effects. Hence, we believe that we have prepared a fluorescent sphere with an environment-independent emission profile.

These results strongly suggest that the procedures described here can be used to produce a unimolecular compartmented structure in which guest molecules are physically locked and for which the diffusion out of the box is unmeasurably slow. Obviously, it is important to know the detailed structure of the guest-in-box systems, but a discussion on this topic is purely speculative at this point. It can be argued that parts of the guest are within the shell domain or even sticking out of the box without, however, the possibility of being extracted. Both shell and core, as a consequence of the unimolecular structure, have a restricted mobility. (Chir)optical studies, including solvatochromic measurements made with probe molecules, will be required to obtain a more detailed insight into the exact nature of these new containers. A number of applications, such as fluorescent markers for pores in the nanometer range and controlled delivery, are foreseen. Moreover, with these systems it may be possible to study the photochemistry and photophysics of isolated molecules in a well-defined cage.

REFERENCES AND NOTES

1. D. A. Tomalia, A. M. Naylor, W. A. Goddard III, *Angew. Chem.* **102**, 119 (1990); *Angew. Chem. Int. Ed. Engl.* **29**, 138 (1990).
2. J. M. J. Fréchet, *Science* **263**, 1710 (1994).
3. G. R. Newkome, C. N. Moorefield, G. R. Baker, M. J. Saunders, S. H. Grossman, *Angew. Chem.* **103**, 1207 (1991); *Angew. Chem. Int. Ed. Engl.* **30**, 1178 (1991).
4. D. A. Tomalia *et al.*, *Polym. J.* **17**, 117 (1985).
5. K. L. Wooley, G. J. Hawker, J. M. J. Fréchet, *J. Am. Chem. Soc.* **115**, 11496 (1993).
6. D. A. Tomalia and H. D. Durst, *Top. Curr. Chem.* **165**, 193 (1993).
7. E. Buhleier, W. Wehner, F. Vögtle, *Synthesis* **1978**, 155 (1978).
8. M. Maciejewski, *J. Macromol. Sci. Chem.* **17**, 689 (1982).
9. A. M. Naylor, W. A. Goddard III, G. E. Kiefer, D. A. Tomalia, *J. Am. Chem. Soc.* **111**, 2339 (1989).
10. L. A. Paquette, J. T. Negri, R. D. Rogers, *J. Org. Chem.* **57**, 3947 (1992).
11. D. J. Cram, R. Jeager, K. Deshayes, *J. Am. Chem. Soc.* **115**, 10111 (1993).
12. N. Branda, R. Wyler, J. Rebek Jr., *Science* **263**, 1267 (1994).
13. M. Saunders *et al.*, *J. Am. Chem. Soc.* **116**, 2193 (1994).
14. Y. Kikuchi *et al.*, *ibid.* **114**, 10302 (1992).
15. Y. Murakami, O. Hayshida, T. Ito, Y. Hisaeda, *Pure Appl. Chem.* **65**, 551 (1993).
16. C. A. Hunter, *Chem. Soc. Rev.* **1994**, 101 (1994).
17. B. J. Whitlock and H. W. Whitlock, *J. Am. Chem. Soc.* **116**, 2301 (1994).
18. K. Fujimoto and S. Shinkai, *Tetrahedron Lett.* **35**, 2915 (1994).
19. M. P. Pileni, *J. Phys. Chem.* **97**, 6961 (1993).
20. E. M. M. de Brabander-van den Berg and E. W. Meijer, *Angew. Chem.* **105**, 1370 (1993); *Angew. Chem. Int. Ed. Engl.* **32**, 1308 (1993).
21. A typical procedure for the construction of the dense shell is as follows. To a stirred solution of 72 mg (0.010 mmol) of amine dendrimer of the fifth generation (64 end groups) in 10 ml of CH_2Cl_2 with 0.1 ml of triethylamine was added 235 mg (0.65 mmol, 1.01 equivalent per NH_2 end group) of *N*-tBOC-L-Phe hydroxy succinimide ester. After it was stirred overnight, the reaction mixture was diluted with CH_2Cl_2 to 50 ml and subsequently washed with water (three times with 30 ml) and saturated Na_2CO_3 (three times with 30 ml), respectively. Drying (with Na_2SO_4) followed by evaporation of the solvent yielded 190 mg (0.0082 mmol, 82%) of the tBoc-L-Phe–modified dendrimer. All spectral data were in accordance with the proposed structure.
22. A. D. Meltzer *et al.*, *Macromolecules* **25**, 4541 (1992).
23. A remarkable sharp triplet was observed for the box containing eight probe molecules. A comprehensive study of the mobility of the EPR probe and the nature of the radical assemblies is in progress (J. F. G. A. Jansen *et al.*, in preparation).

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DNA Sequence from Cretaceous Period Bone Fragments

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DNA was extracted from 80-million-year-old bone fragments found in strata of the Upper Cretaceous Blackhawk Formation in the roof of an underground coal mine in eastern Utah. This DNA was used as the template in a polymerase chain reaction that amplified and sequenced a portion of the gene encoding mitochondrial cytochrome b. These sequences differ from all other cytochrome b sequences investigated, including those in the GenBank and European Molecular Biology Laboratory databases. DNA isolated from these bone fragments and the resulting gene sequences demonstrate that small fragments of DNA may survive in bone for millions of years.

Biological molecules have varying stabilities over extended periods. Immediately after cell death, these molecules begin rapid degeneration. Nucleic acids have limited life expectancies under physiological conditions, and DNA is particularly susceptible to oxidative and hydrolytic damage. Alterations resulting in abasic sites and other base or sugar modifications quickly destabilize the molecule, producing strand breaks and other degradative changes (*1*). Under physiological conditions, it would be extremely rare to find preserved DNA that was tens of thousands of years old. If biological molecules are to be preserved over geologic time periods, they must be removed from physiological conditions soon after biological death and maintained in that condition. At the same time, the molecules must be protected from other extremes that may be responsible for the nonphysiological conditions, such as heat and pressure. These requirements would preclude the recovery of biological molecules from ancient sources in most instances. However, there have been reports of the persistence of amino acids associated with fossils and dinosaur bones in the sedimentary matrix (*2*, *3*), and recent reports of ancient DNA recovered from insects and plants trapped and preserved in amber have demonstrated the possibility of finding extremely old DNA from ancient organisms (*4*). In this report, we present evidence of the isolation and amplification of DNA from bone material recovered from a Cretaceous period coal bed.

Coal beds are the result of large accumulations of peat in ancient bogs that have been covered by silt, sand, and other muds, sealing the peat from further organic decomposition and eventually resulting in coalification. We recovered two bone fragments associated with coal beds from the Upper Cretaceous Blackhawk Formation of the Mesaverde Group that range in thickness from 1.5 to 8.2 m. These strata represent coastal plain and lower delta plain deposits formed along the western shoreline of a large inland sea known as the Mancos Sea (*5*, *6*). This sea covered much of the western interior of North America during the Cretaceous period. The rock formation is approximately 80 to 85 million years old (*7*). The coal is a high-volatile bituminous type that has an estimated depth of burial of

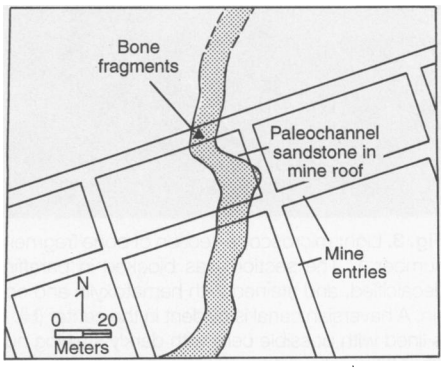

Fig. 1. Map of the mine area and entries, showing the probable path of the sandstone paleochannel in which the bone fragments were found.

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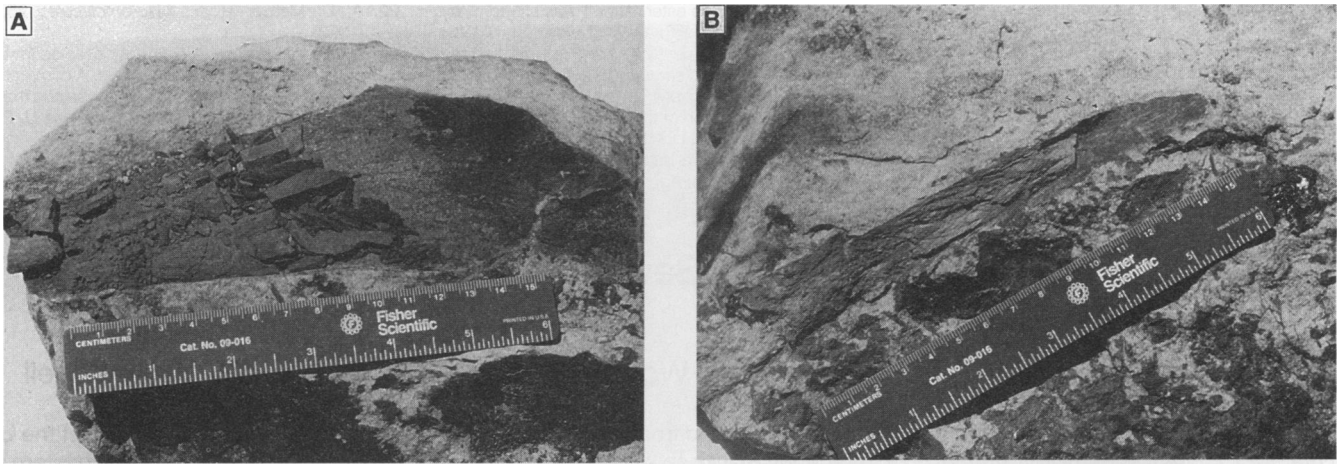

Fig. 2. Bone fragments recovered in the sandstone matrix from the roof of the coal mine. (**A**) Bone number 1. (**B**) Bone number 2. The size of bone fragment 1 is approximately 20 cm by 6 cm by 3 cm and that of bone fragment 2 is 20 cm by 2 cm by 1 cm.

3 km. The temperature of formation may have been 90° to 95°C (6–8).

The strata immediately overlying the coal bed consist of interlaminated very fine– to fine-grained carbonate-cemented sandstone created by overbank and crevasse splay deposition from rivers, and they are sometimes mappable for thousands of feet in underground mine entries (Fig. 1). Fragments from two bones were recovered in one of these channel-fill deposits approximately 30 cm above the top of the coal bed at a depth of 610 m below the surface (Fig. 2). Fractured pieces of siltstone and sandstone containing the bones were placed in sample bags and brought to the surface where they were immediately transported to the laboratory. Bone number 1 appears to be a fragment of a long bone that was at least 6 cm in diameter. Bone number 2 may be a fragment of rib, with the smallest diameter of the remaining fragment being approximately 2 cm. Also exposed in the channel fill in the sample area were coalified plant remains as well as a number of dinosaur tracks. At least 14 different dinosaur species have been differentiated by the tracks found in Blackhawk Formation coal beds (*9*, *10*). The recovery of bone material in the mine has been extremely rare; only a few reports of fossil bone have been made over the life of the mine. On the basis of the circumstantial physical and geologic evidence, it is likely that the bone fragments belong to a Cretaceous period dinosaur or dinosaurs.

The possibility of contamination of ancient samples by contemporary DNA is a serious concern when analyzing ancient DNA sequences. It may not always be possible to determine if a sequence obtained derives from ancient sources by the information contained in the nucleotide sequence alone. It is imperative that every precaution be taken to prevent the introduction of contemporary DNA into the ancient sample.

In the laboratory we removed small pieces of the bone from the surrounding matrix. The bone was well isolated by the siltstone from the accompanying coal and from any fossilized plant material. The bone did not appear to be typically fossilized. The bone texture is brittle and waxy and flaked off when cut with a scalpel. A small piece of bone number 1 was embedded in paraffin, decalcified, and sectioned for light microscopy (Fig. 3). The bone does not seem to have undergone extensive diagenesis, and cell structure remains. Numerous Volkmann or haversian canal-like systems are present and are lined with structures that stain darkly with hematoxylin and eosin and may represent cellular nuclei. Possible osteocytes are present in the matrix along with many lacunae. The lamellae are not arranged in concentric circles, which is sug-

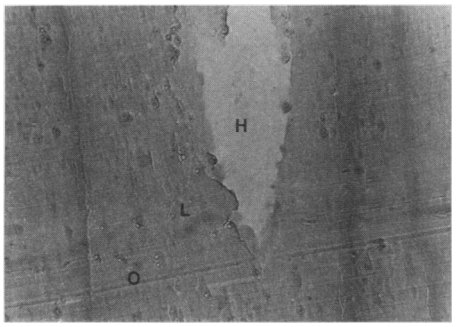

Fig. 3. Light microscope section of bone fragment number 1. The section was blocked in paraffin, decalcified, and stained with hematoxylin and eosin. A haversian canal is evident in the center (H). It is lined with possible cells with darkly staining nuclei. Other osteocytes are present in the matrix (O), also with possible nuclei. Numerous lacunae (L) are also present. An adjacent section stained with trichrome demonstrated the presence of collagen.

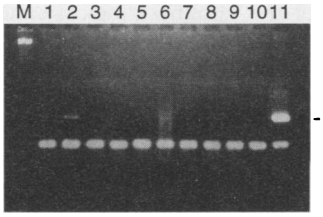

Fig. 4. Amplification product of DNA recovered from the bone fragments, showing the 174-bp fragment produced from primers designed for a conserved region of the gene encoding cytochrome b. Lanes representing negative controls are clean. M indicates the 123-bp marker. Lanes 1 to 3, ancient DNA amplifications with a positive result in lane 2. Lanes 4 to 6, extraction reagent controls. Lanes 7 to 10, amplification-negative controls (all amplification reagents with double-distilled H_2O in place of ancient DNA). Lane 11, positive control.

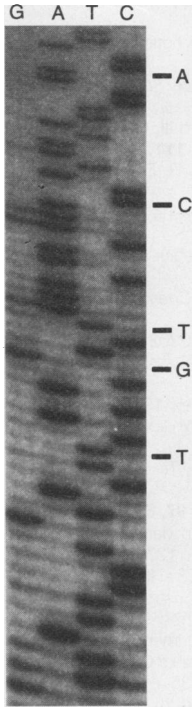

Fig. 5. Sequencing gel of sequence 3-37, showing regions of polymorphisms compared with the consensus sequence.

gestive of woven rather than lamellar bone. Abundant material that stains with trichrome is evident and may represent residual collagen. Fragments from bone number 2 showed identical histology. Other samples were prepared for elemental analysis with a scanning electron microscope. There was no indication of replacement of the organic material by inorganic minerals, which would occur during permineralization associated with fossilization. The bone has a 2:1 ratio of calcium to phosphate and no detectable silicon and aluminum, a composition similar to that expected of contemporary bone.

Using flame-sterilized instruments, we removed 15 fragments of bone and 3 fragments of the surrounding sandstone matrix for 42 different DNA extractions. Bone extracts were from the interior portions of the fragments that were freshly fractured and had not previously been exposed to any exterior conditions. A small portion of bone or surrounding matrix, approximately 3 mm^3, was placed in a sterile 15-ml conical polypropylene centrifuge tube and crushed with a sterile glass rod. DNA from approximately 0.03 to 1.5 g of powdered material was extracted with the use of either Chelex 100 or guanidine thiocyanate (*11*). Multiple blank extractions using all reagents but not containing any bone were always done in parallel. All reagents for polymerase chain reaction (PCR), including primers, were prepared in double-distilled autoclaved water and filtered through 30,000 molecular weight cellulose filters (Millipore). Preparation of all reagents and the amplification mixtures was done in a protective hood, the interior of which is maintained under ultraviolet irradiation when not in use.

Primers for PCR from various regions of mitochondrial DNA were designed with the use of a consensus of 20 different mammalian sequences, including six sets from conserved regions of the gene encoding mitochondrial cytochrome b (*12*). All of these sets produced fragments of the expected size [all less than 200 base pairs (bp)] with the use of a range of mammalian, avian, and reptilian DNA. They did not amplify a number of different bacterial DNAs. Only one of the six cytochrome b sets was successful in amplifying DNA extracted from the bone. These primers correspond to bases 15,603 to 15,622 and 15,758 to 15,777 of the human mitochondrial sequence (*13*). The lack of amplification with the other primer sets is consistent with the sample being free of contaminating contemporary DNA. Amplification with the noted primer set produced a 174-bp fragment (*14*) (Fig. 4). Negative controls consisting of either blank extractions (all of the reagents used in the extraction of the ancient DNA without any ancient tissue) or double-distilled H_2O were done in parallel to the experimental extractions to monitor contamination in any of the reagents or instruments used in the extraction and amplification of the DNA. The positive amplification products were directly sequenced after asymmetric amplification (*15*). It was only rarely that a positive amplification product was observed. The frequency of successful positive amplifications was 1.8% (9 out of 494).

The PCR is inhibited in some ancient extracts (*16*, *17*). This problem also occurred with some of the bone extracts demonstrated by inhibition of a primer artifact usually seen in control reactions. This phenomenon provided an additional control for detection of contamination in the amplification reagents. A series of 1:2 dilutions of the original extracts in filtered double-distilled H_2O were used as templates for PCR. We observed that amplifications usually did not occur until the dilutions were between 1:2 and 1:8. Amplification was never observed after a 1:32 dilution. If there were contaminants in the reaction reagents, they would not have been diluted and we would have observed amplifications in the extract reactions diluted more than 1:32.

A total of 42 separate extracts were made from different individual samples of the bone fragments or the surrounding matrix. From these extracts, 104 experimental protocols representing over 2880 individual amplifications (including negative controls) were attempted. Of these, we were successful in obtaining amplification in nine reactions. Six of these successful amplifications were from the guanidine thiocyanate extractions. In addition, there were three positive amplifications from experimental protocols in

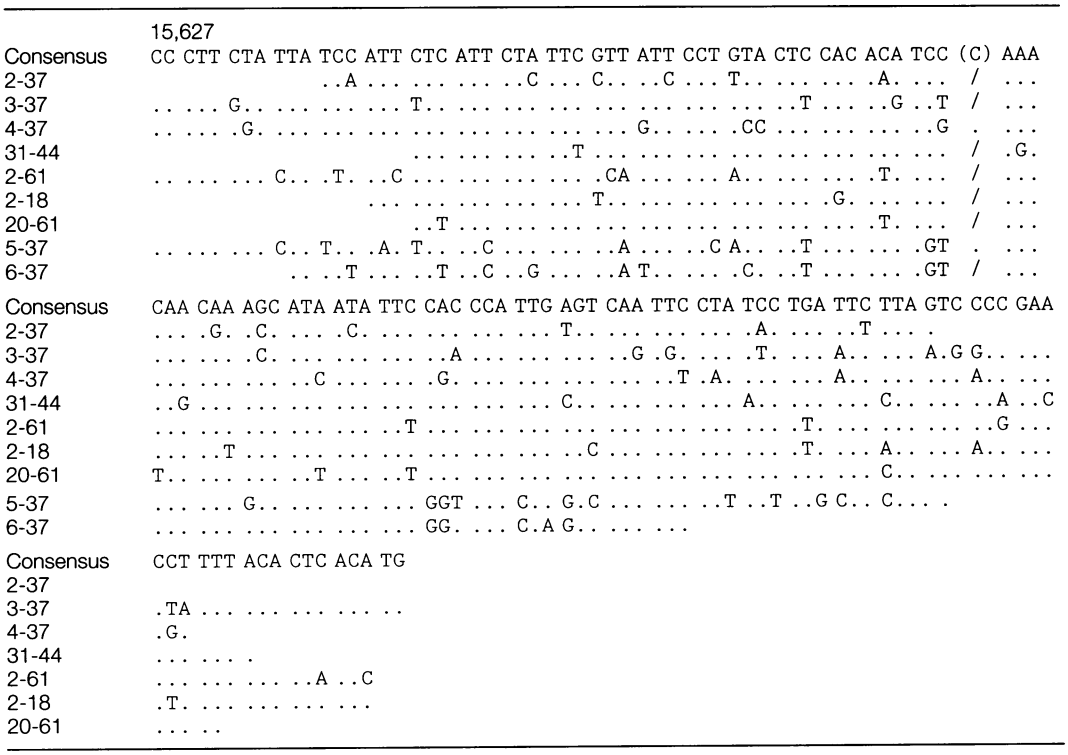

Fig. 6. DNA sequences obtained from the nine amplification products. Seven are from bone fragment 1 and two (5-37 and 6-37) are from bone fragment 2. The consensus sequence was determined from the seven fragments from bone 1 by majority rule. There are nine nucleotide sites that the two bone 2 sequences share that are different from those in the consensus bone 1 sequence. The sequence starts with base 15,627, as numbered from the human mitochondrial sequence (*13*). Slashes indicate a missing base.

which some of the negative controls also demonstrated an amplification product. In all of these latter cases, the contaminating DNA was of human origin, as demonstrated by sequencing. Included in the negative controls were 64 amplifications attempted from extracts of the surrounding sandstone matrix. No amplification or evidence of inhibition was seen in these extracts.

Nucleotide sequences were obtained from the nine clean reaction protocols (Fig. 5), and the resulting sequences are shown in Fig. 6. Seven of these sequences resulted from DNA extracted from bone number 1. The other two sequences derived from bone number 2. There was a significant amount of variation (single base substitutions) within each set of sequences derived from the same bone. The amount of within-group sequence variation sequences from bone 1 was 9.2% (808 sites, 74 differences); in sequences from bone 2, it was 8.9% (168 sites, 15 differences). A consensus sequence for the seven sequences derived from bone 1 was determined by majority rule. The two sequences from bone 2 were compared with the consensus sequence from bone 1 and were determined to differ from it if both bone 2 sequences matched each other and differed from the bone 1 sequence at the same nucleotide position. The resulting consensus sequences differed from each other at 9 of 84 sites (10.4%). The amount of difference between the two consensus sequences is nearly the same as that observed between individual sequences; however, an important observation is that the two bone 2 sequences agree with each other at the nine sites that differ from the bone 1 consensus sequence. Although these bone fragments were found in the same ancient river bed, they were not found as part of what could be determined to be a continuous skeleton. It is possible that the bones represent two different animals.

None of the nine sequences are identical, a phenomenon that may be expected when using a rare and likely damaged template (*18*). If the probability of finding a template for amplification is rare, which is likely in these ancient DNA extractions, it may be that all of the resulting daughter amplicons can trace their lineage to a single original molecule. If this molecule was slightly damaged, also likely under the conditions of this experiment, the random insertion of bases during repair or replication by the polymerase could result in a different nucleotide sequence for each positive amplification product (*19*).

We compared each of the nine sequences independently with all published sequences of cytochrome b using the current GenBank and European Molecular Biology Laboratory databases (updated 2 June 1994) with the National Center for Biotechnology Information BLAST network service (version 1.3.13 MP 3-Mar-94) (*20*). Sequences represented in the database include those from mammals, birds, reptiles, amphibians, and insects. In addition, other partial sequences not represented in these databases were obtained in our laboratory for ostrich, rhea, emu, turkey, box turtle, garter snake, and alligator. We also used the two consensus sequences described above, one representing each of the two bone fragments, as the query sequence in the same database. The best match was between the consensus sequences for the two bone fragments, with a 10.4% difference over 84 bases compared. This is comparable to the difference seen between many species of mammals for this region of cytochrome b (*12*). When compared with the bone fragment sequences, most contemporary database sequences had a nearly equal number of differences, grouping around 30%, with the largest difference, 62%, found between the honey bee sequence and one of the bone fragment sequences. The nucleotide sequences from the bone were not significantly closer to the bird or reptile sequences, as compared with mammal sequences. It seems that this limited region of the gene encoding cytochrome b is too short for use in phylogenetic analysis of distantly related organisms (*21*). When the putative amino acid sequence is determined on the basis of the consensus nucleotide sequence, it is interesting that mammals and birds are represented approximately equally in the nearest matches. However, these matches are only in the range of 60% homology.

Although we were not successful in obtaining DNA sequences from a large number of different loci, some of which are better suited for phylogenetic analysis, this result demonstrates that the recovery of DNA from well-preserved Cretaceous period bone may be possible.

REFERENCES AND NOTES

1. T. Lindahl, *Nature* **362**, 709 (1993).
2. L. R. Gurley, J. G. Valdez, W. D. Spall, B. F. Smith, D. D. Gillette, *J. Protein Chem.* **10** (no. 1), 75 (1991).
3. G. B. Curry, in *Molecular Evolution and the Fossil Record*, T. W. Broadhead, Ed. (Short Courses in Paleontology: No. 1, Paleontological Society of America, Knoxville, TN, 1988), pp. 20–33.
4. R. DeSalle, J. Gatesy, W. Wheeler, D. Grimaldi, *Science* **257**, 1933 (1992); R. J. Cano, H. N. Poinar, D. W. Roubik, G. O. Poinar Jr., *Med. Sci. Res.* **20**, 619 (1992); R. J. Cano, H. N. Poinar, N. J. Pieniazek, A. Acra, G. O. Poinar Jr., *Nature* **363**, 536 (1993); H. N. Poinar, R. J. Cano, G. O. Poinar Jr., *ibid.*, p. 677.
5. J. K. Balsley, *Am. Assoc. Pet. Geol. Guide* (1982), pp. 263–275.
6. H. H. Doelling, *Utah Geol. Surv. Monogr. Ser. 3* (1972), p. 571.
7. L. F. Hintze, *Brigham Young Univ. Geol. Stud. Spec. Publ. 7* (1988), pp. 53–60.
8. The determination of the precise temperature of formation is problematic. This estimate is based on a vitrinite reflectance of 0.65. Vitrinite reflectance is both temperature- and time-dependent.
9. L. R. Parker and J. K. Balsley, in *Dinosaur Tracks and Traces*, D. D. Gillette and M. G. Lockley, Eds. (Cambridge Univ. Press, Cambridge, 1989), pp. 353–359.
10. L. R. Parker and R. L. Rowley Jr., *ibid.*, pp. 361–366.
11. The 3-mm^3 pieces of bone were placed in a 15-ml sterile disposable conical centrifuge tube and crushed with the end of a sealed glass rod that had been flame sterilized. Approximately 0.5 to 1.5 g of the resulting power was either extracted overnight at 56°C with rotation in 2 ml of 4 M guanidine thiocyanate, 0.1 M NaCl, 0.014 M beta-mercaptoethanol, 0.025 M EDTA, and 0.5% sarkosyl, or 0.03 g was used in a Chelex 100 (Bio-Rad, Richmond, CA) extraction (*22*). DNA was precipitated from the guanidine thiocyanate after low-speed centrifugation to sediment the particulate matter by addition of an equal volume of 7.5 M ammonium acetate and 2.5 volumes of 100% ethanol. It was placed at −20°C for 1 hour, centrifuged at 2000*g* for 30 min, and suspended in 500 μl of sterile TE [0.01 M tris (pH 7.8) and 0.001 M EDTA].
12. D. Irwin, T. D. Kocher, A. C. Wilson, *J. Mol. Evol.* **32**, 128 (1991).
13. S. Anderson *et al.*, *Nature* **290**, 457 (1981).
14. Forty cycles consisting of 93°C for 30 s, 48°C for 2 min, and 72°C for 1 min were done on a Perkin- Elmer Cetus 480 thermal cycler in a volume of either 100 μl or 25 μl with either 50 μl of template extract or 5 to 7 μl of template extract and overlaid with 60 μl of decane. The reaction mixture contained 67 mM tris (pH 8.8), 2 mM $MgCl_2$, 16.7 mM $(NH_4)_2SO_4$, 10 mM beta-mercaptoethanol, 0.2 mM deoxynucleotide triphosphate, 1 μM primer (5′-CCAAACAAACTAGGAGGCGT-3′, 5′-ACTGGTTGGCTGCCAATTCA-3′), and 1 μM DNA (5 to 50 μl of extract). Analysis of 20 μl of the final products was done on 2.5% NuSieve agarose (FMC Bioproducts, Rockland, ME). Bands were excised and melted in 500 μl of sterile double-distilled H_2O at 70°C for 10 min. Two microliters of this was subjected either to an additional round of balanced amplification for use in cloning or to asymmetric amplification with one of the original primers at one-twenty-fifth to one-fiftieth of the original concentration.
15. Template for sequencing was generated by asymmetric amplification (*23*). Primers were the same as for the amplification and were used at a concentration of 100 pmol per reaction. Dideoxy sequencing with Sequenase was done as outlined by the manufacturer with the use of ^{32}P (U.S. Biochemical, Cleveland, OH). The 8% polyacrylamide gel was dried and exposed to XAR film for periods ranging from 2 hours to overnight. The gel was read and entered into the database by hand.
16. E. Hagelberg, B. Sykes, R. Hedges, *Nature* **342**, 485 (1989).
17. S. R. Woodward, M. J. King, N. M. Chiu, M. J. Kuchar, C. W. Griggs, *PCR Methods Appl.* **2**, 244 (1994).
18. S. Paabo, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1939 (1989).
19. If the phenomenon of jumping PCR occurred in these samples, it would have been between two mitochondrial molecules derived from a single bone of an individual. These molecules should be essentially homologous; any sequence differences would reflect random damage to the molecules. If jumping occurred between two or more randomly damaged molecules, it would be difficult to differentiate from other types of artifactual sequences generated by polymerase error.
20. S. F. Altschul *et al.*, *J. Mol. Biol.* **215**, 403 (1990).
21. T. D. Kocher *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6196 (1989).
22. P. S. Walsh, D. A. Metzger, R. Higuchi, *Biotechniques* **10** (no. 4), 506 (1991).
23. U. B. Gyllensten and H. A. Erlich, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7652 (1988).

24. We thank the mine personnel who discovered and provided the bone samples. R. P. Evans provided assistance with the sequence analysis. R. A. Heckman and the electron optics laboratory provided help with the microscopy and analysis.

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