Isolation and characterization of DNA from archaeological bone

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SUMMARY

DNA was extracted from human and animal bones recovered from archaeological sites and mitochondrial DNA sequences were amplified from the extracts using the polymerase chain reaction. Evidence is presented that the amplified sequences are authentic and do not represent contamination by extraneous DNA. The results show that significant amounts of genetic information can survive for long periods in bone, and have important implications for evolutionary genetics, anthropology and forensic science.

1. INTRODUCTION

Until recently, the study of evolutionary genetics has relied largely on extrapolation of information from living organisms to reconstruct past history. The discovery that DNA can be recovered from the remains of extinct animals has provided a new source of primary evidence to add to the genetic information gained from extant species. Higuchi et al. (1984) were the first to report the retrieval of informative DNA sequences from an extinct animal after they succeeded in cloning mitochondrial DNA (mtDNA) sequences from a museum specimen of the quagga, a member of the genus Equus that became extinct more than 100 years ago. This was followed by the cloning of repetitive nuclear DNA sequences from the skin of an ancient Egyptian mummy (Paabo 1985). However, only a very low proportion of the DNA was original, most of it being derived from extensive microbial contamination of the specimens.

The early technical difficulties of cloning DNA from the remains of ancient organisms were overcome after the development of the polymerase chain reaction (PCR) (Saiki et al. 1985). A single intact copy of a target DNA sequence is sufficient for PCR, making it an ideal tool for the study of ancient DNA (Pääbo 1989; Pääbo et al. 1989), and for forensic science (Higuchi et al. 1988; Jeffreys et al. 1988), as it can be used for biological samples containing very little or degraded DNA. PCR has been used for the analysis of mitochondrial DNA from a variety of soft tissue remains, including a 7000 year old human brain preserved in a peat bog (Pääbo et al. 1988) and preserved museum skins (Thomas et al. 1989), and chloroplast DNA from a 17–20 million-year-old magnolia leaf (Golenberg et al. 1990). Animal mtDNA has several useful features which have already been exploited in phylogenetic and evolutionary studies, including its small size, fast rate of evolution and maternal mode of inheritance. In addition, the entire human mitochondrial genome of 16,569 bases has been sequenced (Anderson et al. 1981). The high number of mitochondria in mammalian cells (10^3–10^4 copies per cell) means there is significant likelihood of detecting mtDNA, even in highly degraded samples.

Whereas these studies showed that DNA persists in ancient remains, the study of archaeological bone and teeth samples provides the opportunity for population analysis because they are so much more abundant than soft tissue remains and generally better preserved. The amplification of mtDNA from ancient bone was demonstrated recently by a number of workers (Hagelberg et al. 1989; Horai et al. 1989; Hanni et al. 1990). However, all of these studies used human skeletal material and there is a real possibility that the results could have been artefacts caused by contamination of the bones with traces of modern human DNA.

We show in this paper that authentic DNA can be recovered from bones, and describe in detail the methods used to extract and amplify DNA sequences from ancient bone, showing examples of analysis of human and animal samples from various ages and contexts. The results open up new research possibilities in many areas of biology, particularly anthropology, population genetics and molecular evolution. Practical applications in forensic science are already on record and will be described elsewhere.

2. MATERIALS AND METHODS

(a) Cleaning of samples

It is essential to minimize contamination of the bones by modern DNA. The bones were always handled with gloved hands or forceps to avoid contamination by skin cells or sweat. Whenever possible, work was done on freshly excavated, unwashed bones, as it has been noted that careless washing and drying of bones, and storage of the bones while still damp, can lead to the growth of mould and accelerate
the decay process. In any event, it is not known what effect even careful washing and drying has on the preservation of DNA.

With unwashed bones, excess soil was first removed by scraping with a scalpel blade. The bone pieces were cleaned with an abrasive jet of fresh aluminium oxide grit (fine quality, size No. 1), in an Airabrasive 6500 unit (S.S. White Industrial Products, Piscataway, New Jersey). The powdered bone and aluminium oxide were removed from the cleaned bone using a soft brush, and the pieces broken into small fragments (0.5–1 cm) with a hammer or pliers, depending on the shape or hardness of the sample. The small fragments of bone could then be reduced to fine powder in a freezer mill (Spex Industries Inc., Edison, New Jersey) refrigerated with liquid nitrogen, in a sample tube with a capacity of 2–4 g of bone. The sample tubes and impactors were cleaned and sterilized between use to prevent carryover between bone samples. After grinding, the bone powder was stored in sterile containers at −20 °C.

(b) DNA extraction

Three different methods for the extraction of DNA from tissue samples were tested. In the first method (Graham et al. 1978), the bone powder was incubated in a homogenizing solution containing ethylene diamine-tetra-acetic acid (EDTA) to chelate high concentrations of calcium, sodium dodecyl sulphate (SDS), sodium perchlorate, chloroform, and phenol, followed by low speed centrifugation and re-extraction of the aqueous phase with chloroform, and finally precipitation with two volumes of ice-cold absolute ethanol. The second method was that used by Pääbo et al. (1988) in which samples were first incubated for 3 h with agitation in a lysis buffer containing collagenase, after which SDS, dithiothreitol, and proteinase K were added and incubation continued for approximately 20 h. This was followed by extraction with phenol and chloroform and finally by centrifugation-driven dialysis using Centricon 30 microconcentrators (Amicon Division, W. R. Grace & Co., Danvers, Massachusetts) to concentrate the extract.

The third method tested was a variant of the method described by Maniatis et al. (1982), in which the sample was incubated in a lysis solution consisting of EDTA, proteinase K, and the detergent N-lauroylsarcosine, followed by extraction with phenol and chloroform and finally dialysis centrifugation as above. All three methods described yielded DNA from bone, but the variant of the method of Maniatis et al. was the simplest and fastest, and seemed to give the best quality DNA for subsequent amplification. This was the method used throughout this study and described below in detail.

Bone was decalcified by suspending powdered or very thinly sectioned bone in 50 volumes of 0.5 M EDTA, pH 8.5, and incubating with agitation for 72 h with two fresh changes of EDTA. Complete decalcification of the bone samples did not prove necessary for successful extraction of DNA, and in several cases actually reduced the yield of DNA. However, short EDTA washes were useful to remove soluble contaminants and some of the dark brown colouration (probably due to humic acids or iron from the soil) from the powdered bone before incubation in the lysis buffer. DNA was extracted by incubating 1–1.5 g bone at 37 °C for 18–24 h in 10 volumes of a lysis solution consisting of 0.5 M EDTA (pH 8.5), 100 µg ml⁻¹ proteinase K, and 0.5% N-lauroylsarcosine, followed by one extraction with phenol (freshly opened water-saturated phenol, glass distilled grade, Rathburn Chemicals Limited, Wakeburn, Scotland; equilibrated with 20 mM Tris-HCl, pH 7.5), two extractions with phenol/ chloroform (1:1), and finally chloroform to remove all traces of phenol. The aqueous phase was desalted and concentrated by centrifugation-driven dialysis at room temperature, using Centricon 30 microconcentrators as recommended by the manufacturers, in a Beckman GPR benchtop centrifuge with a GA-24 fixed angle rotor at 6000 r.p.m. The final volume of the extracts was 100–200 µl, and 10 µl portions were electrophoresed through 1% agarose gels with DNA markers of known size, followed by ethidium bromide staining to visualize the DNA under ultraviolet light.

Disposable sterile containers and pipette tips were used throughout, as well as sterile reagents and solutions dedicated solely for work on ancient DNA. All the solutions used were prepared with sterile water for irradiation obtained in sealed 11 bottles (Baxter Healthcare Ltd., Thetford, Norfolk). Blank control extractions (containing no bone) were performed in parallel with the bone extractions to monitor possible sources of contamination from reagents and equipment, for instance from aerosols inside laboratory centrifuges. Whenever possible, equipment used for other purposes in the laboratory was washed or sterilized before use. The dialysis centrifugation of the bone extracts was carried out in a lidded rotor used solely for this purpose.

(c) Polymerase chain reaction

DNA amplifications were done by the method recommended by Perkin Elmer Cetus in 25 µl reactions containing two units Thermus aquaticus (Tag) DNA polymerase. The reaction buffer consisted of 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.1% gelatin, 200 µM each dNTP and 20 pmol of each primer. Bovine serum albumin (160 µg ml⁻¹) was added to the reactions to help overcome the effect of a powerful PCR inhibitor of unknown origin which was present in many of the bone extracts. The commercial GeneAmp® 10× PCR buffer, MgCl₂, and nucleotides were purchased from Perkin Elmer Cetus as an additional safeguard against contamination of the reagents with DNA from the laboratory.

The primers used in this work were the highly conserved mtDNA primers (Kocher et al. 1989) which specify a 375 base pair (b.p.) fragment of the cytochrome b gene (primers L14841, H15149) and 499 b.p. fragment of the 12S rDNA gene (primers L1091, H1478). These primers amplify homologous sequences in a wide range of animal species and have been used to generate sequence data for phylogenetic analysis (Kocher et al. 1989; Thomas et al. 1989; Irwin et al. 1991). Other primers were those used to amplify and sequence a 422 b.p. fragment of the hypervariable region of human mtDNA (primers L29, H408 (Vigilant et al. 1989), and a 121 b.p. fragment in a small noncoding region (region V) containing a useful anthropological marker (primers A, B) (Wrischnik et al. 1987). The primers were synthesized in an Applied Biosystems DNA synthesizer model 381A and used for PCR and sequencing without further purification.

Two µl of bone DNA extract, representing about 1–2% of the total DNA extracted from 1–1.5 g bone, were used in each amplification reaction. A blank reaction containing no DNA was set up in each experiment to monitor any possible contamination of the PCR reagents. The amplifications of the cytochrome b, 12S rRNA and region V fragments consisted of 35 cycles of denaturation at 94 °C (1 min), annealing at 55 °C (1 min), and extension at 72 °C (1 min). The denaturation step of the first cycle was lengthened to 5 min to ensure complete denaturation of the genomic DNA. The conditions for amplification of the hypervariable region were identical, but the extension temperature was increased to 74 °C. PCR amplifications were performed in a Perkin Elmer Cetus DNA Thermal Cycler.

Portions (10 µl) of the PCR reactions were generally electrophoresed on 1.5% agarose minigels and stained with
ethidium bromide to visualize the DNA fragments under ultraviolet light. The PCR products obtained with primers A and B were electrophoresed on 1 mm thick 7.5% polyacrylamide gels and visualized by silver staining (Bio-Rad Silver Stain, Bio-Rad, Richmond, California).

(d) Asymmetric PCR and DNA sequencing

The amplification products were sequenced directly using a modified unbalanced PCR method (Gyllensten & Erlich 1988) to generate a single-stranded product suitable for sequencing. First, a normal PCR amplification of the DNA sample was performed using equal concentrations of both primers. A second preparative reaction was carried out, using 1 µl of the first reaction as the template and omitting one of the two primers altogether. The limiting primer, which is carried over from the first PCR reaction, is used up after several PCR cycles and the reaction then proceeds using the second primer only, leading to the formation of an excess of single-stranded PCR product that is a suitable substrate for sequencing. The second round of PCR was carried out in triplicate 100 µl reactions to increase product yield. The three reactions were pooled after the asymmetric PCR, and unincorporated primers and nucleotides removed by four repeated steps of dialysis centrifugation, washing with 2 ml sterile water after each step. The final volume was approximately 50 µl, and sequencing was done by using 7 µl of the concentrate and the limiting primer from the second amplification. A commercial kit (Sequenase; US Biochemical Corp.) was used for dideoxy chain-termination sequencing (Sanger et al. 1977) and the products were electrophoresed on 6% denaturing polyacrylamide gels and autoradiographed for 2–4 days.

3. RESULTS

The results from four typical bone specimens (three of which are shown in figure 1) are presented, as follows: (1) a human male femur from a medieval cemetery at Abingdon, Oxfordshire, with a radiocarbon date of 750 ± 80 years before present (BP); (2) a recent forensic sample, a human tibia from a mass grave in Argentina, buried for approximately 13 years; (3) a pig bone, originally from a leg of pork, part of the provisions aboard the ship Mary Rose, which sank off the south coast of England in 1545; (4) a fragment of a human fibula from Polynesia, pre-1778 (before European contact). All four samples had been excavated by archaeologists, and had been stored for various lengths of time in cardboard boxes or plastic bags.

A fragment about 2 cm² was removed from each bone sample and DNA was extracted as described above. We have shown that in the order of 1–10 µg DNA are recovered typically from 1–1.5 g bone (Hagelberg et al. 1989), and although the bulk of the DNA, including the high molecular mass component, stems from microbial contamination, it serves as a useful carrier for the highly degraded authentic bone DNA. MtDNA fragments were amplified successfully from all four bone DNA extracts. Figure 2 shows the results of PCR of the first three DNA samples with the universal cytochrome b and 12S rRNA primers, and with the human D-loop (hypervariable region) primers. All the extraction blank controls and PCR controls were negative, indicating that there was no detectable contamination of the samples during the bone extractions or setting up of the amplification reactions. PCR with the cytochrome b primers yielded the expected 375 b.p. fragment from the three DNA samples, although the medieval human bone produced a fainter band than the pig or modern bone DNA. The 12S rRNA primers produced a certain amount of non-specific amplification from the medieval bone but gave the expected product with the remaining two samples. The relatively poor results of the medieval human bone DNA with the primer systems in this study were assumed to be due to poorer state of preservation of this bone and greater DNA damage, as shorter DNA fragments (100–200 b.p.) could be amplified quite efficiently.

On one occasion very slight amplification of the pig sample was noticed with the human D-loop primers (figure 2). This had never been observed before with

Figure 1. Three bone specimens from which DNA was recovered. (a) Human femur fragment, radiocarbon date 750 ± 80 years BP, from the medieval cemetery at Abingdon, Oxfordshire; (b) human tibia fragment, buried approximately 13 years, from a mass grave in Argentina; (c) 16th century pig bone, originally from a leg of pork, recovered from the wreck of the Mary Rose.

Figure 2. PCR amplifications of mtDNA from samples numbered (1) to (5), as follows: (1) DNA from medieval human bone; (2) DNA from 20th century human bone; (3) DNA from 16th century pig bone; (4) extraction blank control, containing no bone; (5) PCR blank control. The amplified products were: cyt b, 375 b.p. (mtDNA bases 14808–15182); 12S rRNA, 449 b.p. (mtDNA bases 1057–1505); D-loop, 442 b.p. (mtDNA bases 8–429).
Figure 3. Direct sequencing of a fragment of pig cytochrome b gene amplified from DNA extracted from a 16th century pig bone. Sequencing was done with primer H15419. This sample and was probably due to slight contamination of the pig bone DNA with extraneous human DNA from the laboratory in this particular PCR experiment. The D-loop primers also produced non-specific amplification with the Argentinian sample, particularly a strong band at 800–900 b.p. The product of the pig cytochrome b PCR was sequenced to establish that it was indeed authentic bone DNA and not the result of contamination. Figure 3 shows the sequencing autoradiograph after asymmetric amplification and direct sequencing, and the 136 bases which were read (bases 14931 to 15066 in the human reference sequence (Anderson et al. 1981)). The sequence obtained (read independently by three observers) corresponded to the pig cytochrome b sequence (Irwin et al. 1991) in a region where there are 33 base differences (24%) between the human and pig sequences. This is unequivocal evidence that the sequence was amplified from authentic bone DNA and not contamination. Modern genomic pig DNA had never been handled in this laboratory.

DNA from the Polynesian bone was amplified with the region V primers A and B. The usual amplification product of these primers is 121 b.p. and carries two 9 b.p. repeats, one of which is frequently deleted in people of East Asian origin (Wrischnik et al. 1987), giving a 112 b.p. fragment after amplification. In modern populations from central and eastern Polynesia, mtDNA with the 9 b.p. deletion is the predominant, often sole molecular species (Hertzberg et al. 1989). The PCR product of the bone extract was shown on a polyacrylamide gel to be shorter than the usual 121 b.p. band, and on sequencing was found to carry the 9 b.p. deletion (figure 4).

4. DISCUSSION

The studies described here show that it is relatively straightforward to amplify mtDNA sequences a few hundred bases long from well-preserved archaeological bone. Moreover, even larger sequences up to 1 kb were amplified from the forensic bone sample (data not shown). We have also amplified polymorphic nuclear sequences less than 100 b.p. from other recent (ca. 10 years) forensic bones, but so far have not attempted to repeat this on older bones.

The most contentious aspect of PCR bone analysis is the question of the ultimate source of the amplified DNA: endogenous bone DNA or extraneous con-
contamination? The sequencing data of the pig and Polynesian bones show that endogenous bone DNA is being amplified; nevertheless, the extreme sensitivity of PCR presents difficulties as extraneous or contaminating DNA sequences, for instance from microorganisms in the tissue remains, from shed cells of archeologists handling the specimens, or from the products of previous PCR reactions carried over in laboratory reagents and equipment, can be amplified as readily as the target sequences. Although the problem of carry-over contamination between PCR reactions might be controlled to a certain extent by a recently described method of post-PCR sterilization (Cimino et al. 1991), all sources of contamination need to be monitored carefully. Non-specific amplification products also occur occasionally with ancient DNA extracts, probably due to the high levels of extraneous microbial DNA, as shown with the example of the Argentinian bone sample. However, the real problem when working with ancient human tissue is contamination with modern human DNA. Pääbo et al. (1988) addressed the problem of verifying the authenticity of ancient human DNA and found, as we have, that laboratory contamination can be monitored by control extractions and amplifications, and by performing repeated independent extractions from the same individual. These workers also observed that the size of the amplified products was a good indicator of the degree of contamination, as ancient DNA yielded only fragments in the order of 100 b.p. whereas DNA from modern contamination almost always gave rise to longer PCR products.

We also monitor contamination by checking the repeatability and reproducibility of the results, and have replicated up to 10 independent extractions of bone samples from the same individual with identical results, but in our hands the size of the amplified product is not a good guide of contamination as we consistently recover sequences of 300–400 b.p. from ancient bone, significantly longer than reported for soft tissue remains (Pääbo et al. 1989). In addition to the precautions taken to avoid contamination in the laboratory and the measures taken to monitor such contamination when it does occur, we recommend the examination of animal bones recovered from the same archaeological context and handled in the same manner as human bones as a prudent control to detect possible contamination from handling.

It is difficult to define what constitutes a well-preserved bone from the point of view of DNA survival, as a systematic survey of DNA in archaeological bones remains to be done. It is clear that the age of the bone has relatively little to do with DNA content and the burial conditions will probably be shown to be crucial. But even here we have found inconsistencies, as bones from the same site have given disparate results in DNA recovery; for example, of five 17th century bone samples from the English Civil War cemetery in Abingdon, two were very friable and did not yield amplifiable DNA, although all were recovered from the same mass grave and were from young men of similar age (as determined by the state of epiphyseal fusion).

In conclusion, we have established that DNA can be recovered from ancient bone, often less degraded than the DNA recovered from soft tissue remains, and that mtDNA sequences can be amplified consistently from many archaeological samples. The contamination problems outlined above and the possibility of PCR artefacts such as 'jumping PCR' (Pääbo et al. 1990) mean that caution will be necessary in the interpretation of the results of any population surveys utilizing ancient bone. In addition, archeologists and conservators will need to learn of the potential for genetic information in excavated skeletal remains, and to develop appropriate methods for the removal and storage of samples for future study.

We are now beginning to work on a wider range of bone samples, and although the oldest bones we have studied so far date to only about 5000 years BP (Hagelberg et al. 1989), the analysis of chloroplast DNA from a 17–20 million-year-old magnolia leaf (Golenberg et al. 1990) suggests that in exceptional circumstances DNA may survive indefinitely, which augurs well for the study of hominid evolution. If, as seems likely, nuclear DNA sequences can also be recovered from ancient samples, the amount of information accessible will be much increased. These technological advances will have important implications for research in anthropology as first-hand genetic data will add another dimension to the more traditional sources of information on past populations, such as linguistics, culture and morphology. Forensic work is an obvious application of these techniques, and nuclear polymorphisms in DNA recovered from forensic bone have already proved sufficiently informative for kinship relations to be determined. Although archaeology, anthropology and forensic science will probably profit most from PCR bone analysis, these methods will also be applicable to studies of evolutionary and population biology, for example in resolving questions of the phylogenetic relations between extinct and living taxonomic groups.

We thank the Oxford Archaeological Unit, the Argentine Team of Forensic Anthropology, the Mary Rose Trust and the American Museum of Natural History for kindly donating bone samples for this study. We are grateful to Rosalind Harding and Douglas Higgs for helpful comments on this manuscript. This work was supported by a research grant under the NERC Special Topic in Biomolecular Palaeontology.

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