# IDENTIFICATION OF THE SKELETAL REMAINS OF TWO 12-YEARS OLD BODIES BY NUCLEAR DNA POLYMORPHISMS ANALYSIS.

***P. Martín, A. Alonso, C. Albarrán and M. Sancho.***

Sección de Biología. Instituto de Toxicología. Mº de Justicia e Interior. Madrid. SPAIN.

## INTRODUCTION

The ability to analyse by PCR-based methods trace amounts of human DNA isolated from old bone material (Hagelberg et al. 1989; Hochmeister et al. 1991) offers the opportunity to identify unknown skeletal remains by a comparative genetic analysis with their presumptive relatives (Hagelberg et al. 1991; Gill et al. 1994).

We here report the successful identification of two 12-years-old bodies by comparative typing of nuclear DNA polymorphisms in the remains and in their presumptive parents. We also describe the main methodological challenges that we have found during this investigation.

## CASE BACKGROUND

Two skeletons buried with quicklime were found in a grave near Alicante (Eastern Spain) in 1985. Autopsy findings revealed that both skulls had bullet wounds as well as other signs of violence and mistreatement before death, but the cadavers were kept unidentified. On 1995, the police obtained some kind of evidence which indicated that the two bodies would corresponded with those of two individuals, disappeared in 1983, that presumably collaborated with a terrorist group from the Basque Country. The anthropological analysis carried out by forensic experts allowed to perform stature and age estimation as well as sexing of the remains, but no clinical or odontology records were available for comparison. To confirm the identification, DNA analysis was requested.

## MATERIALS AND METHODS

### *Laboratory Organization*

The following conditions were fullfilled to minimize the risk of contamination: Bone extractions were set up in a laminar flow cabinet with dedicated equipment (treated with 5% Sodium hypochlorite and UV light). Amplifications were set up in a laminar flow cabinet with a strict physical separation from the laboratory area where bone DNA was extracted. Negative controls were used in all experiments. At least two extractions per sample on different occasions and from different parts were performed. The DNA profile of the investigators involved in the case was available.

### *DNA Extraction and Quality Control*

The outer surfaces of a femur and a tibia from each cadaver were removed by sanding and three cleaned bone fragments from each body were independently pulverized to obtain three portions of aproximately five grams of bone powder. DNA was extracted from one of the bone powder portions using the protocol described by Hochmeister et al. (1991) that included a decalcification step for three days in 0.5 M EDTA at pH 7.5. DNA was extracted from the two remainning bone powder portions following the protocol described by Gill et al. (1994) without previous decalcification. In all cases the mixture that resulted from the Proteinase K treatment was extracted three times with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1). DNA extracts were washed and concentrated with TE buffer. using Centricon-100 microconcentrator devices. Total DNA was determined by agarose gel electrophoresis and ethidium bromide staining and by fluorimetry. Human DNA was quantitated by slot-blot hybridization with the human-specific D17Z1 probe using the Quantiblot system (Perkin-Elmer). About 1-3 µg/µl of total DNA were recovered in each extract, but only a small proportion (20-60 pg/µl) was of human origin. DNA was extracted from the blood of the presumptive parents by the standard phenol/chloroform extraction procedure.

### *PCR Amplification and Typing*

The amplification of STR loci was performed by single-locus PCR reactions in the case of HUMFES/FPS, HUMVWA, HUMF13B and HUMF13A1 or by a multiplex PCR reaction in the case of HUMTH01, HUMTPOX and HUMCSF1PO according to the manufacturer's recommendations using the GenePrint STR System (Promega Corporation, Madison, WI, USA). PCR products were typed by denaturing polyacrylamide gel electrophoresis followed by silver stain (Budowle et al. 1991; Martín et al. 1995). The amplification and typing of HLA-DQA1 and PM systems were performed according to the manufacturer's recommendations using the Amplitype PM and HLA-DQalpha forensic DNA Amplification and Typing kits (Perkin-Elmer Corporation, Norwak, CT).

### *Statistical Analysis*

The statistical weight of the evidence linking the skeletal remains to the presumptive parents was evaluated using Spanish allele frequencies. The likelihood ratio (LR) of the probabilities of obtaining the precise combination of alleles seen in the presumptive parents and in the skeletal samples, under the alternative assumptions that the skeletal remains are either derived, or not derived, from an offspring of their presumptive parents, was determined for each locus.

## RESULTS AND DISCUSSION

Given the low quantity (20-60 pg/µl) and the extent of degradation of the nuclear human DNA recovered from the bone samples we opted for a PCR-based typing of polymorphic markers with very short alleles (STRs, HLA-DQA1 and PM systems) as the unique suitable typing approach of nuclear DNA in this case.

All 13 loci were reproducibly amplified and typed from at least two independent bone DNA extracts (Fig.1 illustrates the STR profiling) and the typing results were fully consistent with the bone DNA from each body being derived from an offspring of their respective presumptive parents. It should be noted, however, that in one of the three DNA extracts obtained from one of the bodies, in addition to the bone-alleles, other PCR-products which could not be attributed to none of the presumptive parents were also detected (Fig. 2). A comparison of the typing results obtained from this DNA extract with the DNA profile of the researchers involved in this investigation allowed to demonstrate in this sample a contamination with modern human DNA originating from the investigator who performed DNA extraction. Apart from contamination, another methodological challenge found in this investigation during STR typing was the difficulty to detect some alleles (specially larger alleles which showed lower amplification efficiency) due to the presence in the bone DNA extracts and consistently in the PCR-reaction mix of vast amounts of irrelevant microbial DNA that often produced a high background when silver stain detection was attempted. In these cases, typing was confirmed by reamplification of a dilution ($10^{-3}$-$10^{-6}$) of the original PCR-product (Fig. 3).

The statistical weight of the evidence linking the skeletal remains to their respective presumptive parents was evaluated using Spanish allele frequencies. The cumulative likelihood ratio for all 13 loci was very high in both cases (LR/cadaver 1 = 35973; LR/cadaver 2 = 657117) establishing with a high degree of confidence the identification of the cadavers.

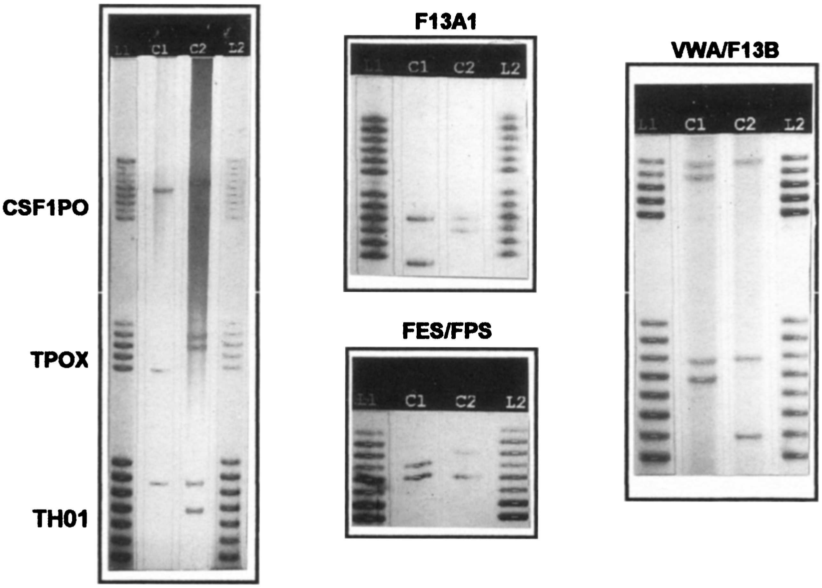

**Figure 1.** Representative STR profiles obtained from the bone DNA extracts. (C1): cadaver 1; (C2): cadaver 2, (L): allelic ladder.

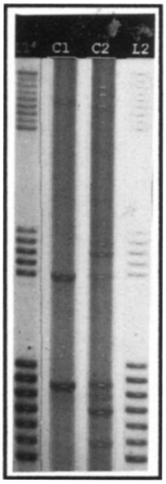

**Figure 2.** Example of STR typing (TH01, TPOX and CSF1PO loci) showing a DNA contamination in the bone extract recovered from cadaver 2.
(C1): cadaver 1
(C2): cadaver 2
(L): allelic ladder

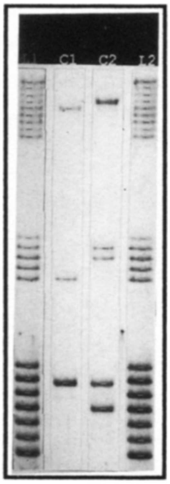

**Figure 3.** STR profiles (TH01, TPOX and CSF1PO loci) obtained by reamplification of a $10^{-4}$ dilution of the original PCR-products.
(C1): cadaver 1
(C2): cadaver 2
(L): allelic ladder.

## REFERENCES

Budowle B, Chakraborty R, Giusti AM, Eisenberg AJ, Allen RC (1991) Am J Hum Genet 48: 137-144
Gill P, Ivanov PL, Kimpton C, Piercy R, Benson N, Tully G, Evett I, Hagelberg E, Sullivan K (1994) Nature Genet 6: 130-135
Hagelberg E, Gray IC, Jeffreys AJ (1991) Nature 352: 427-429
Hagelberg E, Sykes B, Hedges R (1989) Nature 342: 485
Hochmeister MN, Budowle B, Borer UV, Eggmann U, Coney CT, Dirnhofer R (1991) J Forensic Sci 36: 1649-1661
Martín P, Alonso A, Budowle B, Albarrán C, García O, Sancho M (1995) Int J Leg Med (in press)