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Extraction, Evaluation, and Amplification of DNA from Decalcified and Undecalcified United States Civil War Bone

REFERENCE: Fisher, D. L., Holland, M. M., Mitchell, L., Sledzik, P. S., Webb Wilcox, A., Wadhams, M., and Weedn, V. W., **"Extraction, Evaluation, and Amplification of DNA from Decalcified and Undecalcified United States Civil War Bone,"** *Journal of Forensic Sciences*, JFSCA, Vol. 38, No. 1, January 1993, pp. 60–68.

ABSTRACT: Deoxyribonucleic acid (DNA) was extracted from documented skeletal specimens of U.S. Civil War soldiers to determine the need for decalcification prior to extraction. The polymerase chain reaction (PCR) was performed to determine if the calcification state had an effect on the ability to amplify the extracts and to determine how successful amplification would be with these aged specimens. Bone samples were pulverized to a fine powder and divided into two sets. One set of samples was decalcified and the other set left undecalcified. Both sets were extracted using an organic procedure. The results demonstrate that decalcification is not a necessary step in the extraction process and that the yield of DNA is generally two times greater when decalcification is omitted. Furthermore, the calcification state had no effect on the ability to perform the PCR. Although the extracted DNA was very degraded, a 410 base pair (bp) segment of the mitochondrial DNA (mtDNA) control region was amplified. These results suggest that DNA can be extracted and amplified from 125 year old bone without decalcification, which may assist in the identity of modern and historic forensic specimens.

KEYWORDS: physical anthropology, deoxyribonucleic acid (DNA), human bone DNA, historical skeletal remains, forensic DNA typing, mitochondrial DNA (mtDNA)

As DNA typing technology matures, the potential to retrieve information from human skeletal remains assists scientists in answering questions ranging from the migration pattern of American Indians [*1*] to solving forensic questions of human identity [*2–6*].

Received for publication 5 March 1992; revised manuscript received 7 May 1992; accepted for publication 11 May 1992.

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Studies have shown that bone can withstand various harsh environmental conditions and yield DNA that can be correctly typed by techniques using the polymerase chain reaction (PCR) [*5,7*]. Furthermore, DNA has been obtained from bone specimens greater than 5500 years old [*8–10*]. Lee et al. [*3*] reported recovering 1.5 to 10 μg DNA/mg from spongy bone and 0 to 500 ng DNA/mg from compact bone using a conventional organic extraction procedure. Although high levels of DNA were recovered without using a decalcification step, the specimens were less than one year old. Several groups have reported using a decalcification procedure prior to extracting DNA from aged and ancient bone specimens [*5,8,9,11,12*]. Decalcification is a timely step and can take up to 20 days to complete [*12*].

A large portion of human skeletonized remains requiring DNA analysis will be aged (that is, greater than one year of age). Therefore, it is important to determine if decalcification is a necessary step prior to the extraction of DNA from aged bone. The National Museum of Health and Medicine, Armed Forces Institute of Pathology (formerly the Army Medical Museum), maintains a collection of more than 2000 documented skeletal specimens from Confederate and Union Civil War soldiers killed or injured in battle [*13*]. These specimens were acquired during a collection program initiated by the Surgeon General of the Army in 1862. Specimens were forwarded to the museum by surgeons or picked up on battlefields several years after the war. A majority of these specimens were sent with tissue adhering; maceration and preparation for dry mounting was conducted at the museum. Documentation relating to the specimens usually includes name, date of injury, date of death or surgical intervention, rank, regiment, surgeon's records, and types of medical treatment received. Eight bone specimens from U.S. Civil War soldiers were obtained from the National Museum of Health and Medicine to determine the significance of decalcification on DNA isolation from bone and to evaluate the efficiency of amplification by PCR.

The condition of extracted DNA determines the types of analyses that are feasible. Although undegraded bone may yield sufficient quantities of high molecular weight DNA suitable for restriction fragment length polymorphism (RFLP) analysis, bone samples yielding degraded DNA, can only be typed by a method using the PCR [*3–6*]. However, it is not always possible to obtain amplification of nuclear genes, perhaps due to insufficient template DNA. In these instances, mitochondrial DNA (mtDNA), which can be present in several thousand copies per cell, may be amplified and can yield valuable information [*14*]. Mitochondrial DNA is unique in that it is maternally inherited [*15,16*]. The noncoding portion of mtDNA, the control region, contains two hypervariable segments rich in polymorphism among unrelated individuals [*2,17*]. Although mtDNA typing is noninformative in paternity cases, sequences obtained from this region can be useful in cases of questioned identity [2]. Furthermore, maternal relatives, even those several generations removed, can serve as appropriate references.

In this study, segments of mtDNA of 97, 210, 343, 410, and 478 base pairs (bp) were amplified. The success of amplification will be presented.

Materials and Methods

The specimens used in this study were donated by the National Museum of Health and Medicine. Bone sample location and bone type information pertaining to the eight specimens is presented in Table 1.

Extraction

All specimens were cleaned with a sterile cotton tipped swab wetted first with distilled water then with 95% ethanol. The samples were crushed with a chisel and hammer, and

TABLE 1—*Specimen data.*

Specimen	Bone Sample Location	Bone Type
1	Proximal left femur	Cortical
2	Right temporal (mastoid)	Cortical and cancellous
3	Right sphenoid	Cancellous
4	Left temporal (squamosal)	Cortical
5	Left temporal (petrous)	Cortical
6	Humeral shaft	Cortical
7	Left femoral head	Subchondral
8	Proximal left tibia	Cortical and cancellous

then pulverized to a fine powder with a Tekmar Tissumizer (Tekmar Company, Cincinnati, OH). It was not possible, however, to completely pulverize the femoral cortical bone due to its extreme hardness. The specimens were then divided into two 1 g sets. One set was decalcified by the Hochmeister method: Samples were incubated overnight with approximately 8 mL of 0.5 M EDTA, pH 7.5 on a platform rocker. Following centrifugation, the supernatant was tested for the presence of calcium using saturated ammonium oxalate, pH 3.0. EDTA washes were continued until the absence of calcium oxalate precipitate indicated the completion of decalcification [*5*]. This step took from three to five days. Both sets were then coextracted for DNA by the procedure previously published by Lee et al. [*3*]: proteinase K digestion, phenol/chloroform/isoamyl alcohol (25:24:1, v/v) extraction, ethanol precipitation, and resuspension of the pelleted DNA in TE buffer (0.01 M Tris, 1 mM Na_2EDTA, pH 7.5). For this study, the Lee et al. [*3*] procedure was altered by adjusting the volumes to accommodate one gram samples and adding an additional aliquot of proteinase K subsequent to the 56°C overnight incubation, and continuing the incubation for an additional 3 to 4 h. A reagent blank was carried through the extraction procedure beginning with the decalcification step.

DNA used as a positive control in the PCR reactions was extracted from whole blood as previously published [*18*]: 1X SSC (0.15 M NaCl, 0.015 M $Na_3Citrate\text{-}2H_2O$, pH 7.0) washed cells were digested with proteinase K, extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol (two consecutive precipitations), and the pellet resuspended in TE buffer.

Evaluation of DNA

One percent of the total extract was electrophoresed on a 1% agarose gel with Tris-Borate (TBE) Buffer (0.89 M Tris, 0.89 M boric acid, 0.002 M EDTA). Equal amounts of DNA extracts were electrophoresed on a 3% NuSieve GTG, 1% SeaKem GTG (FMC Bioproducts) gel in TBE. The tank buffers contained ethidium bromide allowing for visualization of the DNA fragments on a transilluminator following electrophoresis. Lambda Hind III fragments (BRL) and the 123 bp Ladder (BRL) were used as molecular weight markers to determine the average fragment size.

Dilutions were prepared and their absorbencies determined at 260 and 280 nm on a Beckman DU-65 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) using an 8 mm high Micro Cell (100 μL cell). The quantity of DNA was estimated by using the absorbance at 260 nm in the equation $A_{260} \times 50 \times$ dilution factor = μg/μL. The A_{260}/A_{280} ratios were used to evaluate the quality of extracted DNA. Two samples had A_{260}/A_{280} values lower than 1.5 and were re-extracted with phenol/chloroform/isoamyl alcohol, precipitated and resuspended according to the above protocol.

Aliquots of the extracted DNA were transferred to a new tube and the sample volume was adjusted to 20 μL with sterile Milli-Q water. Sheared human placental DNA standards

containing 200, 100, 50, 25, 12.5 and 6.25 ng of DNA were used for comparison. Each sample was boiled for 3 min to denature the DNA and then placed on ice. A Scheicher and Schuell Manifold II Slot Blotter was assembled, a vacuum was applied, and the standards were loaded in duplicate to bracket the bone extracts. The DNA was crosslinked to the nylon membrane using a Stratalinker 2400. A human-specific single locus probe, MS 31 [*19*] was labeled using the random primer method [*20*]. Prehybridization and hybridization were performed as described previously [*19*].

The Polymerase Chain Reaction

Amplifications of mtDNA were attempted using primers which would result in PCR products 97, 210, 343, 410 and 478 bp using the polymerase chain reaction. Amplifications were performed with 500 ng of total DNA extracted from the bone specimens and 4 ng of control DNA. Extraction and PCR reagent blanks were used as negative controls.

Primers M13232 (5′-CGCCCTTACACAAAATGACATCAA-3′-13232), and M13286 (5′-GTGTGGTTGGTTGATGCCGA-3′-13286) were used to amplify a 97 bp product of region V of the mitochondrial genome [*21*]. The numbers at the 3′ end refer to the Anderson et al. [*22*]. Amplification was performed in 50 μL reactions containing 67 mM Tris, pH 8.8, 2 mM $MgCl_2$, 1 mM DTT, 250 μM dNTPs, 160 μg/mL BSA, 2 U Taq Polymerase, and 20 pmole of each primer. Samples were amplified on a Perkin Elmer Cetus DNA Thermal Cycler for 40 cycles of 92°C denaturation for 30 s, 60°C primer annealing for 30 s, and 72°C template extension for 1 min. Amplification was followed by a 4°C soak. Amplification products were electrophoresed on a 3% NuSieve GTG, 1% SeaKem GTG gel in TBE and visualized on a transilluminator.

Primers A (5′-TACTTGACCACCTGTAGTAC-3′-16159), B (5′-TTGACTGTA-ATGTGCTATGT-3′-16331), C (5′-ATTTAAACTATTCTCTGTTCTTTCATGG-3′-16034), and D (5′-TTCACGGAGGATGGTGGTCA-3′-16397), were used to amplify 210, 343, and 410 bp target sequences of a hypervariable segment of the mitochondrial control region. Amplifications were performed in 25 μL reactions of 200 μM dNTPs, 200 μg/mL BSA, 10 pmole of each primer, Taq polymerase (BMB), in Reaction Buffer (BMB). Initially, amplification of the 210 bp fragment was performed with 2 U Taq/reaction. However, amplification was relatively unsuccessful and 6.25 U Taq was required to achieve PCR product detectable on an agarose gel of most samples. The 343 and 410 bp fragments were amplified using 6.25 U per reaction. Amplification of the 210, 343, and 410 bp product were performed with primers A and B, C and B, and C and D, respectively. Samples were amplified on a Perkin Elmer Cetus DNA Thermal Cycler 9600 with the following parameters: 94°C for 30 s, followed by 37 or 40 cycles for the 210, and 343 and 410 bp products, respectively, of 94°C for 10 s, 50°C for 15 s, and 72°C for 15 s. A 10 min extension at 72°C was followed by a 4°C soak.

Primers E (5′-GCAGTATCTGTCTTTGATTC-3′-140) and F (5′-TTGAGGAGGTA-AGCTACATA-3′-580) were used to amplify a 478 bp target sequence of the mtDNA control region. Reactions were performed in 25 μL of 200 μM dNTPs, 200 μg/mL BSA, 10 pmole of each primer, 6.25 U Taq (BMB), in Reaction Buffer (BMB). Samples were amplified on a Perkin Elmer Cetus DNA Thermal Cycler 9600 for 40 cycles of 94°C for 10 s, 55°C for 15 s, and 72°C for 15 s.

The 210, 343, 410 and 478 bp amplification products were electrophoresed on 1% agarose gel (BRL Ultra Pure) in TBE containing ethidium bromide and visualized on a transilluminator.

Results

The results of this study indicate the following: 1) decalcification is not required to extract mtDNA from historic skeletal remains, 2) the yield of DNA is approximately

two times greater when decalcification is omitted, 3) DNA can be successfully extracted from 125 year old cortical, cancellous, and subchondral bone, 4) all represented bone types yield amplified mtDNA sequences of varying efficiency, and 5) elevated levels of Taq polymerase may increase the efficiency of amplification.

After the overnight incubation the decalcified bones were approximately 70 to 80% dissolved. Following the second proteinase K digest, all, or nearly all of the decalcified bone was dissolved. No dissolution of the calcified specimens occurred.

Gel electrophoresis was performed to evaluate the quality of DNA extracted. Due to the extremely degraded state of the extracted DNA, a 1% agarose gel was not adequate to resolve the range of fragments. Resolution of the low molecular weight fragments was achieved on a 3% NuSieve/1% SeaKem gel. One percent of the extract was loaded onto the gel, which was a sufficient amount of DNA to visualize all but a few samples. The gel showed the average size of the DNA to be less than 100 bp (Fig. 1).

To determine the concentration of DNA and contaminating protein present in the extract, UV spectrophotometric analysis was performed. Dilutions of the extracts were prepared and their absorbencies at 260 and 280 nm were determined (Table 2). The total amount of DNA extracted ranged from 6 μg to greater than 100 μg with the average being 30 μg. As the data show, on average, twice the amount of DNA was extracted

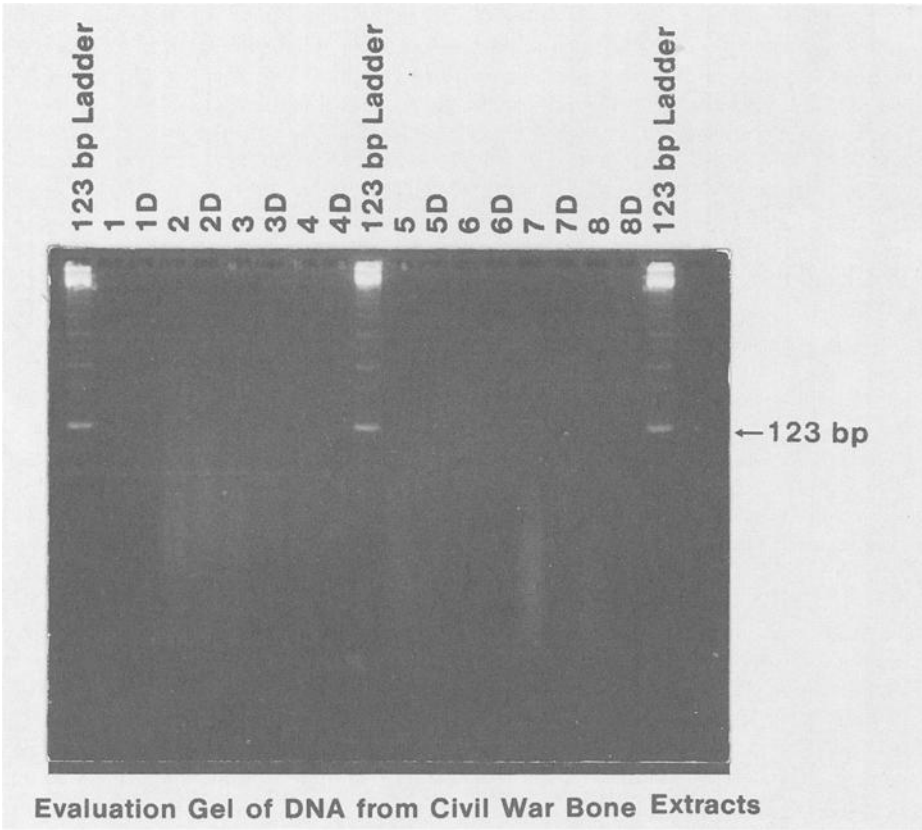

FIG. 1—*3% NuSieve/1% SeaKem GTG (FMC BioProducts) agarose gel of DNA Extracts. Samples are grouped in pairs. The numbers with a "D" represent bone samples that have been decalcified prior to extraction. Decalcification was omitted prior to extracting DNA from the remainder of the specimens.*

TABLE 2—*Quantity and quality of DNA evaluated by UV spectrophotometry.*

Specimen	Decalcified Bone Specimens		Calcified Bone Specimens	
	260/280[a]	Total DNA (μg)[b]	260/280[a]	Total DNA (μg)[b]
1	1.76	17	1.46[c]	7[c]
			(1.25)	(27)
2	1.51	24	>2	45
3	1.58	19	1.84	32
4	>2[c]	9[c]	>2	28
	(1.15)	(22)		
5	1.54	23	1.98	44
6	1.57	17	>2	22
7	1.84	30	1.68	106
8	>2	6	1.90	19
$\bar{X}$	1.73	18	1.86	38
	(1.62)	(20)	(1.83)	(40)

[a]Absorbance at 260 nm/absorbance at 280 nm.
[b]Calculated from absorbance at 260 nm.
[c]Values from second phenol:chloroform extraction. Values from first phenol:chloroform extraction are in parenthesis. See text.

from the bone specimens when the decalcification step was omitted. The average amount of DNA extracted from calcified bone is 40 μg, versus 20 μg from the decalcified bone. The samples with A_{260}/A_{280} ratios less than 1.5 were re-extracted with organics, and the A_{260}/A_{280} redetermined. The final A_{260}/A_{280} readings ranged from 1.46 to greater than 2.0. This value, however, did not appear to correlate with the presence or absence of PCR inhibitor present in the specimen, and hence, did not affect the ability to perform the PCR without elevated levels of Taq polymerase.

The percent of human nuclear DNA as determined by slot-blot analysis was found to be approximately 1% for all specimens. This is consistent with findings from similar studies [*23*]. The total amount of human nuclear DNA extracted from 1 g of starting material ranged from 60 ng to 1 μg with an average of 300 ng.

To demonstrate the authenticity of ancient DNA versus contamination from modern DNA, attempts were made to amplify increasingly larger lengths of DNA fragments [*21*]. Table 3 displays the efficiency of amplification of the DNA extracts for the variously sized PCR targets. The efficiency was determined by evaluating the intensity of the PCR product relative to the control DNA following gel electrophoresis. No sequencing or hybridization was performed on these products. However, the PCR products were the correct size based on molecular weight markers and positive controls. The data shows an inverse relationship between the amplification efficiency of the bone extracts and the length of the PCR target sequence. This is strong evidence that the DNA amplified in this study was ancient human DNA and not contaminant DNA. As the data show, the calcification state of the bone does not affect the ability to perform the PCR. Only three of the sixteen extracts failed to yield any PCR product. A 410 bp PCR product was obtained from three of the extracts. The extraction and PCR reagent blanks yielded no amplification product.

Discussion

Rapid evolution, maternal inheritance, and high copy number make mtDNA an ideal method of studying genealogical relationships [*1,24*]. Retrieval, amplification, and evaluation of mtDNA from museum specimens has permitted the phylogenetic classification

TABLE 3—*Efficiency of amplification by the polymerase chain reaction.*[a]

Specimen	97 bp	210 bp	343 bp	410 bp	478 bp
Control DNA	High	High	High	High	High
1	High	Low	Low	None	None
1D[b]	High	None	None	None	None
2	High	Medium	None	None	None
2D	High	Medium	Low	None	None
3	None[c]	None	None	None	None
3D	None	None	None	None	None
4	None	Low	Low	Low	None
4D	None	None	None	None	None
5	Low	None	None	None	None
5D	High	Medium	None	None	None
6	Low	None	None	None	None
6D	Low	None	None	None	None
7	High	None	None	None	None
7D	Low	None	None	None	None
8	Low	High	High	Low	None
8D	Low	None	Low	Low	None

[a]The efficiency was determined by evaluating the intensity of the PCR product relative to the control DNA following gel electrophoresis.

[b]Decalcified bone samples.

[c]No amplification due to inhibition, amplification was achieved by increasing the levels of Taq to 12.5 U/50 μL reaction.

of the extinct quagga [*25*] and the isolation of several human sequences in remains nearly 7000 years old [*21*]. As a rich source of DNA, museum skeletal specimens can be used to study the migration patterns and prevalence of diseases of ancient human populations [*1*]. It should be remembered, however, that extracting and evaluating DNA from ancient skeletal specimens can be a difficult task.

Amplification of the 97 bp product can serve as a diagnostic test for the presence or absence of Taq polymerase inhibitors. This is a robust amplification system because the PCR product is a small segment of mtDNA. The primers used to amplify the target DNA in this system form a detectable dimer that is easily separated from the amplified target DNA on a 3%/1% NuSieve/SeaKem agarose gel. Although primer-dimer formation makes the primers less available to prime the extension of the target DNA [*26*], the presence or absence of a primer-dimer allows the analyst to determine if inhibition or insufficient template DNA is the cause for weak or unsuccessful amplification. In this study, the extracts were first amplified with 2 units of Taq/50 μL reaction. BSA, known to help overcome Taq inhibition [*21*] was used in all PCR reactions. Four samples (numbers 3, 3D, 4 and 4D) did not yield PCR product. In each of these cases the primer-dimer was absent. One of these samples (number 3) was amplified with increasing amounts of Taq; successful amplification was achieved with 12.5 U Taq/50 μL reaction. Hence, the PCR of this target sequence may assist in determining whether Taq inhibitors are present. If amplification is unsuccessful due to insufficient template DNA, it is unlikely that a larger target, present in lower copy number, will amplify.

This study indicates that increased levels of Taq may not only allow amplification in the presence of inhibitors, but may also aid in amplifying large PCR products relative to the average fragment size of the DNA extract. The DNA extracted in this study was extremely degraded, indicating the likelihood that the number of intact copies of template DNA is inversely related to the size of the target. Successful amplification of the mtDNA target sequences 210 bp and greater was achieved with some of the extracts when the amount of Taq polymerase was increased from 2 U/25 μL to 6.25 U/25 μL. Increased

levels of Taq may facilitate amplification by increasing the likelihood that primed target molecules are recognized and extended in the initial cycles of PCR. Hence, when increasing the concentration of DNA is not feasible, increasing the concentration of Taq is an alternative.

Decalcification of bone specimens prior to the extraction of DNA is a time-consuming step that can take several days to complete. However, our study demonstrates that decalcification of bone specimens is not necessary for the extraction or amplification of DNA. UV spectrophotometric analysis reveals that on average twice the amount of DNA is recovered and the A_{260}/A_{280} values are slightly higher when decalcification is omitted.

The quantity of bone DNA added to the PCR reactions in this study was controlled to determine if the ability to obtain product could be predicted based on UV spectrophotometry and gel electrophoresis. The A_{260}/A_{280} values do not correlate with the ability to perform the PCR. If it is assumed that bone contaminants that may coextract with DNA are present in equal amounts from the same amount of starting material (1 g), then a greater amount of bone contaminant would be present in those PCR reactions with lower concentrations of DNA. However, the concentration of DNA did not correlate with the ability to amplify product. It was possible to amplify segments of the mtDNA control region, some as large as 410 bp, from cortical, subchondral, and cancellous bone. However, no generalizations can be made regarding which bone type will amplify the most efficiently. Although gel electrophoresis showed that the extracted DNA was extremely degraded, approximately one percent of the total DNA was of human origin. As such, the gel could not be used to predict how samples may or may not amplify.

Bone type, UV spectrophotometry, and yield gel electrophoresis results cannot always be used to determine the condition of the extracted DNA. However, amplification of the mtDNA 97 bp fragment, as presented in this paper, or a similar marker, can provide valuable diagnostic information concerning the condition of extracted DNA.

Further studies to determine the efficiency of amplification of nuclear DNA from historical period skeletal remains will be undertaken in the future.

References

[1] Rogan, P. and Salvo, J., "Study of Nucleic Acids Isolated from Ancient Remains," *Yearbook of Physical Anthropology*, Vol. 33, 1990, pp. 195–214.

[2] Stoneking, M., Hedgecock, D., Higuchi, R., Vigilant, L. and Erlich, H., "Population Variation of Human mtDNA Control Region Sequences Detected by Enzymatic Amplification and Sequence-specific Oligonucleotide Probes," *American Journal of Human Genetics*, Vol. 48, 1991, pp. 370–382.

[3] Lee, H., Pagliaro, E., Berka, K., Folk, N., Anderson, D., Gualberto, R., Phil, M., Keith, T., Phipps, P., Herrin, G., Garner, D., and Gaensslen, R., "Genetic Markers in Human Bone: I. Deoxyribonucleic Acid (DNA) Analysis," *Journal of Forensic Sciences*, JFSCA, Vol. 36, No. 3, May 1991, pp. 639–655.

[4] Hagelberg, E., Gray, I., and Jeffreys, A., "Identification of the Skeletal Remains of a Murder Victim by DNA Analysis," *Nature* (London), Vol. 352, Aug. 1, 1991, pp. 427–429.

[5] Hochmeister, M., Budowle, B., Borer, U., Eggman, U., Comey, C., and Dirnhofer, R., "Typing of Deoxyribonucleic Acid (DNA) Extracted from Compact Bone from Human Remains," *Journal of Forensic Sciences*, JFSCA, Vol. 36, No. 6, Nov. 1991, pp. 320–330.

[6] Gaensslen, R. and Lee, H., "Genetic Markers in Human Bone Tissue," *Forensic Science Review*, Vol. 2, No. 2, Dec. 1990, pp. 125–146.

[7] Lee, H., "Analysis of Human Bone DNA by PCR." Presented at Forensic Applications of PCR Technology, sponsored by the Laboratory Division of the Federal Bureau of Investigation, FBI Academy, Quantico, VA, May 1991.

[8] Horai, S., Hayasaka, K., Murayama, K., Wate, N., Koike, H., and Nakai, N., "DNA Amplification from Ancient Human Skeletal Remains and Their Sequence Analysis," *Proceedings of the Japan Academy*, Vol. 65, Ser. B, 1989, pp. 229–233.

[9] Hanni, C., Laudet, V., Sakka, M., Begue, A., and Stehelin, D., "Amplification of Mitochondrial DNA Fragments from Ancient Human Teeth and Bones," *C. R. Academy of Science*, Paris, Vol. 310, Series III, 1990, pp. 365–370.

[10] Hagelberg, E. and Clegg, J., "Isolation and Characterization of DNA from Archaeological Bone," *Proceedings of the Royal Society of London*, Vol. 244, 1991, pp. 45–50.
[11] Hagelberg, E. and Sykes, B., "Ancient Bone DNA Amplified," *Nature* (London), Vol. 342, Nov. 30, 1989, p. 485.
[12] Hummel, S. and Herrman, B., "Y-Chromosome-Specific DNA Amplified in Ancient Human Bone," *Naturwissenschaften*, 78, 1991, pp. 266–267.
[13] Sledzik, P. and Moore-Janse, P., "Dental Disease in 19th Century Military Skeletal Samples." In: Kelley, M. A. and Larsen, C. S. (Eds.), Advances in Dental Pathology, Wiley-Liss, New York, 1991, pp. 215–224.
[14] Budowle, B., Adams, D. E., Comey, C. C., and Merrill, C. R., "Mitochondrial DNA: A Possible Genetic Material Suitable for Forensic Analysis." In: Lee, H. C. and Gaensslen, R. E. (Eds.), *DNA and Other Polymorphisms in Forensic Science*, Year Book Medical Publishers, Inc. Chicago, London, Boca Raton, and Littleton, Mass., 1990, pp. 76–97.
[15] Giles, R., Blanc, H., Cann, H., and Wallace, D., "Maternal Inheritance of Human Mitochondrial DNA," *Proceedings of the National Academy of Sciences, USA*, Vol. 77, No. 11, Nov. 1980, pp. 6715–6719.
[16] Hutchison, C. III, Newbold, J., Potter, S., and Edgell, M., "Maternal Inheritance of Mammalian Mitochondrial DNA," *Nature* (London), Vol. 251, Oct. 11, 1974, pp. 536–538.
[17] Horai, S. and Hayasaka, K., "Intraspecific Nucleotide Sequence Differences in the Major Noncoding Region of Human Mitochondrial DNA," *American Journal of Human Genetics*, Vol. 46, 1990, pp. 828–842.
[18] Budowle, B. and Beachtel, F. S., "Modifications to Improve the Effectiveness of Restriction Fragment Length Polymorphism Typing," *Applied and Theoretical Electrophoresis*, Vol. 1, 1990, pp. 181–187.
[19] Feinberg, A. P. and Vogelstein, B., "A Technique for Radiolabelling DNA Restriction Endonuclease Fragments to High Specific Activity," *Analytical Biochemistry*, Vol. 132, 1983, pp. 6–13.
[20] Wong, Z., Wilson, V., Patel, I., Povey, S., and Jeffreys, A. J., "Characterisation of a Panel of Highly Variable Minisatellites Cloned from Human DNA," *Annals of Human Genetics*, Vol. 51, 1987, pp. 269–288.
[21] Paabo, S., Gifford, J., and Wilson, A., "Mitochondrial Sequences from a 7,000-year Old Brain," *Nucleic Acids Research*, Vol. 16, 1988b, pp. 9775–9787.
[22] Anderson, S., Bankier, A., Barrell, B., de Bruijn, M., Coulson, A., Drouin, J., Eperon, I., Nierlich, D., Roe, B., Sanger, F., Schreier, P., Smith, A., Staden, R., and Young, I., "Sequence and Organization of the Human Mitochondrial Genome," *Nature* (London), Vol. 290, April 9, 1981, pp. 457–465.
[23] Hagelberg, E., "Sequencing from Ancient Bone." Presented at Ancient DNA: The Recovery and Analysis of DNA Sequences from Archaeological Material and Museum Specimens, sponsored by the British Museum (Natural History), the Natural Environment Research Council, Cetus Corporation, and Perkin-Elmer, University of Nottingham, Nottingham, England, July 1991.
[24] Paabo, S., Higuchi, R., and Wilson, A., "Ancient DNA and the Polymerase Chain Reaction," *Journal of Biological Chemistry*, Vol. 264, No. 17, June 15, 1989, pp. 9709–9712.
[25] Higuchi, R. E., Wrischnik, L. A., Oakes, E., George, M., Tong, B., and Wilson, A. C., "Mitochondrial DNA of the Extinct Quagga: Relatedness and Extent of Postmortem Change," *Journal of Molecular Evolution*, Vol. 25, 1987, pp. 283–287.
[26] Innis, M. A. and Gelfand, D. H., "Optimization of PCRs." In: Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J. (Eds.), *PCR Protocols: A Guide to Methods and Applications*, Academic Press, Inc., San Diego, CA, 1990.

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