Mitchell M. Holland,[1] Ph.D.; Deborah L. Fisher,[1] M.P.H.; Lloyd G. Mitchell,[2] M.D.; William C. Rodriquez,[3] Ph.D.; James J. Canik, B.S.; Carl R. Merril,[2] M.D.; and Victor W. Weedn,[1] M.D., J.D.

Mitochondrial DNA Sequence Analysis of Human Skeletal Remains: Identification of Remains from The Vietnam War

REFERENCE: Holland, M. M., Fisher, D. L., Mitchell, L. G., Rodriquez, W. C., Canik, J. J., Merril, C. R., and Weedn, V. W., **"Mitochondrial DNA Sequence Analysis of Human Skeletal Remains: Identification of Remains from The Vietnam War,"** *Journal of Forensic Sciences*, JFSCA, Vol. 38, No. 3, May 1993, pp. 542–553.

ABSTRACT: Deoxyribonucleic acid (DNA) sequence analysis of the control region of the mitochondrial DNA (mtDNA) genome was used to identify human skeletal remains returned to the United States government by the Vietnamese government in 1984. The postmortem interval was thought to be 24 years at the time of testing, and the remains presumed to be an American service member. DNA typing methods using nuclear genomic DNA, HLA-DQ alpha [*1*] and the variable number of tandem repeat (VNTR) locus D1S80 [*2*], were unsuccessful using the polymerase chain reaction (PCR) [*3*]. Amplification of a portion of the mtDNA control region was performed, and the resulting PCR product subjected to DNA sequence analysis. The DNA sequence generated from the skeletal remains was identical to the maternal reference sequence, as well as the sequence generated from two siblings (sisters). The sequence was unique when compared to more than 650 DNA sequences found both in the literature and provided by personal communications. The individual sequence polymorphisms were present in only 23 of the more than 1300 nucleotide positions analyzed. These results support the observation [*4*] that in cases where conventional DNA typing is unavailable, mtDNA sequencing can be used for human remains identification.

KEYWORDS: physical anthropology, DNA typing, bone DNA extraction, human identification, Vietnam War

The identification of soldiers lost in battle is of utmost importance to the United States Government and to the families and friends who must bear the burden of grief. During the Asian and World Wars, thousands of men and women were reported "missing in action" (MIA) or "killed in action and the body not recovered" (KIA-BNR). When bodies cannot be found or identified, families are left with the pain of not knowing with

The opinions expressed are those of the authors and do not necessarily reflect the views of the U.S. Army or the Department of Defense.

Received for publication 4 May 1992; revised manuscript received 17 Aug. and 8 Oct. 1992; accepted for publication 17 Nov. 1992.

[1]Research Scientist, DNA Analyst, Administrator, and Chief, respectively, The Armed Forces DNA Identification Laboratory, The Armed Forces Institute of Pathology, Washington, DC.

[2]Postdoctoral Fellow and Chief, respectively, The Laboratory of Biochemical Genetics, The National Institutes of Mental Health, Neuroscience Center, Washington, DC.

[3]Forensic Anthropologist, The Office of the Armed Forces Medical Examiner, The Armed Forces Institute of Pathology, Washington, DC.

certainty how or whether their loved one has died. Many individuals with losses dating back to World War I still suffer with this pain.

As of March 1992, 2267 Americans were still unaccounted for as a result of conflicts in Southeast Asia (Vietnam, Cambodia, Laos, and China) [5]. Of these, 1095 were Americans KIA-BNR. These numbers are small when compared to the more than 86 000 Americans still unaccounted for as a result of World War II and the Korean War. Intensive efforts by the U.S. Government are ongoing to determine the fate of every American still unaccounted for. As a consequence of these efforts, hundreds of remains have been recovered and returned to the U.S. Army Central Identification Laboratory in Honolulu, Hawaii (CILHI) [6]. Many of these remains have been successfully identified by methods other than DNA typing (anthropologic and odontologic). However a number of cases cannot be identified unequivocally by conventional means. In these cases, DNA based analysis can be used to lend support to the overall identification process.

The putative skeletal remains of an American Marine Corps aviator were returned to the United States Government by the Vietnamese Government in 1984, 17 years after the soldier was declared MIA and subsequently KIA-BNR. Following extensive anthropologic investigation by CILHI, an identification was recommended. Upon instigation of the family, the Marine Corps contacted the Armed Forces DNA Identification Laboratory (AFDIL), requesting DNA analysis of the skeletal remains to confirm the identification. The remains were exposed to extreme environmental conditions (heat and humidity for approximately 17 years and unknown conditions for an additional seven years), and substantial DNA loss and degradation were observed. Nuclear DNA typing methods were attempted unsuccessfully (DQ-alpha and D1S80). Therefore, DNA sequence analysis of mitochondrial DNA (mtDNA) was examined as a possible means of identification.

The mitochondrial genome is 16 569 base pairs (bp) in length and circular, consisting of conserved coding regions and a noncoding, hypervariable control region [7]. The coding region consists of genes encoding for enzyme protein subunits involved in oxidative respiration. The control region spans more than 1100 bp, and includes origins for replication and transcription, as well as the displacement loop. Three characteristics of the control region make it a good candidate for identification purposes. First, there are two hypervariable segments within the control region which have been studied in detail and have been shown to evolve rapidly (mtDNA) polymorphisms occur 5 to 10 times faster than in the nuclear genome) [8–10]. Second, mtDNA is passed through the maternal lineage [11], from mother to child, so that each individual is haploid and has only one mtDNA type. Third, human cells contain hundreds to thousands of copies of the mtDNA genome, but only two copies of the nuclear genome [12]. These additional copies make the likelihood of recovering mtDNA greater when the biological specimen contains limited quantities of DNA or degraded DNA.

In this article, we describe the use of mtDNA sequencing to confirm the identification of skeletal remains, with a postmortem interval of 24 years; the origin of the remains had been in question for seven years. Difficulties encountered with DNA extraction and amplification will be discussed.

Materials and Methods

DNA Sources

A total of seven bone specimens were collected from the skeletal remains; six midshaft specimen measuring approximately 25 mm in length, including a fragment of the right humerus, the right ulna, the right radius, the left tibia, the left fibula, and the left radius, and a 25 mm wedge of bone from the ischium of the right hip bone. The amount of bone collected from each sample ranged from 3 to 15 g. The bone fragments were stored at −70°C. DNA specimens were collected from four family members (mother,

father, and two sisters) by taking three separate oral swabs from each member. The swabs were stored dry at −70°C. DNA was previously extracted from the laboratory personnel for use as positive controls and as controls against contamination.

DNA Extraction

DNA was extracted from all seven bone specimens using the procedure described by Hochmeister et al. [*13*]. Approximately 2 g of bone were used for each extraction. The bone was cleaned aggressively to remove the outer layer of foreign material, broken into small pieces of approximately 0.3 cc, and then pulverized into a fine powder using a Tekmar Tissumizer®. The bone powder was placed into a 15 mL conical tube and decalcified with three successive washes in 8 mL of 0.5 M EDTA, pH 7.5, for 8 to 12 h. The powder was washed three times with sterile deionized water to remove excess EDTA, and DNA was extracted with 3 mL of prewarmed extraction buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2% sodium dodecyl sulfate, and 10 mM EDTA) containing 0.5 mg/mL Proteinase K; the extraction was carried out at 56°C for 12 to 18 h. The concentration of Proteinase K in the extraction suspension was elevated to 1.0 mg/mL and the incubation continued at 56°C for an additional 5 h. The undigested bone powder was pelleted in a centrifuge and the supernatant decanted into a clean 15 mL conical tube. The extract was deproteinated with chloroform/phenol/isoamyl alcohol (25:24:1) and trace phenol extracted with ether. The ether was subsequently removed by incubation at 56°C for 15 min. The DNA was further purified by membrane-based size exclusion using an Amicon Centricon® 30 spin column. As an alternative to ethanol precipitation, the DNA samples were evaporated to dryness in a vacuum microcentrifuge. The dried pellets were resuspended in 100 μL of TE buffer (10 mM Tris-base, pH 7.6, 1 mM EDTA).

DNA from the oral swabs obtained from each family member was extracted using an organic extraction method. The cotton portion of each swab was placed in a 15 mL conical tube and extracted with 3 mL of extraction buffer containing 0.1 mg/mL Proteinase K, and incubated at 56°C for 12 to 18 h. The swab was removed and the extract was deproteinated with chloroform/phenol/isoamyl alcohol (25:24:1). The phenol was extracted with ether, the ether removed by incubation at 56°C for 15 min, and the DNA precipitated in 0.3 M sodium acetate and a two fold excess of 100% ethanol at −20°C for 2 h. The DNA was pelleted by centrifugation, washed with 70% ethanol, dried, and resuspended in 100 μL of TE buffer. The concentration of DNA extracted from both bone and oral swab was quantified by agarose gel electrophoresis and ethidium bromide staining, and U.V. spectrophotometry.

Quantification of Human DNA

Sheared human placental DNA standards, containing 200, 100, 50, 25, 12.5, and 6.25 ng of DNA, were used for comparison to the bone DNA extracts. Samples were boiled for three minutes to denature the DNA and then placed on ice. A Scheicher and Schuell Manifold II Slot Blotter® was assembled (using Hybond-N nitrocellulose membrane), a vacuum was applied, and the standards were loaded in duplicate to bracket the bone extracts. The DNA was crosslinked to the membrane in a Stratalinker 2400 using ultraviolet light. A human-specific single locus nuclear DNA probe, M31 [*14*] was labeled using the random primer method [*15*]. Prehybridization and hybridization were performed as described previously [*14*]. DNA quantities were determined by comparison of the standards to the unknowns. (It should be emphasized that M31 is not a probe for mtDNA, and will not reflect the potential content of mtDNA in the DNA extracts.)

HLA-DQ-alpha Analysis

Amplification of the HLA-DQ-alpha locus was performed according to the specifications of the Cetus Corporation AmpliType HLA-DQ-alpha Forensic DNA Amplification and Typing Kit® with the following modifications. The amplification was performed on a Perkin Elmer-Cetus GeneAmp 9600 Thermal Cycler® using the following PCR parameters—94 C for 10 min, followed by 94 C for 10 s, 60 C for 10 s, and 72 C for 10 s, for 32 cycles, followed by a 10 min soak at 72 C. Reaction mixtures included 25 μL of Cetus premix, 8 μL of 25 mM magnesium chloride, and 17 μL of DNA sample. PCR product was analyzed by agarose gel electrophoresis and the HLA-DQ-alpha type determined according to the procedure provided by Cetus.

Polymerase Chain Reaction Amplification

Two segments of the control region of the mtDNA genome were amplified using the polymerase chain reaction (PCR) [*3*]. The first segment encompassed nucleotides 121 to 340 (219 base pair (bp) product), and the second from 16140 to 16350 (210 bp product); based on the numbering system of Anderson et al. [*16*]. The conditions for amplification of the 219 bp product were 0.2 mM dATP, dCTP, dTTP, and dGTP, 0.4 μM F121 primer (5′-GCA GTA TCT GTC TTT GAT TC, 121-140, 5′-end biotinylated by The Midland Certified Reagent Company, F = forward), 0.4 μM R340 (5′-GTG TTT AAG TGC TGT GGC CA, 321-340, R = reverse), 0.2 mg/mL BSA, buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, and 1.5 mM $MgCl_2$), and 6.25 units of Taq DNA polymerase (Cetus, Boehringer Mannheim Biochemicals®) in a 25 μL reaction volume. The amplification was performed for 32 cycles in a Perkin Elmer Cetus GeneAmp PCR 9600® system: 94 C for 10 s, 50 C for 20 s, and 72 C for 45 s. The conditions for amplification of the 210 bp product were the same as those for the 219 bp product with these exceptions; the primers used were F16140 (5′-TAC TTG ACC ACC TGT AGT AC, 16140-16159, 5′-end biotinylated by Midland) and R16350 (5′-TTG ACT GTA ATG TGC TAT GT, 16329-16350), the reaction volume was 50 μL, and the amplification parameters were 94 C for 10 s, 50 C for 15 s, 72 C for 15 s, for 27 cycles, with an isothermal hold at 72 C for 10 min at the end of the program. Two rounds of amplification were performed in all cases; 1 μL of the first PCR product was used for the second round of amplification. DNA-free negative control reactions, containing reagent blank extract or PCR reagents alone, were included in every PCR performed (including the second round amplification of 1 μL of the initial DNA-free PCR). In addition, positive control reactions were included in every PCR performed to ensure that the PCR reaction products were the correct size. Reaction products were analyzed by agarose gel electrophoresis and ethidium bromide staining prior to DNA sequencing.

DNA Sequencing

The two strands of DNA from the 210 bp PCR product were separated using the biotin group located at the 5′-end of one of the two strands; the 219 bp fragment did not amplify. The PCR product was passed through a Sephadex G-50® spin column (5 Prime-3 Prime Inc.) to remove unincorporated primers. The double stranded DNA was bound to Dynal streptavidin magnetic beads (Dynal AS®) (20 μL of washed beads was used for each sequencing reaction) in the presence of 0.5 M NaCl/TE buffer, washed to remove unbound DNA, and the unbiotinylated strand removed in the presence of 0.2 M NaOH [*17*]. The unbiotinylated strand was collected, neutralized with sodium acetate, and concentrated by ethanol precipitation; the dried DNA pellet was resuspended in 7 μL of sterile, deionized water. The bound strand was washed several times with TE buffer, pH 7.4,

and suspended in 7 μL of sterile, deionized water. The two complimentary strands of DNA were sequenced separately using Sequenase, S35-dATP, and the protocol provided with the Sequenase DNA Sequencing Kit (U.S. Biochemicals); the sequencing primers for the 210 bp product were F16140 for the unbound strand and R16350 for the bound strand at a concentration of 0.2 mM. The reaction products were electrophoresed through an LKB Macrophor DNA sequencing gel system. The dried gel was exposed to Kodak X-ray film for 1-7 days before development. Sequence information was determined by visual evaluation.

Results

Bone specimens were collected from skeletal remains that were returned to the United States by the Vietnamese Government in 1984, and subsequently presented to the Armed Forces DNA Identification Laboratory at the Armed Forces Institute of Pathology in July of 1991. Specimens were taken from the midshaft of six long bones, as well as a wedge of hip bone. DNA was extracted from all seven bone fragments, using decalcification and organic extraction, and analyzed by agarose gel electrophoresis (Fig. 1). Quantification of total extracted DNA was based on UV spectrophotometric evaluation (Table 1). Quantification of human nuclear DNA by slot–blot analysis resulted in detections of less than 1% human DNA for all bone extracts. In fact, nuclear DNA was not detected for many of the extracts. This analysis, however, does not reflect the content of mitochondrial DNA (mtDNA). Our laboratory, as well as others (personal communication), are in the process of investigating potential probes for the quantification of

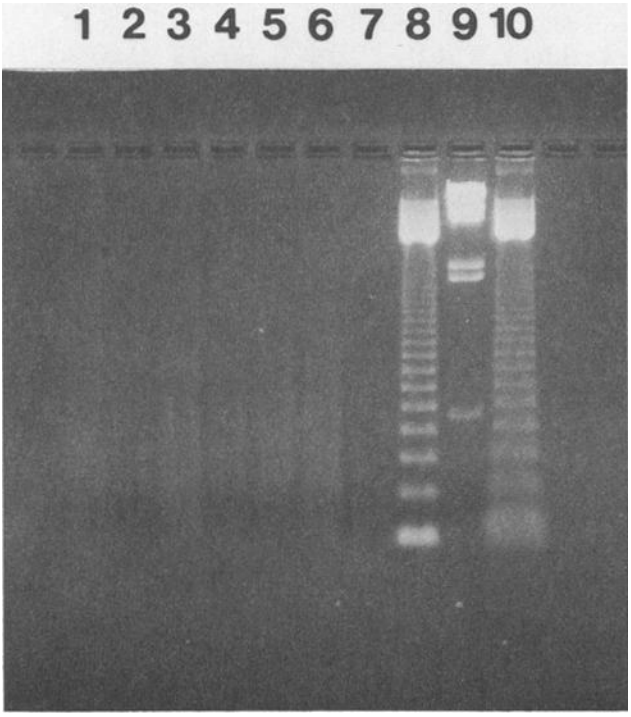

FIG. 1—*DNA was extracted from all seven bone fragments, using decalcification and organic extraction, and analyzed by agarose gel electrophoresis.*

TABLE 1—*DNA quantitation: total and human nuclear DNA.*

Sample Description	Total DNA, μg	Human DNA
Bone DNA:		
Right humerus[a]	4.09	<1%
Right ulna	1.19	<1%
Right radius[a]	92.40	<1%
Left tibia[a]	23.00	<1%
Left fibula[a]	19.20	<1%
Left radius	6.85	<1%
Right hip bone	82.30	<1%

[a]DNA sequence analysis.

mtDNA. The high copy number of potential mtDNA targets may provide higher detection sensitivities.

DNA was also extracted from oral swabs of the family members: maternal, paternal, and two sibling references. High molecular weight DNA was obtained with a concentration of 10 μg/mL; based on agarose yield gel analysis (data not shown).

The maternal and paternal HLA-DQ alpha genotype, using the kit provided by the Perkin Elmer-Cetus Corporation, was "2,3" for both individuals. The HLA-DQ alpha type of the DNA extracted from the bone could not be determined. In fact, no nuclear sequence could be amplified from bone DNA (HLA-DQ alpha or D1S80/MCT118). Many attempts were made to amplify the HLA-DQ alpha locus, modifying the concentration of DNA, the amount of Taq DNA polymerase, the addition of BSA, and altering the PCR parameters, with no success. Consequently, segments of the mtDNA control region were amplified from the DNA of all four family members and from the bone DNA. Two rounds of amplification were necessary to produce sufficient product for sequence analysis. Using mtDNA specific primers (one biotinylated), a 210 base pair (bp) segment of the control region was amplified (16140 to 16350); a 219 bp segment could not be amplified (121 to 340).

Only four of the seven bone extracts yielded 210 bp PCR product (Table 1). Total DNA extraction yield, as well as the quality of the extracted DNA, decreased dramatically as the bone specimen became more porous. The six long bone fragments were not overly porous and yielded significant quantities of total DNA (Fig. 1). Presumably, the reason for a lack of PCR product in two of the six extracts was due to insurmountable inhibition, however the lack of sufficient mtDNA for analysis cannot be ruled out. The results of mixing DNA extracted from right humerus with the BRL 123 bp DNA Ladder (Fig. 1) suggested that the bone DNA extracts contained high levels of salt or protein which retarded the migration of the 123 bp ladder. Regardless, the source of the contamination could not be removed, even by extensive purification using Amicon Centricon 30 size exclusion dialysis. The most likely source of the inhibitor was the foreign material on the outer surface of the bone which may not have been properly removed during the cleaning process. The wedge of hip bone was extremely porous and presumably did not yield sufficient quantities of intact mtDNA for PCR analysis.

A portion of the 210 bp PCR product (10%) was analyzed by agarose gel electrophoresis, and the remaining product prepared for DNA sequence analysis using the method of Mitchell and Merril [*17*]. The entire PCR product was consumed for DNA sequence analysis. The PCR product generated from the four bone DNA extracts, the DNA extracted from the maternal, paternal, and sibling references, and the DNA extracted from the laboratory personnel (two individuals) was subjected to DNA sequence analysis; sequence was obtained in both directions to ensure complimentarity (Fig. 2). A comparison of the sequence from bone DNA and that of the maternal reference resulted in

FIG. 2—*A sequence was obtained in both directions to ensure complimentarity.*

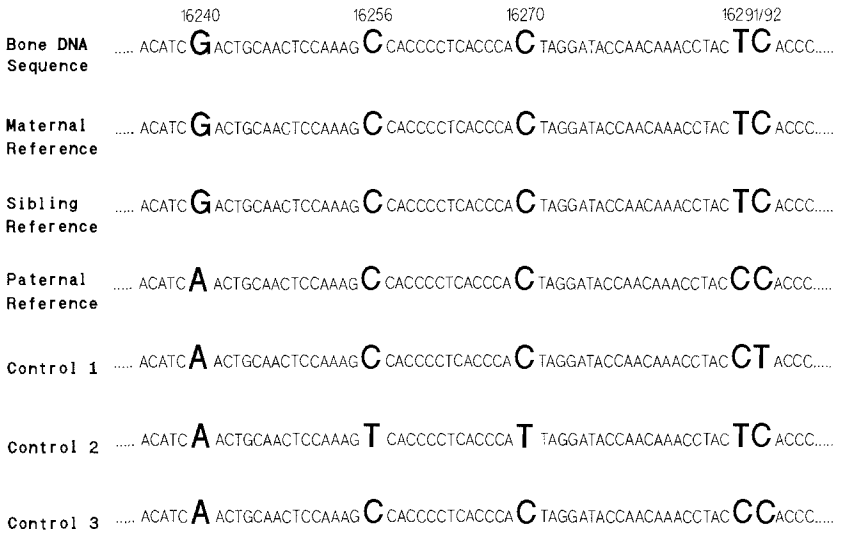

FIG. 3—*A comparison between bone DNA and maternal reference.*

an identical match for the 170 bases of overlapping sequence (Fig. 3). Two polymorphisms were identified within the 170 bp segment (polymorphisms with respect to the published sequence of Anderson et al.) [*16*], one at position 16240, a transition from adenine to guanine, and the second at position 16291, a transition from cytidine to thymine. In addition, the sequence from both siblings matched the bone and the maternal reference, and the sequence from the paternal reference was different. Given the difficulties involved with amplification, the 210 bp DNA sequence was obtained from the DNA of the laboratory personnel who handled the bones and performed the extraction (Fig. 3). The resulting 210 bp DNA sequences were different from all other sequences generated suggesting that the sequence obtained from the bone DNA and the DNA extracted from the oral swabs was not due to contamination. The DNA sequence generated from the bone was compared to sequences available in the literature and through personal communications, and the results illustrated in Table 2.

Discussion

The positive identification of human remains is primarily established by either fingerprint analysis, dental or skeletal radiographic comparison, and in certain instances by the presence of unique markings, such as tatoos. In cases, however, where soft tissue is severely decomposed, or when dental and skeletal comparisons cannot be made, the use

TABLE 2—*Comparison of sequence polymorphisms between racial groups.*

Racial origin	Number of sequences	Changes at position 16240[a]	Changes at position 16291[b]	Changes at both positions
Caucasions	237	1	6	0
Blacks	325	2	12	0
Asians	97	0	0	0

[a]16240 A → G.
[b]16291 C → T.

of DNA based identification is a viable option, and in some instances the only method for identification.

The vast majority of remains returned to the U.S. Army Central Identification Laboratory in Honolulu, Hawaii (CILHI) for identification can be identified by conventional forensic methods (anthropologic and odontologic). However, when commingled or incomplete sets of skeletal remains are recovered, some identifications are based on strong circumstantial evidence. Given recent advances in DNA extraction from bone, identification of a number of these latter cases can now be supplemented with DNA analysis.

The extraction of DNA from bone has advanced rapidly in the past few years [*18,19*]. Hochmeister et al. were able to extract more than 12 μg of human DNA/gram of compact bone that was several months old [*13*]. In addition, 500 ng of human DNA could be extracted from an eleven year old mummified leg. In both examples, amplification of nuclear DNA was achieved; HLA-DQ alpha and various VNTR loci. The DNA yield dropped dramatically, to 0.05 μg/g of bone, when fresh bone was buried in soil for three months. However, amplification of nuclear DNA was not affected. In certain cases, short tandem repeat (STR) loci can be analyzed to type DNA extracted from bone several years old [*20*]. STR loci commonly contain two to four nucleotide repeat units [*21,22*], which are useful for typing extremely degraded DNA and are generally more informative than HLA-DQ alpha. When skeletal remains are exposed to extreme environmental conditions (heat, humidity, and soil) for more than ten years, extraction of sufficient quantities of nuclear DNA for amplification becomes less likely, even for STR analysis. Alternatively, minute amounts of mitochondrial DNA have been recovered from ancient bone specimen, 5500 years of age and older, and amplification of the control region successfully achieved [*19,23*].

The polymorphic nature of the mtDNA control region has been recently exploited to identify human remains [*24*]. Stoneking et al. used sequence-specific oligonucleotides (SSO) to directly probe for polymorphisms in the hypervariable segments of the control region in DNA extracted from five year old skeletal remains found in a desert. The nine "hot spots" selected for polymorphic variance provided diversity values for each population tested (African, Asian, Caucasian, Japanese, and Mexican), of at least 0.95; greater than HLA-DQ alpha and D1S80. The probability of two unrelated individuals having the same SSO-type was 1/19—1/8, suggesting that any one SSO-type across all nine regions would exclude 88.3 to 94.6% of the population. This ability to discriminate between individuals based on mitochondrial SSO-typing is excellent, however, when limited quantities of DNA exist, or the quality of the mtDNA to be analyzed is extremely poor, DNA sequence analysis of short segments of the control region may prove to be equally, or perhaps more informative.

In our attempts to amplify mitochondrial DNA for sequence analysis, we encountered inhibition problems. A very strong inhibitor copurified with the DNA, which was able to inhibit positive control amplification (data not shown). For certain reactions, we were able to overcome the inhibition by the addition of 200 μg/mL bovine serum albumin [*23,25*], and by purification of the initial DNA extract using an Amicon Centricon 30 spin column. Nevertheless, in all cases two rounds of amplification were necessary to generate acceptable quantities of PCR product for DNA sequence analysis. The absence of PCR product in the DNA-free negative control reactions suggested that the product generated after the second round was not due to contamination.

A 210 bp segment of the mitochondrial DNA control region, bases 16140 to 16350 [*16*], was amplified from the DNA extracted from the four bone specimen and from the four references (maternal, paternal and sibling DNA). A 219 bp segment of the bone mtDNA, bases 121 to 340 [*16*], could not be amplified. Our validation studies revealed that this region amplifies well for all control samples tested. Extensive degradation of the mtDNA found in bone of this age may result in the amplification of only certain

segments of the control region. Therefore, particular segments of the control region could be amplified for DNA sequence analysis when the generation of a complete SSO-type is not possible.

The DNA sequence of the 210 bp segment was determined and the 170 bp of overlapping sequence was identical in the bone, the maternal DNA sequence and the two sibling DNA sequences. Furthermore, the polymorphisms were unique when compared to more than 650 sequences in the same region. Contamination was ruled out as a source of the unique sequence based on sequence analysis of the paternal DNA, and the DNA of the laboratory personnel performing the analysis; all were different from each other and different from the bone DNA sequence (Fig. 3). The two polymorphisms, at positions 16240 and 16291, were not seen together in more than 650 DNA sequences currently available; of these 650 sequences, 237 were Caucasian and 97 Asian. The individual polymorphisms were only seen 23 times in the 1300 nucleotides reported at those positions. Consequently, given the unique nature of the sequence, the probability of another such sequence in the general population is presently not more than 1 in 650, or 0.15% of the general population, and not more than 1 in 237, or 0.42% of the Caucasian population.

These results, along with strong circumstantial evidence, make a very compelling argument for the identification of the skeletal remains. Even though the extracted DNA was poor in quality and quantity, DNA sequence information proved useful in the identification process. In addition, the extraction of DNA and the DNA analysis could be done on as little as 2 g of compact bone, even when that bone had been exposed to extreme environmental conditions for an extended period of time; heat and humidity for greater than 15 years.

It is important to note that mtDNA sequence analysis, or SSO-typing of the mtDNA control region is only useful as an identification tool if a relevant sequence database is generated, and the integrity of maternal inheritance is validated. A portion of the sequence database presently available is from isolated subpopulations [*24*]. Therefore, databases must be compiled from the general population (Caucasians, Blacks, Hispanics, etc.) in order to report more appropriate frequency values. In addition, limited research has been done on the integrity of maternal inheritance of the primary structure of DNA [*11*,*26*,*27*]. Although it is generally accepted that maternal inheritance is a faithful process, studies undertaken to validate maternal inheritance at the level of the DNA sequence for five or more generations, within several different maternal lineages, would be valuable.

Finally, advances in automation have made DNA sequencing a potentially routine method of analysis. The DNA sequence of tens of thousands of DNA bases are presently being generated on a weekly basis using automated DNA sequencing with fluorescently labeled reaction products [*28*]. In the future, identification of human skeletal remains could be made in a relatively rapid, and efficient manner by DNA sequence analysis. Efforts are currently underway in our laboratory, as well as other laboratories [*29*], to study the feasibility of using DNA sequence analysis of the mtDNA control region as a source of routine identification.

Acknowledgments

We would like to thank Drs. Mark Stoneking and Kevin Sullivan, and Mr. Charles Ginther, currently working with Mary Claire-King, for their contribution of mitochondrial DNA sequence information and numerous useful discussions. We would also like to thank Mr. Mark Wadhams at Cellmark, Inc. for performing the human DNA quantification analysis. Thanks to Mr. Robert Gale for his technical support in preparing the manuscript.

References

[1] Saiki, R. K., Walsh, P. S., Levenson, C. H., and Erlich, H. A., "Genetic Analysis of Amplified DNA with Immobilized Sequence-specific Oligonucleotide Probes," *Proceedings of the National Academy of Sciences*, Vol. 86, Aug. 1989, pp. 6230–6234.

[2] Kasai, K., Nakamura, Y., and White, R., "Amplification of the Variable Number Tandem Repeat (VNTR) Locus (pMCT118) by the Polymerase Chain Reaction (PCR) and Its Application to Forensic Science," *Journal of Forensic Sciences*, Vol. 35, No. 5, September 1990, pp. 1196–1200.

[3] Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A., and Arnheim, N., "Enzymatic Amplification of beta-Globin Genomic Sequences and Restriction Analysis for Diagnosis of Sickle Cell Anemia," *Science*, Vol. 230, 1985, pp. 1350–1354.

[4] Budowle, B., Adams, D. E., Comey, C. T., and Merril, C. R., "Mitochondrial DNA: A Possible Genetic Material Suitable for Forensic Analysis," *DNA and Other Polymorphisms in Forensic Science*, H. C. Lee and R. E. Gaensslen, Eds., Year Book Medical Publishers, Chicago, IL., 1990, pp. 76–97.

[5] Personal communication; The United States Army Central Identification Laboratory, Hickman Air Force Base, Hawaii.

[6] The United States Army Central Identification Laboratory, Honoluhi, Hawaii, Annual Report, 1991.

[7] Wallace, D. C., "Report of the Committee on Human Mitochondrial DNA," *Cytogenetics and Cell Genetics*, Vol. 51, 1989, pp. 612–621.

[8] Brown, W. M., George, M., and Wilson, A. C., "Rapid Evolution of Animal Mitochondrial DNA," *Proceedings of the National Academy of Sciences*, Vol. 76, No. 4, 1979, pp. 1967–1971.

[9] Aquadro, C. F. and Greenberg, B. D., "Human Mitochondrial DNA Variation and Evolution: Analysis of Nucleotide Sequences from Seven Individuals," *Genetics*, Vol. 103, 1983, pp. 287–312.

[10] Cann, R. L., Stoneking, M., and Wilson, A. C., "Mitochondrial DNA and Human Evolution," *Nature*, Vol. 325, No. 6099, 1987, pp. 31–36.

[11] Giles, R. E., Blanc, H., Cann, H. M., and Wallace, D. C., "Maternal Inheritance of Human Mitochondrial DNA," *Proceedings of the National Academy of Sciences*, Vol. 77, No. 11, 1980, pp. 6715–6719.

[12] Robin, E. D. and Wong, R., "Mitochondrial DNA Molecules and Virtual Number of Mitochondria per Cell in Mammalian Cells," *Journal of Cell Physiology*, Vol. 136, 1988, pp. 507–513.

[13] Hochmeister, M. N., Budowle, B., Borer, U. V., Eggmann, U., Comey, C. T., and Dirnhofer, R., "Typing of Deoxyribonucleic Acid (DNA) Extracted from Compact Bone from Human Remains," *Journal of Forensic Sciences*, Vol. 36, No. 6, December 1991, pp. 1649–1661.

[14] Wong, Z., Wilson, V, et al., "Characterization of a Panel of Highly Variable Minisatallites Cloned from Human DNA," *Annuals of Human Genetics*, Vol. 51, 1987, pp. 269–288.

[15] 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.

[16] Anderson, S., Bankier, A. T., Barrell, G. B. et al., "Sequence and Organization of the Human Mitochondrial Genome," *Nature*, Vol. 290, No. 5806, 1981, pp. 457–465.

[17] Mitchell, L. G. and Merril, C. R., "Affinity Generation of Single-Stranded DNA for Dideoxy Sequencing Following the Polymerase Chain Reaction," *Analytical Biochemistry*, Vol. 178, 1989, pp. 239–242.

[18] 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 Sciences, Paris*, Vol. 310, Series III, 1990, pp. 365–370.

[19] 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.

[20] Hagelberg, E., Gray, I. C., and Jefferys, A. J., "Identification of the Skeletal Remains of a Murder Victim by DNA Analysis," *Nature*, Vol. 352, No. 6334, 1991, pp. 427–429.

[21] Litt, M. and Luty, J. A., "A Hypervariable Microsatellite Revealed by In Vitro Amplification of a Dinucleotide Repeat within the Cardiac Muscle Actin Gene," *American Journal of Human Genetics*, Vol. 44, 1989, pp. 397–401.

[22] Moos, M. and Gallwitz, D., "Structure of Two Human beta-Actin-Related Processed Genes One of Which is Located Next to a Simple Repetitive Sequence," *The EMBO Journal*, Vol. 2, No. 5, 1983, pp. 757–761.

[23] Hagelberg, E., Sykes, B., and Hedges, R., "Ancient Bone DNA Amplified," *Nature*, Vol. 342, Nov. 1989, pp. 485.

[24] Stoneking, M., Hedgecock, D., Higuchi, R. G., Vigilant, L., and Erlich, H. A., "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.

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

[26] Orrego, C. and King, M. C., "Determination of Familial Relationships," *PCR Protocols: A Guide to Methods and Applications*, Part Four, Diagnostic and Forensic, 1990, pp. 416–426.

[27] Ashley, M. V., Laipis, P. J., and Hauswirth, W. W., "Rapid Segregation of Heteroplasmic Bovine Mitochondria," *Nucleic Acids Research*, Vol. 17, 1989, pp. 7325–7331.

[28] Hunkapiller, T., Kaiser, R. J., Koop, B. F., and Hood, L., "Large-Scale and Automated DNA Sequence Determination," *Science*, Vol. 254, Oct. 1991, pp. 59–67.

[29] Sullivan, K. M., Hopgood, R., Lang, B., and Gill, P., "Automated Amplification and Sequencing of Human Mitochondrial DNA," *Electrophoresis*, Vol. 12, No. 1, 1991, pp. 17–21.

Address requests for reprints or additional information to
Mitchell M. Holland, Ph.D.
Armed Forces DNA Identification Lab
AFIP
14th and Alaska Sts.
Washington, DC 20306-6000