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The biochemistry of ancient DNA in bone

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Abstract. The amount of DNA in ancient bone was determined by ethidium bromide staining after the removal of the potent Taq inhibitor, fulvic acid. A complete decalcification and a perfusion protocol were used to recover DNA from bone. A variety of purification techniques including molecular sieve, hydroxyapatite binding and 'Magic' preparations yielded DNA that spanned from 3.4μg/g of bone to below detectable limits. Fulvic acid was shown to interfere with the quantification of DNA derived from ancient human skeletal material one hundred to over seven thousand years old. Scanning UV in the 300 to 230 nm range is a simple and sensitive technique for documenting fulvic acid contamination in ancient bone extracts.
Key words. Bone; collagen; fulvic acids; DNA; ancient DNA.

Introduction

Extraction and PCR amplification of ancient DNA are complicated by the mechanisms of decomposition that accompany all buried or aged samples. In this regard, DNA shares the fate of other molecules that make the transition from the biosphere to the geosphere[11,22,23]. One of the most intensely studied molecules in the vertebrate fossil record is collagen. An extensive literature documents not only the decay of this large, helical protein molecule[7,10,23], but also the complications with regard to ^{14}C dating that arise due to closely associated, often soil-derived degradation products known collectively as humus[17].

The presence of a potent inhibitor or inhibitors to Taq polymerase in virtually all ancient DNA extracts is one of the difficulties encountered when attempting to PCR amplify target sequences. Another concern is the amount of DNA that is preserved in fossil bone, which is significant for making informed samples choices of the appropriate size.

Materials and methods

Sample descriptions. Three human cortical bone samples from femurs were obtained during surgery, and stored at −80 °C until extracted. These bone samples were extracted by removing nonmineral associated material in 4M guanidine HCl, and sequentially decalcifying the material in 4M guanidine HCl/0.5M EDTA[9].

Three taphonomic bone samples representing separate animals (two elephants (C75-6 and C75-3) and one wildebeest (C75-1)) collected within a year after death and then some 15 years later were used in this study. More complete descriptions of the experimental approach and changes observed in this fauna over the fifteen year study period are reported in references 2, 3, 19 and 20.

Human skeletal collections from North and Central America, that span a period including a historic cemetery in New Orleans and an archaic Amerind cemetery approximately 7,000 years old, were assayed for DNA content. A short description of each site is given in the table.

Light absorption of ancient bone DNA extracts in the UV (230 nm–300 nm) was compared with authentic DNA, degraded collagen and several soil extracts. Fulvic acids were isolated from a variety of soil types by extraction in 1M sodium hydroxide, and humic acids removed in 1M HCL; the material was exchanged into water by dialysis and lyophilized. Autofluorescent properties of humic and fulvic acids were monitored by means of gel electrophoresis (4–20% TBE), and Taq polymerase (Perkin-Elmer) inhibition was determined by the addition of known quantities of fulvic acids to a control lambda DNA PCR amplification.

Ancient DNA extraction and purification

The general approach taken in DNA extraction from bone is shown in the flow chart of figure 1. Bone was powdered in a Spec mill, and sample holders were cleaned between samples by boiling in water and soaking in 5% sodium hypochlorite. Generally, at least 15 grams of compact bone from the midshaft of the femur were processed. Total decalcification and removal of hydroxyapatite was achieved by dialysis against 0.5 M EDTA (0.5 M Tris, pH 7.2). In one set of experiments, 1 gram of bone powder was extracted in 10 mls of deionized, distilled water, PCR buffer and 30mM phosphate buffer.

The skeletal material from the Windover archaeological site in Florida was a special case. Extensive destructive analysis was not permitted, and the DNA was removed from human femora by drilling a small hole in the midshaft and perfusing each bone with two 50 ml aliquots of 4.0 M guanidine HCl/0.5M EDTA (100 mM Tris, pH 7.2)[20]. Each bone sample was propped on sterile supports, and the guanidine/EDTA solution dripped onto a sterile tray. The solution was reintro-

Inhibitor isolation and identification

Site name and location	Number of individuals	Antiquity	Methods used in purifying DNA
Cypress Grove New Orleans, Louisiana	21	1853–1929 A.D.[1]	'Magic', ETOH
Glorietta Pass New Mexico	7	circa 1860 A.D.	Superose 6
Plains Amerind South Dakota	24	500–2000 years B.P.[13]	'Magic'
Venado Beach Panama City, Panama	6	1000–1750 years B.P.[12]	Superose 6, 'Magic'
Sitio Sierra Central Pacific, Panama	7	1900 years B.P.[5]	'Magic'
Windover Titusville, Florida	16	7000 years B.P.[6]	Superose 6, 'Magic', ETOH Hydroxyapatite

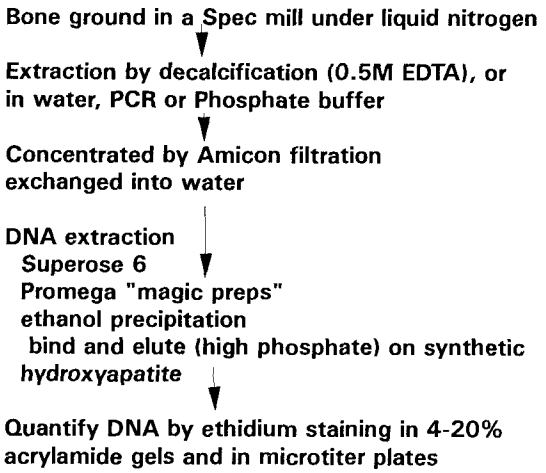

Figure 1. Flow chart of DNA isolation and purification from bone.

duced into the marrow cavity approximately 15 times in order to maximize the removal of DNA from the hydroxyapatite mineral phase. The number of times the guanidine/EDTA solution can be introduced into the midshaft cavity has a practical limit controlled by the gelatinization of this solution by collagen degradation products. Fifty to 100 mls of distilled, deionized water were introduced into the bone cavity and added to the guanidine/EDTA solution. The bone was submerged in water to remove salts, and allowed to air dry.

Solutions were concentrated by stirred cell Amicon filtration (YM30), and exchanged into water. The Amicon cells were washed extensively between each sample and boiled in water for four hours. As a control, a solution of 0.5 EDTA was concentrated, exchanged into water prior to each sample, and used as the reagent blank throughout these experiments.

A variety of techniques was used in purifying DNA from a range of skeletal material. In general, multiple purification techniques were employed when the concentration of fulvic and humic acids were high. No one technique described here removed Taq polymerase inhibition completely from the most highly contaminated extracts. Inhibition was tested in control lambda DNA PCR amplifications by adding 1 ul of ancient DNA extract. FPLC separation based on molecular weight was accomplished using Superose 6 column (Pharmacia) with 100 mM ammonium acetate as the eluent. A proprietary product of Promega, Inc. called 'Magic' was useful in purifying the ancient DNA from several of the skeletal extracts. Ethanol precipitation (100% ethanol, 2 × volume) after 'Magic' purification was used to concentrate some of the DNA samples. The traditional method of phenol/chloroform/isoamyl alcohol extraction, followed by ethanol precipitation[16] was found to result in large and sometimes total loss of ancient DNA when used on unpurified bone extracts. Final purification of ancient DNA could also be achieved by binding extracts post FPLC or 'Magic' to synthetic hydroxyapatite, and eluting DNA in 300 mM sodium phosphate buffer; however losses were evident in this procedure.

For the purpose of DNA quantification, the bone extracts were considered 'pure' when no autofluorescence was observed in the volume of extract to be assayed for DNA, and when 1 ul of extract no longer inhibited a control lambda amplification. The amount of DNA present in each of the bone extracts was determined by comparative ethidium bromide staining in either 4–20% acrylamide gels, or when there was obvious shearing of the ancient DNA. The quantification was done in microtiter plate wells referenced to a set of standard DNA concentrations. Representative samples were subjected to digestion with DNAase I.

Results

Isolation and partial characterization of a Taq polymerase inhibitor in ancient DNA extracts

Every extract of buried bone exhibited an autofluorescence upon electrophoresis and exposure to UV light

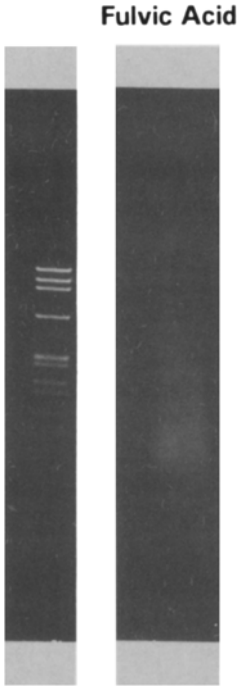

Figure 2. Separation of fulvic acid by 4–20 acrylamide gel electrophoresis in TBE. The fulvic acid was photographed on a UV light box without the addition of any stain. A PhiX174 RF DNA/Hae III (1353, 1078, 872, 603, 310, 271, 281, 234, 194 and 118 base pairs) standard fragment was stained with ethidium bromide for molecular weight comparison.

(254 nm) that was not altered by the addition of ethidium bromide, and is not DNAase digestible. The soil extract that most closely resembled this autofluorescence were the fulvic acids, soluble, heterogeneous, and highly phenolic breakdown products of organic soils (fig. 2). Fulvic acids exhibited a continuous smear of autofluorescence in a 4–20% acrylamide gel with the greatest intensity concentrated at molecular sizes less than 603 bp.

The absorption of fulvic acids in the ultraviolet spectrum is shown in figure 3. On a dry weight basis, relative to DNA, fulvic acids are intensely absorptive in the 260–280 nm range. The presence of fulvic acids in ancient DNA extracts can be most easily monitored by assaying absorption at 240, 260 and 280 nm. Fulvic acids are also potent Taq polymerase inhibitors; addition of 100 ng of fulvic acid completely inhibited amplification of a control lambda DNA target by Taq polymerase.

The amount of DNA in bone

In fresh compact human bone, the DNA bound to the mineral phase, hydroxyapatite, ranged from 1.5 to 3 ug/gram (fig. 4). The amount of DNA removed by the first nondemineralizing, denaturing extract was more variable (approximately 3 to 5.5 ug/g), and reflects the cellular content and associated vasculature.

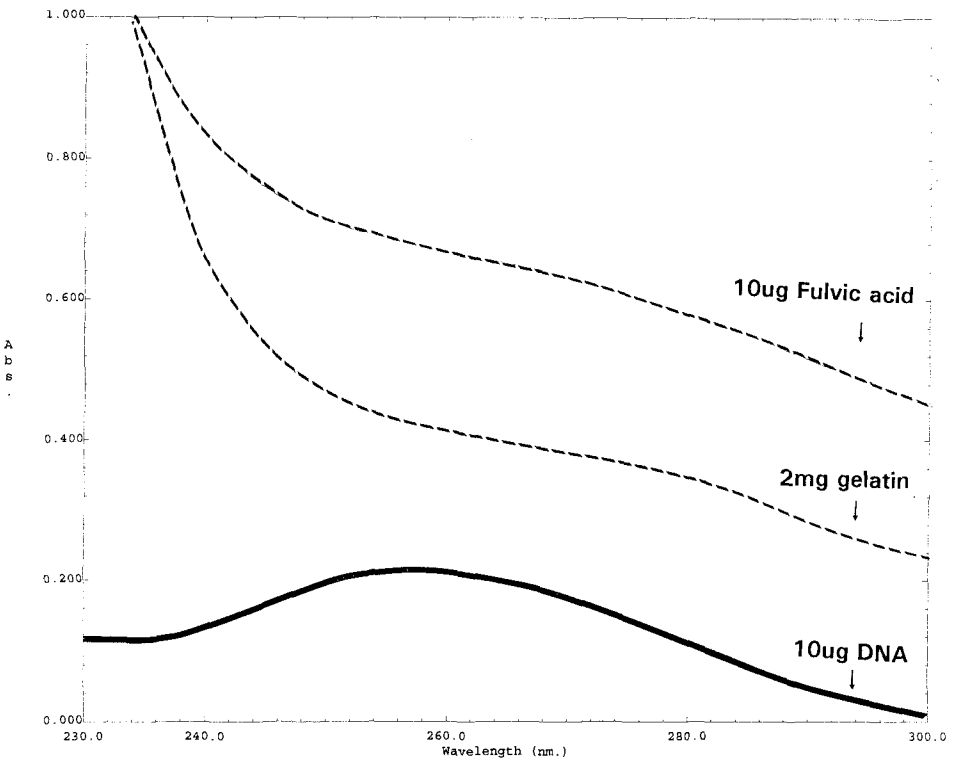

Figure 3. Absorption of (300–230 nm) of 10 ug of DNA, 2 mg of gelatin and 10 ug of fulvic acid.

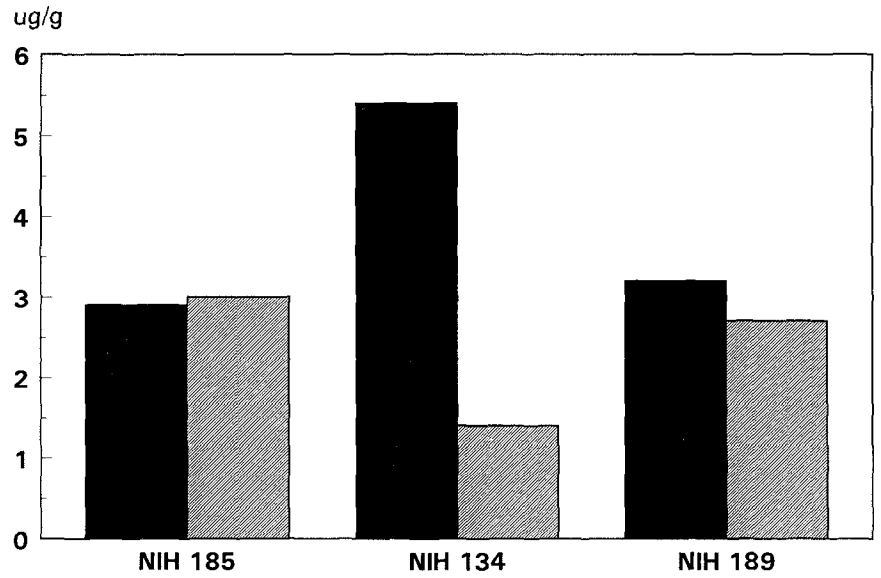

Figure 4. DNA content from 3 samples of modern human compact bone (femur) obtained at surgery that were sequentially extracted in 4M guanidine HCl (solid black) and 4M guanidine HCl/0.5M EDTA (shaded grey).

Skeletal elements from naturally aged and weathered bone (fig. 5) had DNA content that ranged from approximately 2 ug/gram to 0.2 ug/gram. Although these three animal skeletons exhibited a ten-fold difference in DNA content, very little change in the amounts of DNA was observed over the fifteen years that the bones weathered in Africa. Powdered bone from these taphonomic samples was extracted in water, PCR and phosphate buffer, and totally decalcified in EDTA. No ethidium bromide staining was observed in the nondecalcifying extracts of these samples (fig. 6), and the DNA was removed only upon decalcification. However, nondecalcifying extracts did autofluoresce on acrylamide gels, absorb light in the UV at 260 and 280 nm, and most importantly, inhibited the PCR of lambda DNA, suggesting the presence of fulvic acids in nondecalcifying extracts.

The taphonomic bones were collected from animals whose carcasses remained on the surface without the benefit of molecular preservation that rapid burial may offer. These data indicate that a major impact on the amount of DNA in bone is likely in the immediate post-death environment, and that once an animal is skeletalized, the DNA is much more stable, probably as a result of hydroxyapatite binding.

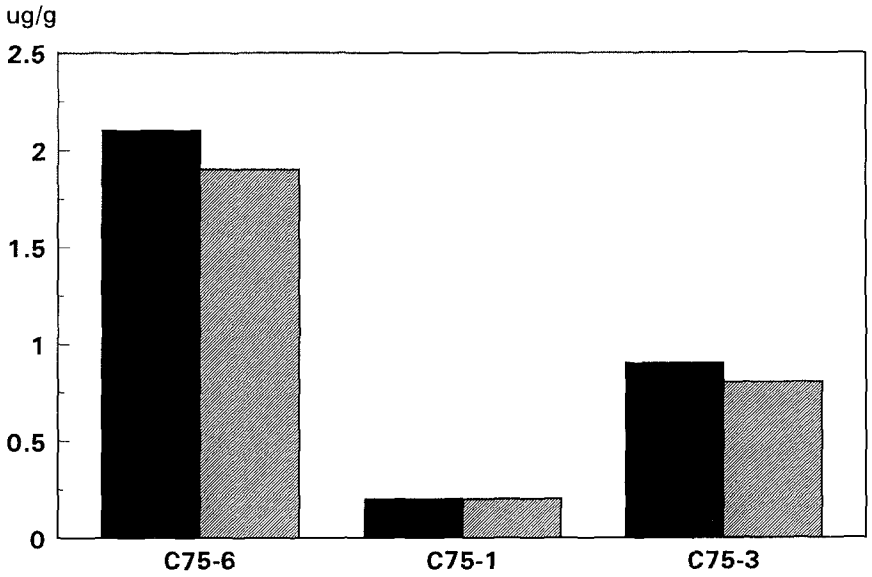

Figure 5. Amount of DNA in EDTA extracts from three animal bones: C75-6 and C75-3 are elephants, C75-1 is a wildebeest. These animals died of natural causes in Kenya in 1975, and a piece of the skeleton was collected yearly for the following fifteen years[2]. The solid dark bar represents the 1975–76 collections and the shaded bar is the 1990 collection.

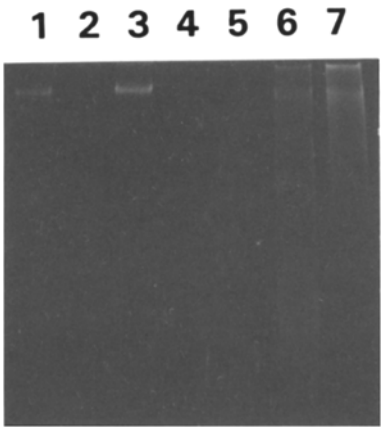

Figure 6. Extracts (4–20% acrylamide TBE gel stained with ethidium bromide) of taphonomic bone C75-6 (elephant). This animal died in Kenya in 1975 and skeletal samples were collected yearly for the next fifteen years. Lane 1) 12.5 ng genomic DNA; 2) blank; 3) 25 ng genomic DNA 4 and 5) PCR buffer extracts of 1 gram of bone powder from samples collected in 1975 and 1990; 6 and 7) EDTA extracts of 100 mg of bone powder from samples collected in 1975 and 1990.

The overall temporal trend in figure 7 is one of DNA content declining with burial time. All of these samples (fig. 7) were recovered from intentionally buried humans, and the major difference in DNA content is found between the historic samples of Cypress Grove and Glorietta Pass, and the prehistoric samples of the Plains, Venado Beach, Sitio Sierra and Windover. A latitudinal gradient also seems to be operating because the greater amount of DNA was found in the Plains samples of South Dakota compared to the tropical cemeteries at Venado Beach and Sitio Sierra. Intrasite variability in DNA content is also observed consistently, even in the most recent historic burials of Girard Street where there were individuals with nearly contemporary levels of DNA, and one individual with no detectable DNA. The range of DNA content found within each site mirrors the variability of protein content found in many archaeological assemblages[4,22].

While measuring the amount of DNA in buried bone does not address the question of whether the molecule is indigenous to the individual, it is interesting to note that the DNA content from intentionally buried humans did not exceed that documented in fresh human bone. Qualitatively, in terms of the amount of apparent strand breakage, the DNA from these buried bones also exhibited a good deal of variability. In the Plains samples, the DNA was of an apparent high molecular weight (evidenced by electropheretic analysis) and comigrated with modern genomic DNA. In contrast, many of the samples from the much younger Cypress Grove Cemetery showed evidence of extensive cleavage of the DNA backbone (fig. 8).

Conclusions

As in ancient soft tissues[15], much bone of holocene age contains DNA. The DNA is contained in decalcifying extracts, that fraction which is usually discarded in the preparation of bone samples for both ^{14}C dating and in the paleodietary analysis involving light-stable isotopes. Both the overall amounts of DNA on a dry weight basis,

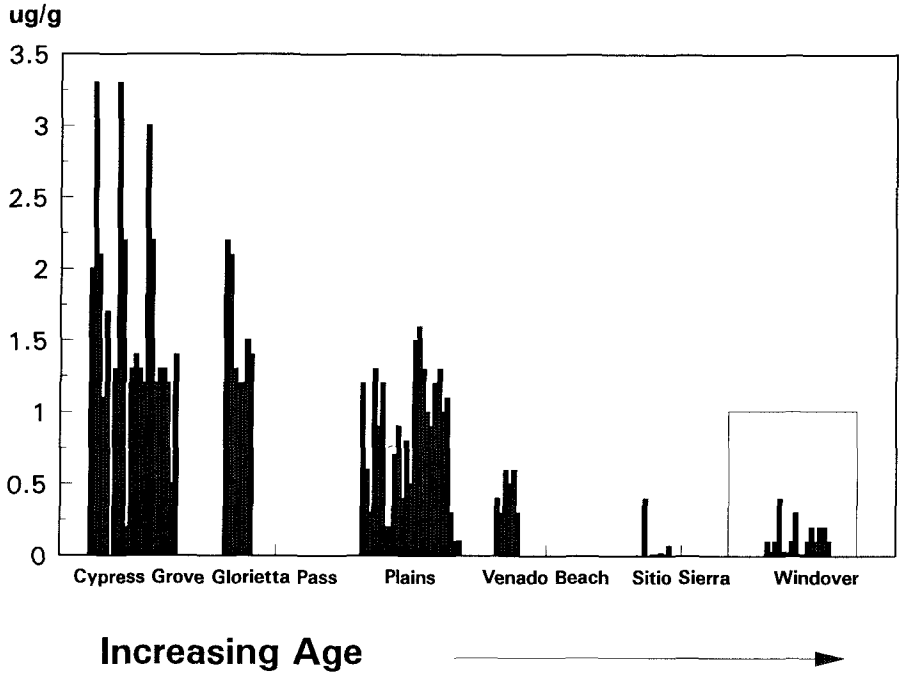

Figure 7. Amount of DNA (ug), EDTA extracted, per gram of compact bone from buried human femurs measured after purification and removal of contaminants. The age of the cemeteries range from 100 to 7,000 years (see 'Materials and methods'). The DNA in the Windover collection was removed by perfusion of the midshaft of the femur, and is reported in ug/femur.

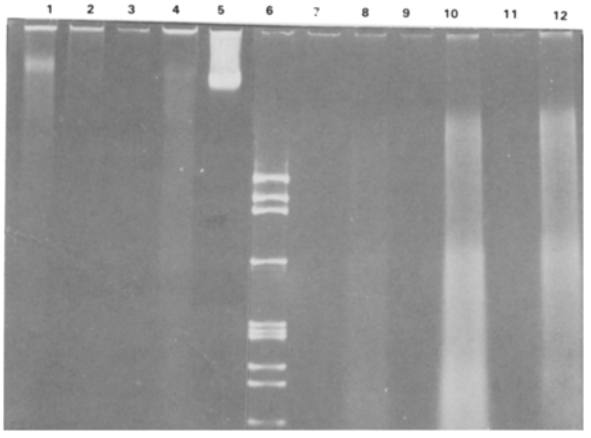

Figure 8. 4–20% acrylamide TBE gels stained with ethidium bromide. Samples 1–4) Plains Amerinds, approximately 1 gram of bone per lane. #1 is the oldest sample ^{14}C dated at 1550 years B.P.; 5) 500 ng of modern genomic DNA; #6PhiX174 RF DNA/Hae III, 1353, 1078, 872, 603, 310, 271, 281, 234, 194 and 118 base pairs; #8, 10 and 12 Cypress Grove bone extracts from approximately 1 gram of bone; #7, 9, 11 DNAase I digestion (Mn++ added) of corresponding even numbered sample to the right.

and the amount of breakage observed in the extracted DNA varied widely in the sample set of eighty-one individuals who had been buried in different environments for up to 7,000 years. Although there was a trend toward lower DNA content with burial time, the impact of the environment on DNA preservation in bone is seen in both, taphonomic fauna that were not intentionally buried, and in the intrasite variability of buried bone. The average molecular weight of DNA in virtually all bones analyzed greatly exceeded that reported in soft tissues[14]. Whether the apparent temporal stability of DNA in bone correlates with decreased base damage requires further investigation.

The inhibitor identified in this study, fulvic acid, is a well known, pervasive and persistent contaminant of the burial environment. Because of its chemical characteristics and negative effect on the polymerase chain reaction, fulvic acid can be removed from bone extracts through a variety of purification techniques. The purification processes required to remove fulvic acid from bone extracts result in losses of DNA that can be substantial if multiple, sequential treatments are required. Unfortunately even trace amounts of fulvic acid in an ancient bone extractions will produce erroneous estimations of DNA content if UV absorption is used to quantify DNA (e.g. Fisher et al.[8]).

The bulk of the vertebrate fossil record is made up of the hydroxyapatite hard tissues, bones and teeth. It is from these calcified tissues that we may learn about the genetic diversity and relationships of past populations and extinct species.

1 Beavers, R. D., Lamb, T.R, and Greene, J. R., A Preliminary Archaeological Investigation of a portion of Cypress Grove II Cemetery Site. Research Report #12. University of New Orleans, New Orleans, Louisiana, 1990.

2 Behrensmeyer, A. K., Taphonomic and ecologic information from bone weathering. Paleobiology *4(2)* (1978) 150–162.

3 Behrensmeyer, A. K., Western D., and Dechant Boaz, D.E., New perspectives in vertebrate paleoecology from a recent bone assemblage, Paleobiology *5(1)*, (1979) 12–21.

4 Bocherens, H., Fogel, M., Tuross, N., and Zeder, M., Preservation of trophic structure and climatic information through isotopic signatures in fossil mammals for a pleistocene cave in southern England. J. archaeol. Sci., in press.

5 Cooke, R., Archaeological research in central and eastern Panama: A review of some of the problems, in: The Archaeology of Lower Central America, pp. 263–302. Eds F.W. Lange and Z. Stone. School of American Research Advanced Seminar Series, University of New Mexico Press, Albuquerque, NM 1984.

6 Doran, G. H., and Dickel, D. N., Radiometric chronology of the archaic Windover Archaeological Site (8BR246), Fla Anthrop. *41* (1988) 365–380.

7 Elster, H., Emmanuel, G., and Weiner, S., Amino acid racemization of fossil bone, J. archaeol. Sci. *18* (1991) 605–617.

8 Fisher, D. L., Holland, M. M., Mitchell, L., Sledzik, P.S., Wilcox, A. W., Wadhams, M. and Weedn, V. W., Extraction, evaluation and amplification of DNA from decalcified and undecalcified United States Civil War bone. J. forens. Sci. *38(1)* (1993) 60–68.

9 Fisher, L. W., Hawkins, G. W., Tuross, N. and Termine, J. D. Purification and partial characterization of small proteoglycans I and II, bone sialoproteins I and II, and osteonectin from the mineral compartment of developing human bone. J. biol. Chem. *262* (1987) 9702–9709.

10 Hassan, A., and Hare, P. E., Amino acid analysis in radiocarbon dating of bone collagen, in: Advances in Chemistry Series, #171, pp. 109–116. ed. G.F. Carter. American Chemical Society, Washington, DC 1978.

11 Logan, G.H, Collins, M. J., and Eglinton, G., Preservation of organic molecules, in: Taphonomy: Releasing the Data Locked in the Fossil Record, vol. 9, pp. 1–24. Eds. P.A. Allison and D.E.G. Briggs, Plenum, New York, NY 1991.

12 Lothrop, S., 14–C dates for Venado Beach, Canal Zone. Panama Archaeologist 3 (1960) 96.

13 Owsley, D., and Jantz, R., (eds), Skeletal Biology of the Plains. Smithsonian Press, Washington, DC 1994.

14 Paabo, S., Molecular genetic investigations of ancient human remains, Cold Spring Harb. Symp. quant. Biol., vol L (1986) 441–446.

15 Paabo, S., Ancient DNA: Extraction, characterization, molecular cloning, and enzymatic amplification. Proc. natl Acad. Sci. USA. *86* (1989) 1939–1943.

16 Sambrook, J., Fritsch, E.F., and Maniatis, T., Molecular Cloning. Cold Spring Harbor Press, Cold Spring Harbor, NY 1989.

17 Swift, M. J., Heals, O. W., and Anderson, J. M., Decomposition in Terrestrial Ecosystems. University of California, Berkeley, CA 1979.

18 Tuross, N., The other molecules in ancient bone: noncollagenous proteins and DNA, in: Molecular Archaeology of Prehistoric Human Bone, pp. 275–294. Eds Joseph Lambert and Gisela Grupe, Springer, Berlin 1993

19 Tuross, N., Behrensmeyer, A. K., and Eanes, D., Strontium increases and crystallinity changes in taphonomic and archaeological bone, J. archaeol. Sci. *16* (1989) 661–672.

20 Tuross, N., and Fogel, M., The archaeological, conservation and scientific challenge of exceptional molecular preservation in the fossil record, in: Proceedings of the Archaeometry Meeting. Ed. David Scott, Getty, Los Angeles, CA 1994.

21 Tuross, N., Fogel, M., and Hare, P. E., Variability in the preservation of the isotopic composition of collagen from fossil bone. Geochim. cosmochim. Acta *52* (1988) 929–935.

22 Tuross, N., and Stathoplos, L., Ancient proteins in fossil bones, in: Methods in Enzymology, Vol. 224, pp. 121–129. Eds E.A. Zimmer, T.J. White, R.L. Cann and A.C. Wilson, Academic Press, London 1993.

23 Wyckoff, R., The Biochemistry of Animal Fossils. Scientechnica, Bristol 1972.