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Forensic DNA Testing on Skeletal Remains from Mass Graves: A Pilot Project in Guatemala

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ABSTRACT: A reliable method for extracting DNA from teeth was developed and successfully applied to a set of 12 skeletons recovered from two 10-year-old Guatemalan mass graves. Attempts to identify the remains by mitochondrial DNA (mtDNA) testing were hampered by low sequence diversity. Our findings demonstrate the feasibility of using DNA typing to identify victims from mass graves.

KEYWORDS: physical anthropology, DNA, human identification, mass graves

In many investigations of government-sponsored violations of human rights, forensic specialists are confronted with the task of identifying skeletal remains from a mass grave. Ordinarily, positive identification is established through the comparison of antemortem dental or medical records with corresponding observations on the skeleton. However, in undeveloped countries with poor standards of health care, such records are usually scanty or non-existent, especially when many years have passed between the victim's death and recovery of the skeleton. Often, the only antemortem information available is that reported by the victim's family, which may consist of general anthropological variables such as sex, age, race, stature, and handedness; and other traits unique to the individual such as bone-affecting injuries or diseases, and skeletal anomalies. In some cases, these data may be supplemented with descriptions of clothing and personal effects known to have been in the victim's possession when last seen alive. With careful evaluation, such information is usually sufficient to establish the identities of victims in cases where the population of the grave is small; reasonably heterogeneous in age, sex, and stature; and restricted to a list of known decedents. However, when the grave contains many skeletons from a relatively homogeneous group (for example, young males of military age) and the list of possible decedents is uncertain, the available antemortem data may not be sufficient for identification.

Genetic typing of DNA extracted from skeletal remains may provide a solution to this problem. Several groups have performed forensic DNA testing on skeletal DNA using nuclear [*1–7*] and mitochondrial markers [*6–11*]. Both bone and teeth have been used successfully for DNA testing, but teeth are more resistant to environmental damage, easier to transport, and frequently yield higher quality DNA [*4,7,10*].

mtDNA testing has several advantages over conventional nuclear DNA testing [*9*] for large-scale forensic projects such as mass graves. Since mtDNA is present in thousands of copies per cell [*13*], it is much easier to recover from forensic samples than nuclear DNA, which is present in only two copies per cell. Unlike nuclear DNA, which is inherited in equal parts from both parents, mtDNA is inherited only from the mother [*14*]. This means that a comparison of the mtDNA genotypes of the deceased and a single maternal relative is sufficient to confirm or exclude identification, whereas DNA testing with nuclear markers usually requires knowledge of both parental genotypes. In addition, the hypervariable regions of the mitochondrial genome are quite small, about 500 base pairs in length [*15–18*]. This is useful since DNA extracted from skeletal remains is frequently less than 1000 base pairs (bp) in length [*1,19*].

In this report, we present reliable methods for extraction, amplification, and automated sequencing of mtDNA from teeth.

Background of the Case

The samples studied in this case were taken from twelve skeletons exhumed in July 1992, from two mass graves in San Jose Pachoa Lemoa (SJPL), Guatemala, a Quiche Indian village located 15 km south of the provincial capital of Santa Cruz de Quiche. Ten years earlier—on February 14, 1982—a local Civil Patrol rounded up the village residents and confined them to a schoolhouse. After preliminary questioning about anti-government activities, the Patrol took twelve of the men to a separate classroom for further interrogation, and subjected them to severe beatings, which were overheard by the other villagers. That evening, the Patrol led the twelve away, bound and blindfolded, never to be seen alive again. The next morning, the villagers discovered what appeared to be two large, freshly dug graves in a nearby ravine.

For ten years after the incident, the villagers, fearing reprisals, did not disturb the suspected graves. In July 1992, the mother of one of the missing men petitioned a Guatemalan court to investigate the case. The court authorized an international team of forensic anthropologists and archeologists from Chile and the United States to assist the newly established Guatemalan Forensic Anthropology Team in the inquiry. The work of the visiting forensic experts was supported and organized jointly by the American Association for the Advancement of Science and Physicians for Human Rights.

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Materials and Methods

Exhumation and Anthropological Examination

Using standard archeological techniques, the team excavated the two graves over a five day period in July 1992. At the same time, the team collected antemortem descriptions of the missing men, and maternally linked relatives of the men were asked to provide hair samples for mtDNA testing. However, family members of only six of the twelve missing men were willing to donate DNA samples.

The twelve skeletons were removed to a provincial morgue for anthropological examination. After dentition was photographed and charted, an intact, undiseased tooth was removed from each skull and submitted for mtDNA testing.

In the morgue, the skeletons were first examined to determine sex, race, age, handedness, and stature. Morphological and discriminant function analysis classified all the individuals as Amerindian males. Estimated ages ranged from the mid-teens to mid-forties, and antemortem stature estimates ranged from 149 to 165 centimeters. The remains were then examined in more detail for signs of disease, injury, or skeletal anomaly that might be reflected in the physical descriptions provided by relatives of the missing men. All of the skeletons displayed extensive, deep cut-marks suggesting that they had been hacked to death by machetes, a common method of execution among the Civil Patrols. When the anthropological profile of each skeleton was compared with antemortem information about the missing men, some matches were immediately apparent. For example, a mother of one of the missing men stated that when her son was about 18 years old, he suffered a severe dislocation of his right shoulder which was never treated, leaving the arm partially disabled. Skeleton 3 displayed a scapulo-humeral pseudoarthrosis of the right shoulder, and since its age and stature also fell in the proper range, it was provisionally identified as that of the woman's son. If a tentative match assigned on the basis of anthropological data could be confirmed by descriptions of the victim's clothing and personal effects, the identification was considered positive.

DNA Extraction from Teeth

Teeth were washed three to five times in deionized water, and three to five times in 1M sodium phosphate buffer (pH 7.2). Each wash was carried out using 30 mL of wash solution with continuous agitation for a minimum of 30 minutes. After washing, teeth were placed inside 15 mL screw-cap polypropylene centrifuge tubes (Falcon) and cracked with a vise. Fractures exposing the entire pulp cavity gave the best extracts, as judged from the amplification results. Tooth fragments were extracted with 5 mL of guanidine-EDTA buffer (GE buffer: 50 mM Tris-HCl pH 8.0, 4 M guanidine HCl, 0.4 M EDTA) until a white suspension formed. The suspension was transferred to a fresh tube, and centrifuged at 5000× g for 10 minutes. The supernatant was discarded, and the white precipitate was redissolved in the minimum required volume of fresh GE buffer, usually 5 to 10 mL. The resulting clear extract was treated with 100 μg/mL proteinase K (Boehringer) overnight. Afterward, the extract was concentrated and exchanged into TE buffer (10 mM Tris-HCl pH 8.3, 1 mM EDTA) by centrifugal ultrafiltration using 30 000 molecular weight cutoff filters (Centriprep-30, Centricon-30, Amicon). The final extract, usually around 100 μL in volume, was treated at 95°C for 10 minutes to inactivate the Proteinase K, and stored at −20°C. In most cases, 2 μL aliquots of the extract were used for PCR.

Hair samples

Root portions of freshly plucked hair samples from SJPL residents were stored in 100% ethanol at room temperature. To prepare DNA, single hair roots were removed from the ethanol, air-dried briefly, and placed in 100 μl of a solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.5% Triton X-100, and 100 μg/mL proteinase K. The hair was digested overnight at 37°C, and proteinase K was inactivated by holding the mixture at 95°C for 10 minutes. Usually, 1 μL of the extract was used for PCR.

PCR

PCR was carried out using 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleotide triphosphate (dATP, dGTP, dTTP, and dCTP), 0.25% Triton X-100, 100 μg/mL bovine serum albumin, and 1.25 units recombinant Taq polymerase (Amplitaq, Perkin Elmer) in a 25 μL total reaction volume. For primary amplification, primers LLL (5′-ACACCAGTCTTG-TAAACCGGAGATG-3′) and LHL (5′-CCTGAAGTAGGAAC-CAGATGTCGG-3′) were each used at a concentration of 0.3 μM. These primers amplify the mtDNA sequence from positions 15909 to 16517, referring to the numbering of the Anderson sequence [*20*]. This region contains hypervariable region I (HVI) as defined by Vigilant et al. 1989 [*18*]. Amplification was carried out using a cycle of 94°C for 30 seconds and 72°C for 1 minute. Hair samples were amplified for 30 cycles, and tooth extracts were amplified for 35 to 40 cycles. Five microliter aliquots of the amplification reactions were analyzed by agarose gel electrophoresis (3% NuSieve, 1% Seaplaque; FMC) to estimate product DNA concentration.

Generation of Sequencing Templates

Sequencing templates were generated by asymmetric PCR [*21*] using the same primers and conditions as for primary amplification. Primer concentrations were 1 μM for the excess primer, and 0.03 μM for the limiting primer. One nanogram of the primary amplification product was used as the initial target. In most cases, a dilution of the primary amplification reaction was used to provide initial target for the secondary amplification. In a few cases, gel purification of the primary amplification product was performed to avoid additional amplification of nonspecific primary amplification products. Gel purification was performed using an agarase method (Gelase, Epicentre). Asymmetric amplifications were carried out for 25 to 30 cycles, and DNA products were purified by isopropanol precipitation [*22*] before sequencing. Usually, template from half of a 25 μL amplification reaction was sufficient for a single set of sequencing reactions.

Automated Sequencing

Fluorescein-labeled primers L15996 (5′-Fluorescein-CTCCAC-CATTAGCACCCAAAGC-3′) and H16403 (5′-Fluorescein-GAT-ATTGATTTCACGGAGGATGG-3′) were used to sequence templates produced in asymmetric reactions containing excess LHL and excess LLL, respectively. The fluorescein labels were added during oligonucleotide synthesis using a fluorescein phosphoramidite (Fluoreprime, Pharmacia LKB Biotechnology). Sequencing was carried out using a T7 polymerase-based kit (AutoRead kit, Pharmacia LKB Biotechnology). Preliminary tests demonstrated that the quality of sequence data obtained using T7-polymerase exceeded that obtained with thermostabile polymerases

(Taq, Tth; single or multiple cycles), which showed uneven peak heights and occasional single base positions where no signal was obtained.

Sequencing was carried out using the Automated Laser Fluorescent (ALF) Sequencer and ALF manager software (Pharmacia LKB Biotechnology) using protocols provided by the manufacturer. Sequence comparisons were made with the Genetics Computer Group version 7.0 package [*23*].

Precautions Against Carryover Contamination

To prevent carryover contamination from previous PCR reactions, DNA extractions and assembly of primary PCR reactions were performed in separate, dedicated laminar flow hoods within a designated preparation room. Equipment and reagents reserved for these procedures were not removed from the preparation room. All subsequent procedures, including amplification, gel electrophoresis, template preparation, and sequencing, were performed in other rooms. Disposable isolation gowns, caps, masks, and gloves were worn when working in the preparation room.

Solutions for DNA extraction were prepared in disposable plastic containers. PCR solutions (Perkin Elmer) and deionized water (EM Omnisolv) were purchased. Air displacement pipettes with aerosol barrier tips (ART, BioRad) were used for preparation of PCR reactions. The preparation room and hoods were irradiated with germicidal lamps when not in use.

To test for the presence of DNA contamination in the reagents, each set of DNA extractions included mock-extracted samples that were processed in parallel with the tooth samples. In all cases, amplification reactions using the mock-extracted samples yielded no specific products.

Results

Strategy for Identification by mtDNA Sequencing

DNA was extracted from teeth taken from the skeletons, and hair samples donated by maternal relatives of the missing villagers. Hypervariable region I of the mtDNA was amplified from each sample, sequenced, and then compared to test for relatedness between the remains and families of the missing men. Additional SJPL residents, who were unrelated to the missing men, were also tested to help estimate the level of mtDNA sequence polymorphism in the population.

DNA Extraction from Teeth

The DNA extraction procedure is described in detail in Materials and Methods. Briefly, the teeth were washed, fractured to expose the pulp cavity, and DNA was extracted with EDTA and guanidine-HCl, agents that partially dissociate the inorganic tooth matrix [*24*]. The method gave adequate yields of mtDNA from all teeth tested, as judged by successful amplification of the 500 base pair (bp) mtDNA target. Extracts from different kinds of teeth (molars, premolars, incisors) gave equivalent amplification results.

mtDNA sequencing

A 341 base pair (bp) region from hypervariable region I (HVI) of the mtDNA [*18*] was sequenced for all samples. We found that the use of nested sequencing primers greatly reduced sequencing artifacts, and allowed use of a rapid isopropanol precipitation procedure [*22*] to purify templates after asymmetric PCR, instead of the more time-consuming ultrafiltration method [*21*].

Six of the SJPL families tested have a transition mutation at position 16189 that creates a long uninterrupted poly dG:dC tract (see Fig. 1). In these families, sequencing signals from the region downstream (3′) of the poly dG:dC tract were weak, indistinct, and consequently, unreadable. Both template strands gave the same result. Alternative sequencing strategies using other sequencing enzymes (Taq, Tth), cycle sequencing, or phage T4 single-stranded DNA binding protein did not alleviate the problem. The fact that the sequence becomes unreadable at the poly dG:dC tract for both template strands suggests that the amplified templates are heterogeneous with respect to the length of the tract. We believe that this heterogeneity is caused by misalignment mutations within the poly dG:dC tracts that occur during amplification.

Since the length of the poly dG:dC tracts in samples carrying the 16189 transition could not be determined with confidence, it was not used as a forensic marker. Analyses of several independently amplified templates showed that the length of the poly dG:dC tract was 12 bp for SJPL mtDNA types C, F, H, and I; 11 bp for type L; and 8 bp for type P.

Analysis of mtDNA Sequences

The maternal lineages of the missing men were poorly defined. Two of the men were brothers. None of the other ten had known common maternal ancestors. However, it was not possible to trace maternal lineages further back than the maternal grandmothers of the missing men, so that earlier common maternal ancestors among the eleven families could not be ruled out.

Among the 30 individuals sequenced in this study, representing 23 unrelated families, a total of 28 variable sites were detected (Fig. 1). Several unrelated families shared the same mtDNA sequence, so that only 16 different sequence types were observed, as shown in Tables 1 and 2.

The distribution of sequence differences for all pairwise comparisons between unrelated SJPL individuals is shown in Fig. 2. The average number of differences is 6.11, a value similar to that observed in previous studies of Native American mtDNA diversity [*25,26*]. The distribution is bimodal, suggesting a relatively small number of mtDNA lineages. Three mtDNA lineage clusters were identified, shown by the grouping in Table 1. The largest cluster includes 16 unrelated families (Table 1, top), and is characterized by C to T transitions at 16111 and 16290, and a G to A transition at 16319. The next largest cluster includes 6 families (middle),

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16022  TGTTCTTTCA TGGGGAAGCA GATTTGGGTA CCACCCAAGT ATTGACTCAC
                      G
16072  CCATCAACAA CCGCTATGTA TTTCGTACAT TACTGCCAGC CACCATGAAT
                           C                    T
16122  ATTGTACGGT ACCATAAATA CTTGACCACC TGTAGTACAT AAAAACCCAA
          A       C
16172  TCCACATCAA AACCCCCTCC CCATGCTTAC AAGCAAGTAC AGCAATCAAC
          T   CCTT T C                                   C
16222  CCTCAACTAT CACACATCAA CTGCAACTCC AAAGCCACCC CTCACCCACT
        T                T          C                    G   T
16272  AGGATACCAA CAAACCTACC CACCCTTAAC AGTACATAGT ACATAAAGCC
       G                  T   G    C              C         A
16322  ATTTACCGTA CATAGCACAT TACAGTCAAA TCCCTTCTCG TC
         C T                                     T  C
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FIG. 1—*Polymorphic mtDNA sites observed in the SJPL population. The top line shows the L strand of the Anderson sequence* [20] *for the region analyzed in this study: 16022–16363 of the mtDNA control region. Sequence changes observed at least once in the SJPL population are indicated beneath the reference sequence.*

TABLE 1—*mtDNA types found in the SJPL region.*

	Position in reference sequence																												
Type	16037	16092	16111	16129	16136	16179	16182	16183	16184	16185	16187	16189	16217	16223	16239	16249	16265	16270	16272	16290	16293	16298	16311	16319	16325	16327	16360	16362	Lineage¶
Ref.	A	T	C	G	T	C	A	A	C	C	C	T	T	C	C	T	A	C	A	C	A	T	T	G	T	C	C	T	
A	*	*	T	*	*	*	*	*	*	*	T	*	*	T	*	*	*	*	*	T	*	*	*	A	*	*	*	C	
B	G	*	T	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	T	*	*	*	A	*	*	*	*	
D	*	*	T	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	*	T	*	*	*	A	*	*	*	*	
E	*	*	T	*	C	*	*	*	*	*	*	*	*	T	*	*	*	*	*	T	*	*	*	A	*	*	*	C	
G	*	*	*	*	C	*	*	*	*	*	*	*	*	T	*	*	*	*	*	T	*	*	*	A	*	*	*	C	II
J	*	*	T	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	T	*	*	*	A	*	*	*	*	
K	*	*	T	*	*	*	*	*	*	*	*	*	*	T	*	*	G	*	*	T	G	*	C	A	*	*	*	*	
L‡	*	*	T	*	*	*	*	*	*	*	*	C	*	T	*	*	*	*	*	T	*	*	*	A	*	*	*	C	
N	*	*	T	*	*	*	*	*	*	*	*	*	*	T	T	*	*	*	*	T	*	*	*	A	*	*	*	C	
O	*	*	T	*	*	*	*	*	*	*	*	*	*	T	*	*	*	*	G	T	*	*	*	A	*	*	T	C	
C†	*	*	*	*	*	*	C	C	*	*	*	C	C	*	*	*	*	*	*	*	*	*	C	*	*	*	*	*	
F†	*	*	*	A	*	*	C	C	*	*	*	C	C	*	*	C	*	*	*	*	*	*	*	*	*	*	*	*	
H†	*	C	*	*	*	*	C	C	*	*	*	C	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	IV
I†	*	*	*	*	*	*	C	C	*	*	*	C	*	*	*	*	*	T	*	*	*	*	*	*	*	*	*	*	
P	*	*	*	*	*	*	*	C	T	T	*	C	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	
M	*	*	*	*	*	T	*	*	*	*	*	*	*	T	*	*	*	*	*	*	*	C	*	*	C	T	*	*	III

NOTE: The table shows the 16 mtDNA types, designated by letters A–P, observed in the 23 independent SJPL families that were used for forensic testing. For each individual, sequence data were obtained from mtDNA positions 16022 to 16363. Only those positions that differ from the Anderson sequence [20] in at least one sample are shown. Asterisks (*) indicate positions of identity with the Anderson sequence. (‡) SJPL mtDNA type L has an 11 base polyC tract surrounding the T to C transition at 16189. (†) SJPL mtDNA types C, F, H, and I have a 12 base polyC tract created by a T to C transition at 16189, and A to C changes at 16182 and 16183. (¶) The relationships of the SJPL sequences to the Amerindian mtDNA lineages defined by Ward et al. [25] are shown.

TABLE 2—*Summary of SJPL mtDNA testing results.*

Type	Skeleton	Family	Unrelated DNA donors	#Independent families	Frequency
A	2,4,8	02.1	01.A,02.A	4	0.17
B	10,11	01.1,06.1		2	0.09
C	1,6	04.1,07.1		2	0.09
D			14.A,32M	2	0.09
E	7		18.A	2	0.09
F	3			1	0.04
G	5			1	0.04
H	9	11.1		1	0.04
I	12			1	0.04
J			05.A	1	0.04
K			06.A	1	0.04
L			15.A	1	0.04
M			16.A	1	0.04
N			17.A	1	0.04
O			19.A	1	0.04
P			21M	1	0.04

NOTE: Sequences from skeletons are indicated by numbers 1 through 12; sequences from maternal relatives of suspected victims are indicated by case number (#.1); and additional sequences from unrelated residents of the SJPL region are indicated by a control number (#.A or #M). "# Independent Families" indicates the number of unrelated families who shared the mtDNA type. "Frequency" is the frequency of the mtDNA type in the 23 independent families studied.

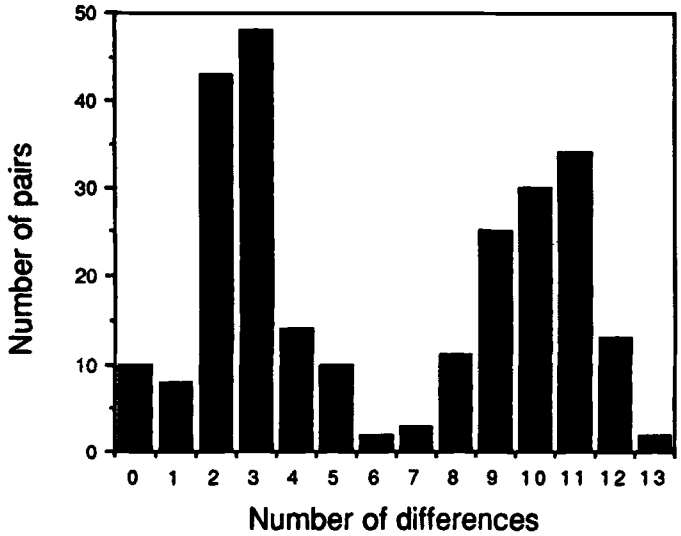

FIG. 2—*Distribution of pairwise mtDNA sequence differences observed in the SJPL population. mtDNA sequences from each of the 23 unrelated families listed in Table 2 were compared. The number of sequence differences for all possible pairwise combinations is plotted. The mean number of differences was 6.11 ± 3.94 (SD) for the 253 possible pairwise combinations.*

and is characterized by T to C transitions at 16189 and 16217. A third lineage is represented by a single family (bottom), and is characterized by a C to T transition at 16223 and T to C transitions at 16298 and 16325. These three lineage clusters are closely related to three of the four major Native American mtDNA lineages recently described [25,27,28].

In Table 2, the skeletons and family DNA donors are classified according to their mtDNA HVI sequence type. One sequence type (A) was observed in four unrelated families, four types (B–E) were observed in two families each, and the remaining eleven types (F–P) were observed in one family each. From these data, the probability that two randomly chosen individuals from the SJPL population will have the same mtDNA HVI type, estimated from the sum of the squares of the frequencies over all observed HVI types [8], is 0.08, or approximately $^1/_{12}$. These data suggest that HVI sequence diversity of the SJPL population is low relative to that of Caucasians, where the corresponding probability has been estimated to be less than $^1/_{300}$ [17]. A low sequence diversity is also suggested by the observation that only 5 of the 12 skeletons (skeletons 3, 5, 7, 9, 12) have HVI types that distinguish them from one another. However, much additional sequence data will be required to accurately estimate the level of mtDNA diversity in this population.

Comparison of mtDNA and Anthropological Results

Using anthropological methods, nine of the twelve skeletons could be positively identified (numbers 1, 3, 4, 5, 6, 7, 9, 10, and 12). The three remaining skeletons (numbers 2, 8, and 11) were similar in age and stature, and because little antemortem data was available for these cases, only tentative identifications could be made.

Table 3 compares the results of the anthropological and mtDNA analyses. There were no discrepancies between the two data sets. For the six cases where mtDNA typing was possible, a one-to-one correspondence between a skeleton and a single family was observed only once (skeleton 9 with family 11.1, Table 3). In four cases (skeletons 1, 6, 10, 11), the skeleton's mtDNA type matched more than one family. Three skeletons (numbers 2, 4, 8) had a common mtDNA type that appears in approximately 17% of the SJPL population (type A, see Table 1). Identifications based on anthropological data indicated that skeletons 2 and 4 belonged to brothers, a finding supported by the fact that they share the same mtDNA sequence. mtDNA typing also confirmed the tentative identifications of skeletons 8 and 11.

From Tables 2 and 3, it is clear that skeletons 3, 5, 7, and 12 could have been distinguished from the others by mtDNA testing, since they have unique mtDNA types. Unfortunately, the families of these four victims were unwilling to donate DNA samples, presumably for fear of reprisals by the Civil Patrol.

TABLE 3—*Comparison of anthropological and mtDNA test results.*

Anthropological results				mtDNA results	
Skeleton #	Quality of ID	Family #	DNA?	Victim's mtDNA type	Families with same type
1	Positive	04.1	yes	C	04.1,07.1
2	Tentative	09.1	no	A	02.1
3	Positive	05.1	no	F	None
4	Positive	09.1	no	A	02.1
5	Positive	03.1	no	G	None
6	Positive	07.1	yes	C	04.1,07.1
7	Positive	08.1	no	E	None
8	Tentative	02.1	yes	A	02.1
9	Positive	11.1	yes	H	11.1
10	Positive	01.1	yes	B	01.1,06.1
11	Tentative	06.1	yes	B	01.1,06.1
12	Positive	14.1	no	I	None

NOTE: "DNA?" indicates whether the family donated a sample for mtDNA analysis. Family 02.1 and skeletons 2, 4, 8 have a common mtDNA type that is present in 17% of the SJPL population (see Table 2). Anthropological evidence indicates that skeletons 2 and 4 belonged to brothers from family 09.1.

Discussion

DNA Extraction from Teeth

Using the extraction procedure developed in this study, we found that teeth are reliable sources of DNA for amplification-based forensic methods. The teeth used in this study had been buried for ten years, but still yielded adequate amounts of mtDNA for analysis. Recently, using the same method, we have successfully extracted DNA from teeth that had been buried for up to 80 years (T. C. Boles, unpublished results). To date, teeth from approximately 25 different skeletons have been extracted using the method, with only one failure. In that single case, the skeleton's teeth were severely decalcified, presumably due to acid ground water at the burial site. The extraction procedure requires no specialized equipment or reagents, and a large number of teeth can be processed in parallel. Our procedure should also be compatible with the sampling methods developed by Smith et al. [7] that allow extraction of the pulp chamber and dentine without destroying crown structure.

We found it important to adopt the anti-contamination measures described in the Materials and Methods. In our experience, it is difficult to avoid carry-over contamination if DNA extraction and PCR product analysis are carried out in the same room. Furthermore, we found that workers performing analyses of amplified DNA can become chronic carry-over contamination "carriers," in agreement with previous reports from other labs [29]. Fortunately, this problem can be effectively controlled by careful use of disposable lab clothing.

On the other hand, we found no evidence that teeth could be contaminated with exogenous DNA from the forensic workers who performed the excavations and anthropological examinations. Presumably, the yield of DNA, estimated to be in the range of 10 to 50 ng DNA per tooth, is large enough to outcompete any exogenous DNA that remains after the wash procedure. However, for the reasons stated in the previous paragraph, forensic workers involved in excavation or handling of skeletal remains should avoid laboratories where amplification-based DNA testing is being carried out, to minimize their risk of becoming contaminated with amplified DNA.

mtDNA Testing in the Guatemalan Population

The probability that two unrelated SJPL residents will have the same mtDNA HVI type estimated from our data, $^1/_{12}$, is insufficient to provide convincing evidence of identification in the absence of other forensic data. Our findings and other recent studies [25–28,30] suggest that mtDNA sequence diversity in Native American populations may be lower than in other populations. For this reason, additional mtDNA sequence data is needed to evaluate the power of mtDNA testing in these populations. Despite these limitations,

our study demonstrates that mtDNA typing can be useful for confirming tentative identifications made on the basis of other data.

Recent studies suggest that the identification power of mtDNA testing is significantly improved when sequences from hypervariable region II (HVII) [*18*] are included in the analysis. For Caucasian populations, inclusion of HVII improves the power of the assay by approximately ten-fold [*6,17*]. If the same improvement can be obtained for the SJPL population, the probability of false identification using both HV regions should drop to approximately $^1/_{100}$, which would make mtDNA testing much more useful. Thus, additional sequence studies that include both HV regions are required to properly evaluate mtDNA testing in this population.

An alternative way to improve the power of the DNA testing is use nuclear markers. Highly polymorphic nuclear microsatellite markers [*31,32*] have been used successfully to type DNA extracted from bone [*1,6*], and therefore, this method should also be applicable to DNA from teeth (see [*7,10*]). Several groups have also successfully used reverse dot blot hybridization with allele-specific oligonucleotide probes (Amplitype HLA DQalpha kit, Perkin Elmer Cetus) to type amplified nuclear loci from bone [*2,3*] and tooth [*7*] extracts. These testing systems offer two advantages over conventional DNA fingerprinting [*12*]. First, the loci used in these tests can be amplified on very short DNA fragments, and therefore, they can be used with skeletal DNA and other highly degraded samples. Second, it is relatively easy to test large numbers of loci using these methods, thereby increasing the power of identification. In principle, if enough loci are tested, it should be possible to identify skeletal remains even on the basis of a single parental genotype.

Automated Sequencing

Automated sequencing clearly facilitated primary data collection and sequence analysis. However, gel preparation was more laborious than for manual sequencing, and substantial training and practice were required before reproducible sequencing results could be obtained. Therefore, sequencing remains the major technical bottleneck in forensic mtDNA testing. In the future, this problem should become less important as forensic DNA typing becomes more routine, and national and international forensic centers are established that can provide provide high volume, low cost sequencing services.

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