of tissues (Ferraro *et al.* 2017). The fossil record of soft tissues provides invaluable insights into their evolutionary biology (Lindgren *et al.* 2014). Animal soft tissues are characterized by nitrogen-bearing macromolecules which are labile and prone to diagenetic alterations (Briggs & Summons 2014; Parry *et al.* 2018). There are several biological and chemical factors that influence the exceptional preservation of soft tissues in deep time (Schweitzer 2011). The mechanisms of soft tissue preservation

often include entombment in concretions of carbonate or amber, or rapid cementation just after deposition of organic remains (Schweitzer 2011; Melendez *et al.* 2013; Grice *et al.* 2019). Collagen has been detected in dinosaur bones of Cretaceous age (Schweitzer *et al.* 2005; Asara *et al.* 2007; Schroeter *et al.* 2017). However, these findings have been widely disputed (Pevzner *et al.* 2008; Manning *et al.* 2009; Buckley *et al.* 2017). The common perception is that collagen cannot survive diagenetic alteration (Service 2017; Saitta *et al.* 2018). Here we demonstrate unequivocally, using online pyrolysis-comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (py-GC×GC-TOFMS) and immunofluorescence analysis, that collagen is preserved in Early Eocene fish vertebrae.

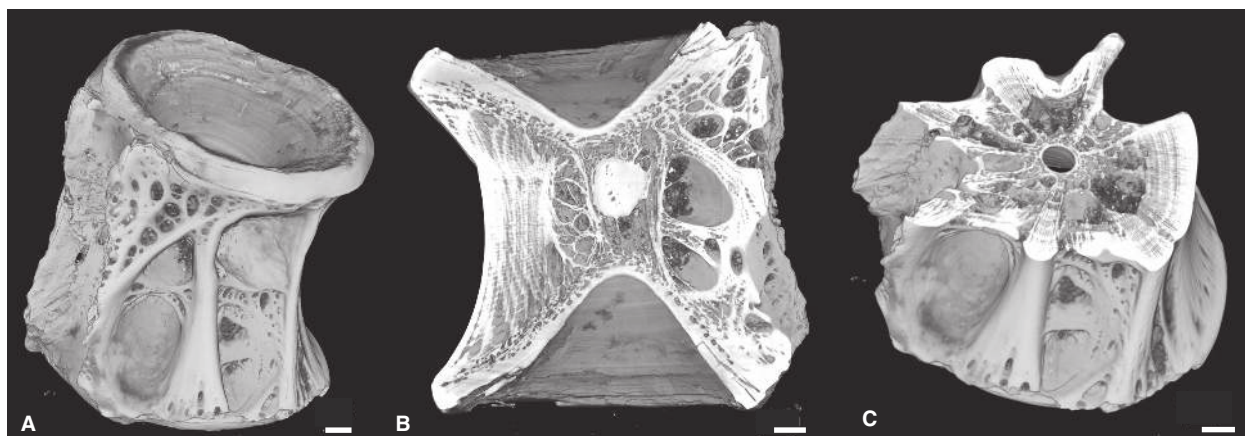


FIG. 1. 3D x-ray microscopy images of a fish vertebra (BSIP 41815). A, three-dimensional view of an entire vertebra. B, longitudinal section of the vertebra. C, transverse section of the vertebra. All scale bars represent 1 mm.

MATERIAL AND METHOD

Fossil and extant fish vertebrae specimens

Several fish vertebrae samples (Fig. 1) were collected from the Vastan lignite mine (21°25'47"N; 73°07'30"E), Cambay Basin, western India. The lignite-bearing sequence is known as the Cambay Formation. The age-diagnostic benthic foraminifera *Nummulites burdigalensis burdigalensis* which occurs in the upper part of the lignite sequence of the mine section places it within the Ypresian Stage (Punekar & Saraswati 2010). The Paleocene–Eocene Thermal Maximum (PETM; *c.* 55.5 Ma) has been reported from the subsurface of the Vastan mine (Samanta *et al.* 2013).

Extant fish vertebrae of *Catla catla* and standard collagen-I obtained from Sigma-Aldrich (<https://www.sigmaaldrich.com>) were also analysed to evaluate the molecular composition of extant collagen. All specimens of fossil vertebrae (BSIP 41814–41817) are housed at the Birbal Sahni Institute of Palaeosciences, Lucknow.

Pyrolysis-comprehensive two-dimensional gas chromatography time-of-flight mass spectrometry (py-GC×GC-TOFMS)

Prior to chemical investigations, fish vertebrae were cleaned several times with dichloromethane to remove soluble organic compounds. Fish vertebrae were pulverized using an agate mortar. We analysed several fish vertebrae to check the reproducibility of the data. Comprehensive GC×GC studies were achieved using the GERSTEL pyrolysis module (PYRO) (<http://www.gerstel.com>) in sequence with a thermal desorption unit (TDU) and cooled injection system (CIS) inlet coupled with Leco Pegasus 4D GC×GC-TOFMS. Each sample (*c.* 5 mg) was placed in a quartz tube. The sample tube was introduced into the coil probe and

pyrolysed for 0.3 min at 610°C. The TDU was kept at 300°C and CIS was temperature programmed from 30°C to 300°C (held for 1 min) at 12°C/s.

The GC×GC system consists of two capillary columns of different configuration connected in series through a dual stage modulator. The primary (1°) column was a Rxi-5 MS with 5% phenyl–95% methylsiloxane (30 m length, 250 µm internal diameter, 0.25 µm film thickness). The secondary (2°) column was a Rxi-17 MS (50%-phenyl–50%-methylsiloxane) (1.05 m length, 250 µm internal diameter, 0.25 µm film thickness). The sample was injected in a solvent vent mode with purge time 80 s and purge flow of 50 ml/min. Helium was used as carrier gas at a flow rate of 1 ml/min. The GC oven was kept at 50°C (held for 2 min) and was then ramped from 50 to 180°C at 10°C/min and again ramped at 5°C/min to 270°C and subsequently raised to 295°C at 3°C/min (held for 10 min). The secondary oven and modulator temperatures were programmed 10°C and 15°C, respectively, higher than the primary oven temperature. The hot jet was pulsed for 0.95 s every 5.5 s with a 1.80 s cooling period between stages. The applied electron ionization energy was 70 eV. The MS transfer line and the source temperature were kept at 280°C and 250°C, respectively. The TOF detector voltage was 1600 V, with a solvent delay of 15 s. All fractions were analysed in a full scan mode over a mass range of 40–700 Da and were sampled at a rate of 100 Hz. The acquisition and the data processing were performed using by ChromaTOF software package (LECO Corporation; <https://leco.com/>).

Immunofluorescence analysis

Prior to immunofluorescence analysis, organic residues from fish vertebrae were isolated using 0.6 (N) HCl acid. Glass coverslips were activated using the method described by Martin *et al.* (2012). Briefly, coverslips were

first cleaned with mild HCl solution and were treated with 10 mg/ml poly-L-lysine (Sigma-Aldrich) for 20 min at room temperature (RT) and then were finally cross-linked with 0.5% glutaraldehyde (Sigma-Aldrich) for 30 min at RT. Activated coverslips were then kept in a 24 well plate and were coated for 1 h at RT with fossil extracted collagen powder solution prepared in PBS and (2) 20% fetal bovine serum as a negative control. Post incubation excess solutions were aspirated and coverslips were washed with PBS two times. Sample coated coverslips were processed using standard immunocytochemistry protocol. Briefly, samples were first blocked with 10% BSA solution for 1 h at RT and then were incubated with rabbit anti-collagen primary antibody at 4°C for overnight. Finally, samples were incubated with FITC tagged anti-rabbit secondary antibody for 2 h at RT and then were mounted on a glass slide for imaging.

Images were acquired using a Zeiss LSM 780 confocal microscope using a 20× objective with the same acquisition parameters (exposure, laser power etc.) for both sample and negative control. Z stack images were taken up to 5–10 μm on top of the coverslip and images represented are the maximum intensity projection of acquired image stacks.

3D x-ray microscopy

A Zeiss Xradia 520 Versa 3D x-ray microscope was used for non-destructive 3D visualization of the internal features of the samples. In this technique, imaging is done by directing X-rays at an object from multiple orientations. Images produced by this method are mainly maps of X-ray attenuation within an object, which is related to its density. Contiguous cross-sectional 2D images or slices of the samples were acquired. The data sets were then imported into ORS visual SI software (Object Research Systems; <http://www.theobjects.com/>) to generate a 3D digital volume of the samples. 3D volumes are reconstructed by stacking the 2D images.

RESULTS

Pyrolysis-gas chromatography-mass spectrometry (py-GC-MS) is a powerful analytical tool for chemical characterization of soft tissues (Stankiewicz *et al.* 1997; Glass *et al.* 2012). A major advantage of two dimensional gas chromatography time-of-flight mass spectrometry over conventional gas chromatography-mass spectrometry is the ability to analyse compounds from a complex mixture and enhanced determination of labile and volatile organic molecules (Eiserbeck *et al.* 2011; Tewari *et al.* 2017). The distribution of nitrogen-bearing compounds in the pyrolysis products of early Eocene fish vertebra is similar to

that of modern fish vertebrae and standard collagen-I (Figs 2, 3, Table 1; Dutta *et al.* 2019, fig. S1).

The prominent pyrolysis products of modern fish vertebrae (including acetic acid, acetamide, succinimide, acetamido pyrone, pyridines, indole, benzonitrile, benzyl nitrile, nonadecanenitrile, docosenamide, diketodipyrrole, 2,5-diketopiperazine (pro-pro), 2,5-diketopiperazine (pro-gly) and other diketopiperazines) are present in 54 myr-old fish vertebrae (Fig. 1). It is noteworthy that pyrolysis products of modern fish vertebrae are also characterized by highly abundant fatty acids which are not detected in fossil vertebrae. The distribution of 2,5-diketopiperazines in the pyrolysis products of fossil and extant fish vertebrae is shown in Figures 2B and 3B. Diketodipyrrole is the most abundant 2,5-diketopiperazine in the pyrolysis products of fossil vertebrae and occurs at higher abundance in comparison to extant fish vertebrae (Figs 2B, 3B). These 2,5-diketopiperazines are formed by dimerization of two amino acids through a peptide chain to form cyclic dipeptides (Stankiewicz *et al.* 1996; Fabbri *et al.* 2012). During the pyrolysis, many of these compounds occur in doublets which are formed due to diastereomerism (Stankiewicz *et al.* 1996). The presence of cyclic dipeptides such as diketodipyrrole, 2,5-diketopiperazine of proline-proline and 2,5-diketopiperazine of proline-glycine (Figs 2B, 3B) which are diagnostic pyrolysis products of collagen (Fabbri *et al.* 2012; van der Werf *et al.* 2017) suggests that the structural protein can survive diagenetic alterations.

Immunostaining also reveals the presence of antigenic materials within the demineralized fossil vertebrae when stained with anti-collagen-I antibody. Maximum intensity projection (MIP) images in the figure shows significantly higher intensity of fluorescence signal compared to the negative control, suggesting clear antigen-antibody interaction in the fossil sample (Dutta *et al.* 2019, fig. S2) and thereby demonstrating the presence of collagen-I in the fossil sample. Collagen epitopes are also preserved in the fossil bone sample. The stained fossil sample is more granular compare to the positive control. These differences in staining pattern may be attributed to the fact that while the positive control was solubilized collagen, the fossil sample was demineralized powder which was not solubilized.

DISCUSSION

Previous reports based on microscopic observations suggest that the collagen fibres are preserved in Mesozoic vertebrates (Bertazzo *et al.* 2015; Plet *et al.* 2017). Recently, Lee *et al.* (2017) reported the preservation of collagen in an Early Jurassic sauropodomorph dinosaur using synchrotron FTIR microspectroscopy. FTIR spectroscopic analysis only examines the functional groups present and does not reveal definite chemical structures. The

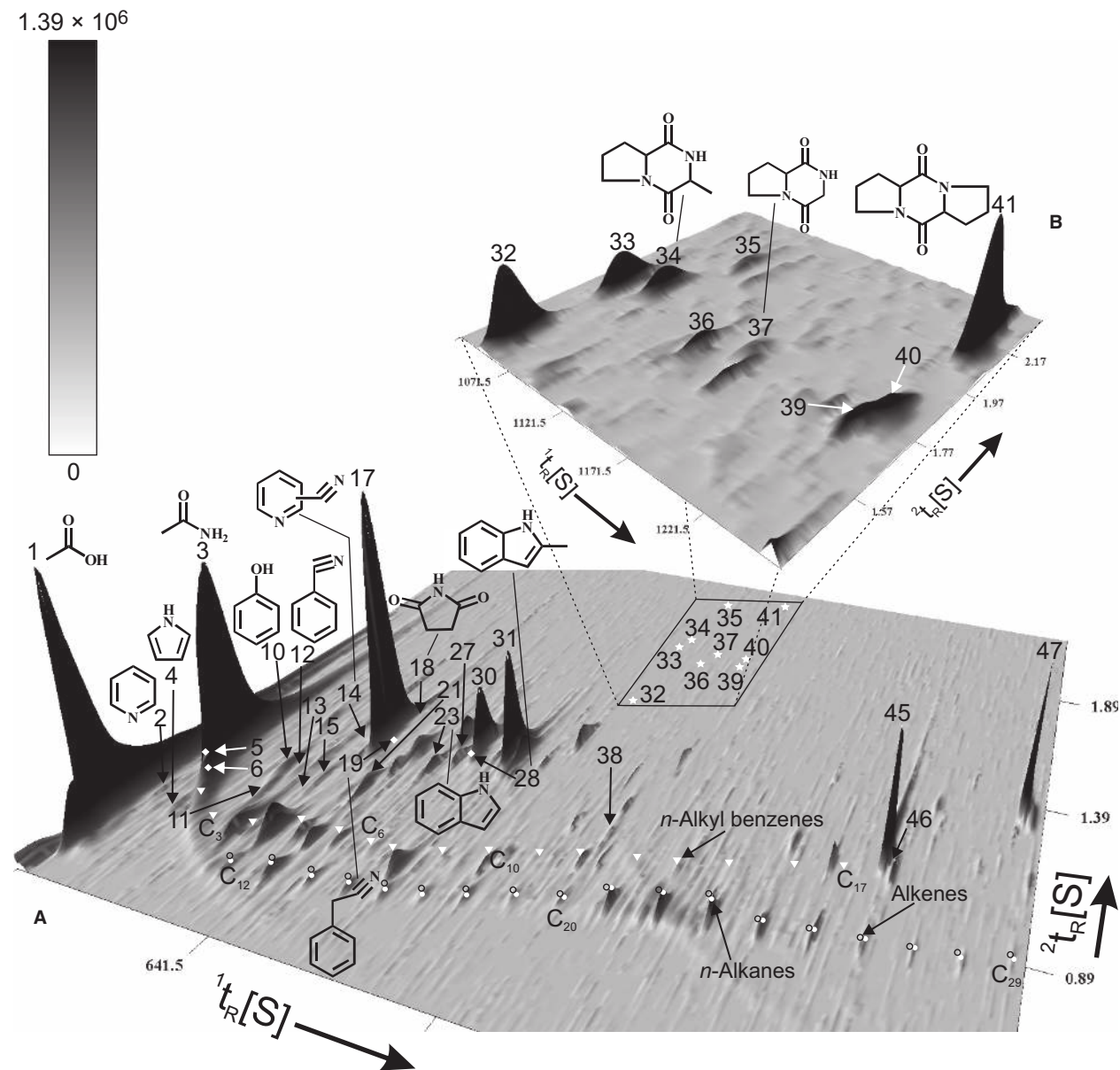


FIG. 2. A, total ion chromatograms of pyrolysis products of the fish vertebra from early Eocene sediments of Cambay Basin, from western India (BSIP 41817). B, selected ion chromatograms at m/z 70 + 90 + 111 of pyrolysis products of Eocene fish vertebra. Peak identifications are given in Table 1; filled circles, open circles and inverted triangles are homologues of n -alkanes, alkenes and n -alkylated benzenes, respectively. Star represents diketopiperazines (DKPs).

present study offers a promising approach to reveal the macromolecular preservation of soft tissues in deep time. Diketodipyrrole, 2,5-diketopiperazine (pro-pro) and 2,5-diketopiperazine (pro-gly) are generated from cyclization and rearrangement of the dipeptide hydroxyproline-hydroxyproline and proline-proline, proline-glycine, respectively (Chiavari & Galletti 1992; Chiavari *et al.* 2003). It is worthwhile to mention that the collagen type I has repeating tripeptide sequence of glycine-X-Y where X is usually proline and Y is hydroxyproline (Lodish *et al.*

2000). A total of 28 different types of collagen have been identified in vertebrates. Collagen I is the most abundant protein in animal bones (Brinckman 2005). It constitutes nearly 20% of bone by mass and more than 90% of the organic matrix of bone (Szpak 2011). Nitrogen-bearing compounds such as nonadecanenitrile and docosenamide are detected in the pyrolysis products of fossil fish vertebrae (Fig. 2A). It has been observed that these compounds become abundant in the pyrolysates of artificially matured protenaceous moieties (Saitta *et al.* 2017). A

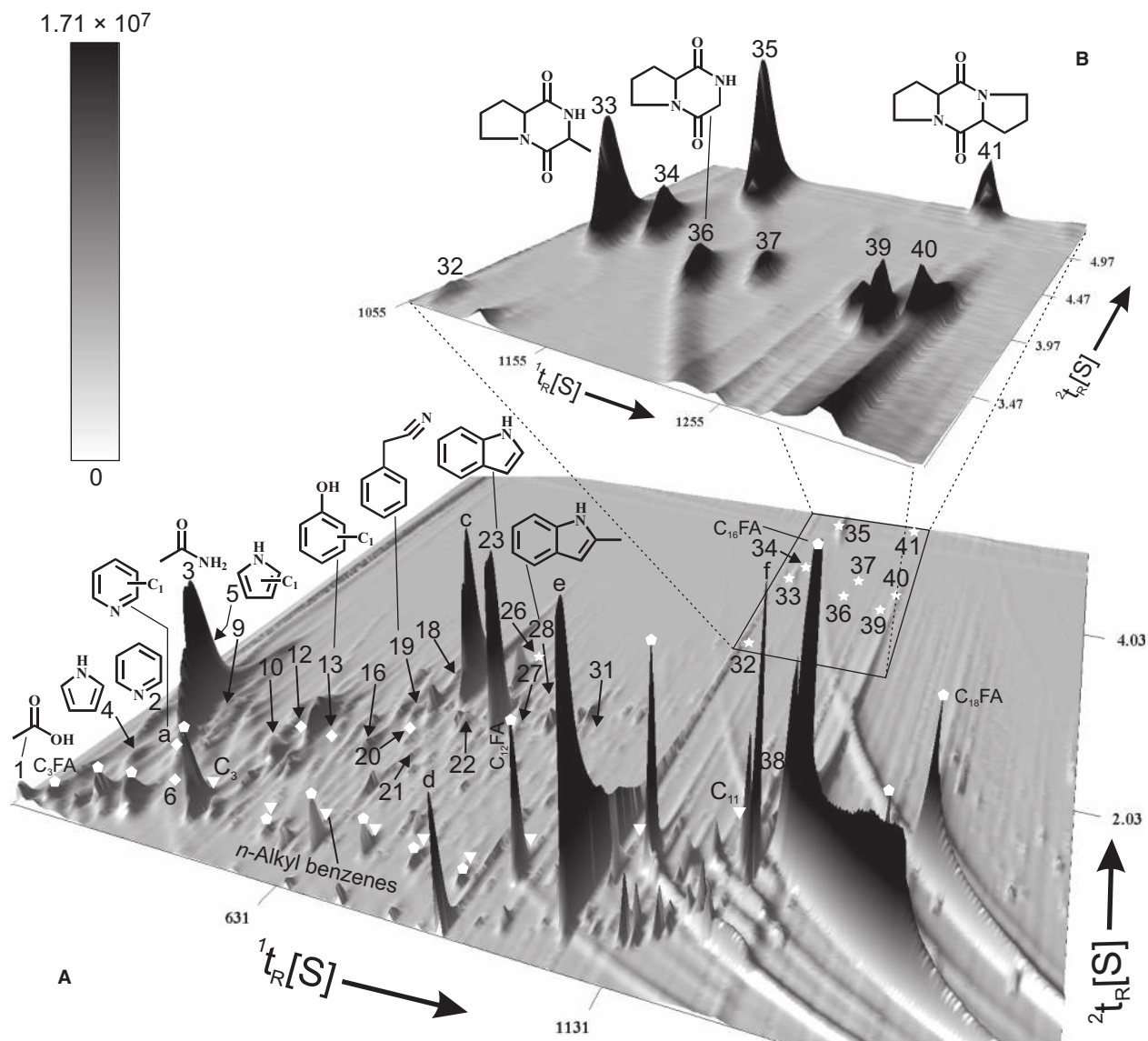


FIG. 3. A, total ion chromatograms of pyrolysis products of modern fish vertebrae. B, selected ion chromatograms at m/z 70 + 90 + 111 of pyrolysis products of modern fish vertebrae. Peak identifications are given in Table 1; inverted triangles and pentagon are homologues of *n*-alkylated benzenes and fatty acid (FA), respectively. Star represents diketopiperazines (DKPs).

series of *n*-alkane/alkene doublets are detected in the pyrolysis products of fossil fish vertebrae. This aliphatic component is probably produced by polymerization of free fatty acids during diagenesis (Stankiewicz *et al.* 2000). The Rock-Eval T_{max} of lignite-bearing Cambay Shale varies from 400°C to 423°C (Dutta *et al.* 2012) which indicates that the host sediments underwent mild thermal alteration.

The preservation of nitrogen-bearing macromolecules in deep time is one of the most controversial topics in palaeontology. It is often believed that the primary control on the preservation of biomolecules in soft tissues is the nature of the depositional environment and low thermal

maturity of the host rock (Stankiewicz *et al.* 1997; Briggs & Summons 2014). It is a well known fact that proteins are relatively unstable biopolymers that easily hydrolyse and are susceptible to diagenesis. Detailed accounts of the diagenesis of collagen have been presented by Collins *et al.* (2002) and Schweitzer (2011). Collagen is possibly preserved in fish vertebrae due to encapsulation of the soft tissue within mineralized hydroxyapatite (see Dutta *et al.* 2019, fig. S3). We also analysed several mammal bone fragments from early Eocene sediments from the Vastan lignite mine, Cambay Basin. However, proteins in those bone fragments are poorly preserved. The pyrolysis products of mammal bones are dominated by *n*-alkane/alkene

TABLE 1. A list of compounds released by pyrolysis of Eocene and extant fish vertebrae.

Peak no.	Compound	Molecular weight
1	Acetic acid	60
2	Pyridine	79
3	Acetamide	59
4	Pyrrole	67
5	Methylpyrrole	81
6	Methylpyrrole	81
7	Unknown	112
8	Unknown	112
9	Methylpyridine	93
10	Phenol	94
11	Methylstyrene	118
12	Benzo nitrile	103
13	Methylphenol	108
14	Pyridinecarbonitrile	104
15	Methylphenol	108
16	Pyridinediol	111
17	Unknown	98
18	Succinimide	99
19	Benzyl nitrile	117
20	Pyrrole-2-carbonitrile	92
21	Napthalene	128
22	Benzenepropanenitrile	131
23	Indole	117
24	Methylnapthalene	142
25	Methylnapthalene	142
26	2,5 Diketopiperazine (Gly-Gly)	114
27	3-Acetamido-4-pyrone	153
28	Methylindole	131
29	Unknown	151
30	3-Acetamido-2-pyrone	153
31	Unknown	167
32	Diketodipyrrole	186
33	2,5 Diketopiperazine (Pro-Ala)	168
34	2,5 Diketopiperazine (Pro-Ala)	168
35	2,5 Diketopiperazine (Pro-Gly)	154
36	2,5 Diketopiperazine (Pro-Arg)	154
37	2,5 Diketopiperazine (Pro-Arg)	154
38	Hexadecenitrile	235
39	2,5 Diketopiperazine (Pro-Leu/Pro-Ile)	154
40	2,5 Diketopiperazine (Pro-Leu/Pro-Ile)	154
41	2,5 Diketopiperazine (Pro-Pro)	194
42	Octadecenitrile	263
43	2,5 Diketopiperazine (Pro-Hyp)	210
44	2,5 Diketopiperazine (Pro-Hyp)	210
45	Nonadecenitrile	277
46	Nonadecanenitrile	279
47	Docosenamide	337
a	Methyl pyridine	93
b	Ethyl pyridine	107
c	Unknown	99
d	C ₁₅ -alkane	212
e	C ₁₇ -alkane	240
f	Unknown	270(?)

doublets and aromatic hydrocarbons with few nitrogen-bearing molecules (Dutta *et al.* 2019, fig. S4). This difference may be explained as a result of microbial degradation and subsequent diagenetic alteration of nitrogen-bearing molecules in mammal bone fragments where the soft tissues are not properly encapsulated. We believe that the hydroxyapatite of the fish vertebra formed a conducive microenvironment by facilitating preservation of collagen while preventing diagenetic alteration including microbial decay. Therefore, one of the important controls of preservation of collagen is the encapsulation of the soft tissue within the mineralized hydroxyapatite in the fish vertebra. Furthermore, this study suggests that characterization of collagen of different body parts of fossilized animal remains can provide useful insights on the macroevolution and physiology of organisms in deep time.

CONCLUSIONS

The present study documents the presence of collagen in early Eocene fish vertebrae. Our results suggest, contrary to common opinion, that the preservation of collagen in fossilized soft tissues is not rare. Identification and distribution of 2,5-diketopiperazines in the pyrolysis products of animal fossil remains can be a useful proxy for recognizing palaeo-proteins. The pyrolysis-GC×GC-TOF MS technique has significant potential for the detection of nitrogen-bearing macromolecules in deep time.

Acknowledgements. SD is grateful to the Department of Science and Technology (DST/SJF/E&ASA-01/2016-17) for providing financial support for this work. The Indian Institute of Technology Bombay central facility is acknowledged for providing access to py-GC×GC-TOF MS and x-ray microscopy facilities. HS is grateful to DST (EEQ/2016/000112) for providing financial support for his field trip. The Director of the Birbal Sahni Institute of Palaeosciences, Lucknow is acknowledged for extending kind support to HS. We thank two anonymous referees and Sally Thomas for their helpful suggestions on an earlier draft of this paper.

DATA ARCHIVING STATEMENT

Data for this study are available in the Dryad Digital Repository: <https://doi.org/10.5061/dryad.d2547d7zf>

Editor. Andrew Smith

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