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Identification of Human Remains Using mtDNA Sequence Analysis in Morocco

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Abstract: Today, mtDNA typing is utilized primarily in cases in which the nuclear DNA is too degraded or cannot be recovered in sufficient quantities to be typed. Laboratory of Genetic of Royal Gendarmerie in Morocco is actively involved in using mtDNA for forensic identifications of human skeletal remains. Reproducible results were obtained for bones and teeth up to 52 years old. The bone and tooth samples were pulverized to fine powder, decalcified and DNA was extracted. 341-bp fragment from HVI (hypervariable region I) and 267-bp fragment from HVII (hypervariable region II) of the mtDNA control region were amplified. After sequencing of the PCR products, mitotypes were compared to the rCRS (revised Cambridge Reference Sequence) and a phylogenetic tree was built.

Key words: Forensic identification, mitochondrial DNA, human, skeletal remains.

1. Introduction

Sequence analysis of human mtDNA (mitochondrial DNA) has been demonstrated to be a valid and reliable tool for the genetic characterization of forensic biological specimens [1-3]. The human mtDNA is a 16569 np (nucleotide pair) closed, circular molecule located within the cytoplasmic mitochondria. mtDNA has a very high mutation rate, approximately 10-fold higher compared to nuclear DNA, probably as a result of poor repair mechanisms as well as a decreased proofreading efficiency of the mtDNA polymerase [4, 5].

In this paper, we report the sequencing of mtDNA of four skeletal remains belonging to four individuals missed up to 52 years old. The application of mtDNA sequence analysis for the identification of these remains was done for the first time in Genetic Laboratory of Royal Gendarmerie in Morocco.

2. Materials and Methods

2.1 Decontamination Procedure

Surface material was removed from the bones and teeth by washing with diluted bleach then with distilled water. The cleaned bone fragments were treated with UV light at 1.0 J/cm2 for 30 min.

2.2 Physical Grinding Procedure

Samples were equally divided and powdered the next day using the same technique. The four samples were powdered in a cryogenic impact grinder (CertiPrep 6770 Freezer Mill, Spex/Mill, Spex, Metuchen, NJ) following the manufacturer's instructions.

2.3 Demineralization Procedure

Demineralization of dentin and bone powder was done by incubating each sample in 700 µL to 1 mL of EDTA 0.5 M pH 8.5 for 48 h at room temperature. Afterwards, EDTA solution was eliminated.

2.4 DNA Extraction

Physically powdered bone (750 mg) was suspended in 1.6 mL of extraction buffer (0.1 M EDTA, 0.5% N-laurylsarcosine-Na salt, 100 mg/mL proteinase K), vortexed and incubated overnight at 37 ℃ with continuous vertical rotation. After phase separation by centrifugation at room temperature at 12,000 r.p.m for 10 min, 250 µl supernatant was transferred to a 1.5 mL

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Eppendorf tube and 3.5 µl 1 µg/µl Dextran Blue (Sigma), 250 µl 4 M NH4-acetate and 500 µl 96% EtOH were added and mixed by vortexing. Dextran Blue precipitates DNA and colors the pellet. The DNA was precipitated at -70 ℃ for 7 min and centrifuged at 14,000 r.p.m at 4 ℃ for 15 min. The remaining extract was stored at -20 ℃. This extract was purified with *DNA IQ*^{TM} DNA isolation System (Promega).

2.5 DNA Amplification and Sequensing

Amplification primers were F15971 (5'-TTAACTCCACCATTAGCACC-3'), R16410 (5'-GAGGATGGTGGTCAAGGGAC-3') for HVI, and F15 (5'-CACCCTATTAACCACTCACG-3'), *R448* (5'-TGAGATTAGTAGTATGGGAG-3') for HVII [6]. PCR was performed in a total volume of 25 µl consisting of 5-10 ng of template DNA, 2.5 µl 10 × PCR reaction buffer (Invitrogen, Karlsruhe, Germany), 1.5 mM $MgCl_2$, 200 mM each dNTP, 10 pmol each primer and 2 U Platinum Taq DNA polymerase (Invitrogen). The amplification was carried out at 94 ℃ (2 min) and then put through 38 reaction cycles: 94 ℃ for 30s, 54 ℃ for 30s and 72 ℃ for 30s. The sequencing reaction was carried out in a final volume of 20 µl containing 4 µl BigDye Terminator RR Mix, 2 µl 5 Big Dye Sequencing Buffer, 10 pmol primer and 4 µl PCR product. Cycling was performed (after a first denaturation step of 96 ℃, 1 min) for 25 cycles of 10s at 96 ℃, 5s at 50 ℃ and 2 min at 60 ℃. Each template was sequenced in both forward and reverse directions using the amplification primers. Sequencing reaction products were purified by Sephadex G-50.

2.6 Detection System

Electrophoretic separation was carried out on an ABI Prism 3130xl Genetic Analyzer (Applied biosystems). All analyses were performed twice from independent bone samples.

2.7 Quality Control

All analyses were done in separate areas for the different steps including bone preparation, DNA extraction, PCR amplification and sequencing. Negative and positive controls were run in parallel.

3. Results and Discussion

3.1 Case#1

By comparison of the mtDNA data from the bone sample to the revised Cambridge Reference Sequence, two transitions were found in HVI both C/T (Table 1). In HVII region seven transitions (4A/G and 3C/T) and one insertion of a cytosine were found (Table 2).

3.2 Case#2

In this case, the mtDNA data from the bone sample to the revised Cambridge Reference Sequence revealed, also two transitions (both C/T) in HVI region (Table 1). Four transitions (2A/G and 2C/T) and one insertion of a cytosine were found in HVII sequence (Table 2).

3.3 Case#3:

This is only case that revealed one transversion A/C in HVI when compared to the revised Cambridge Reference Sequence. In this hypervariable region we found, also, three transitions (2C/T and 1A/G) (Table 1). In HVII region, we found two insertions of a cytosine and two transitions both A/G (Table 2).

3.4 Case#4:

Two transitions were found in both HVI and HVII (respectively 2C/T and 2A/G) when compared to the revised Cambridge Reference Sequence (Tables 1 and 2). Two insertions of a cytosine were also found in HVII region.

4. Global Analysis

To search for compatibilities among mtDNA sequences, these ones were analyzed three times (the revised Cambridge Reference Sequence with) in the dnacomp program of phylip 3.67 software [7].

Sequences of three cases (#1, #2 and #4) showed the shorter time of divergence from the revised Cambridge

Table 1 Observed differences between HVI sequences of the four cases and the one of revised Cambridge reference sequence.

Sites	16069	16124	16126	16172	16219	16223	16224	16258	16278	16311
rCRS	C	T	T	T	A	C	T	A	C	T
Case#1	T	T	C	T	A	C	T	A	C	T
Case#2	C	C	T	T	A	T	T	A	C	T
Case#3	C	T	T	C	G	C	T	C	T	T
Case#4	C	T	T	T	A	C	C	A	C	C

Table 2 Observed differences between HVII sequences of the four cases and the one of revised Cambridge reference sequence.

Sites	73	150	152	185	195	228	263	265	295	309.1	315.1
rCRS	A	C	T	G	T	G	A	T	C	-[a]	-[a]
Case#1	G	C	T	A	C	A	G	C	T	-[a]	C
Case#2	G	T	C	G	T	G	G	T	C	C	-[a]
Case#3	G	C	T	G	T	G	G	T	C	C	C
Case#4	G	C	T	G	T	G	G	T	C	C	C

-[a]: Single base insertion.

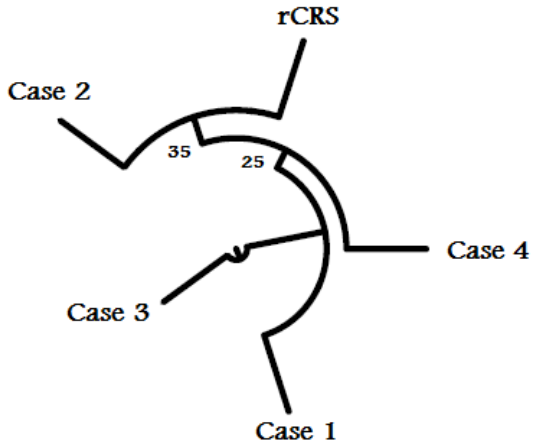

Fig. 1 Phylogenetic tree of HVI sequences. The number of each branch is the bootstrap value obtained for 1000 replicates.

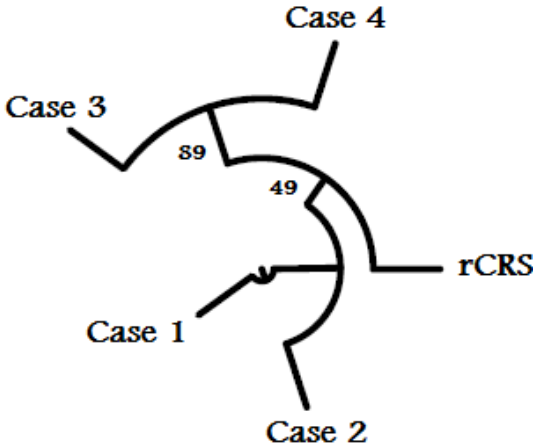

Fig. 2 Phylogenetic tree of HVII sequences. The number of each branch is the bootstrap value obtained for 1000 replicates.

Reference Sequence in opposite of the fourth case#3 sequence which is the most distant from the reference sequence and from the three other cases sequences as illustrated in the phylogenetic tree (Fig. 1).

The second analysis revealed a different schema of compatibilities among sequences. According to the revised Cambridge Reference Sequence, case#1 seems to have the most distant sequence, while sequences of case#3 and case#4 are the closest ones. Case#1 sequence is, also, the most distant from the three other cases sequences which are relatively closer from each other (Fig. 2).

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