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CASE REPORT

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mtDNA as a tool for identification of human remains

Identification using mtDNA

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Abstract Two cases are presented in order to emphasize the importance of mitochondrial DNA in forensic medicine. The first case involved a charred body which could not be identified by morphological means because of severe destruction of all tissues. The parallel use of PCR methods using genomic DNA and sequencing of the mitochondrial d-loop region produced unequivocal and reproducible results. In the second case, various parts of a highly decomposed body were investigated. The application of standard PCR methods for genomic DNA proved unsuitable to answer the question whether the body parts belonged to the same body. However, when sequencing of mitochondrial DNA segments amplified from tissue and bone samples was performed, clearly interpretable results were obtained.

Key words DNA typing · Mitochondrial DNA · Non-coding region · D-loop region · PCR · Sequencing · Identification

Introduction

When the amount of genomic DNA is too low or the DNA is too degraded (e.g. severe putrefaction of tissues or bones), conventional analysis of genomic DNA for human identification may not work even if highly sensitive Amp-FLPs or STR-PCR methods are used. As repeatedly demonstrated, amplification and sequencing of mitochondrial (mt)DNA can be successfully performed even in cases where typing with genomic DNA is no longer possible [1–4]. There are several reasons why typing with mtDNA can be more successful than PCR with genomic DNA. A practical consideration of great importance is the much higher copy number of mt DNA [5]. Each cell contains several hundreds or thousands of copies of mtDNA compared to only two copies of autosomal chromosomes. This greatly increases the chance to recover mtDNA, especially when the sample contains limited quantities of DNA or highly degraded DNA.

In addition to the high copy number, mtDNA offers additional advantages for forensic studies. Firstly, mtDNA is well characterized and the complete sequence of human mtDNA has been determined [6]. Secondly, mtDNA is strictly maternally inherited and thus is not subject to recombination in meiosis [7, 8]. Therefore, an individual usually has a uniform mtDNA population [9]. Consequently, the mtDNA sequences of mother and child, and among brothers and sisters, are identical. A comparison of mtDNA sequences is simple and straightforward and therefore provides a useful tool for the identification of individuals. The generation of mtDNA variability can only occur through new mutations. In vertebrates, the mutation rate of mitochondrial genes is ~10-fold higher compared to nuclear genes [10–13]. The non-coding region is the most polymorphic region of the human mtDNA [10, 14, 15]. This control region contains the origin of replication for one strand, the d-loop region, as well as both origins of transcription [6, 16].

Based on these facts sequencing of amplified mtDNA can be used for identification of human remains in cases where conventional DNA typing is unsuccessful.

On the other hand, the mode of inheritance of mitochondrial DNA also has certain disadvantages. Due to the maternal transmission, brothers and sisters as well as the mother cannot be distinguished from each other by analysing mtDNA and cannot therefore be used for paternity analyses. Thus it has to be decided from case to case whether the use of this method is appropriate. In this context, we present two cases in which we were able to perform typing of body parts using mtDNA sequences.

Case histories

Case 1

A highly charred body was found in a burnt out motor vehicle in a remote forest area. As the fire had severely destroyed the body,

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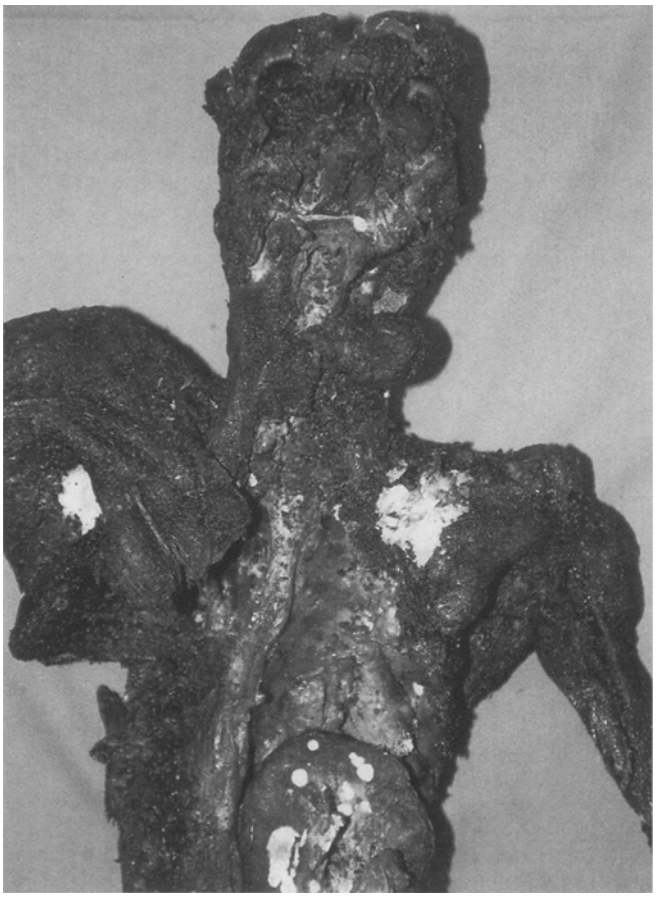

Fig. 1 Charred body found in the burnt out motor vehicle (case 1)

identification by morphological means (eg. dental status) was not possible (Fig. 1). The circumstances as well as the results of the autopsy led to the assumption that the body could be that of a young woman reported missing some days before and who was assumed to have been the victim of a homicide. The identification of the body was attempted with DNA from a bone sample using PCR systems and amplification of mitochondrial DNA sequences. For comparison, blood samples of the parents and a brother of the missing person were obtained.

Case 2

Several putrefied parts of an apparently adult human body (left arm, right arm, trunk) were found in a river within a few days. It was uncertain whether these parts belonged to one and the same person. Identification of the body parts was performed by sequencing amplified mtDNA from bone and muscle tissue. Blood samples of possibly related persons were not available.

Materials and methods

DNA extraction from bone marrow

The bones were cut open and the marrow was removed. After freezing and thawing the tissues sample was ground and incubated overnight in extraction buffer (10 mM Tris-HCl pH 8.0, 10 mM Na_2EDTA pH 8.0, 100 mM NaCl, 20% SDS, 40 mM DTT) with proteinase K (800 µg/ml) at 56° C. After two extractions with phenol/chloroform, DNA was precipitated with ethanol and the dried pellet was resuspended in 80 µl bidistilled water. The total DNA concentration was determined by gel electrophoresis (0.8% agarose gel) in comparison to a defined DNA reference standard. In addition, the ratio of human DNA was defined by the dot-slot technique and hybridization with the human-specific probe D17Z1 (ACES 2.0+ Human DNA Quantitation System, Gibco BRL) [17].

DNA extraction from bones

Bone samples were pulverized in a bone mill (Retch, type MM2) under cooling with liquid nitrogen and 0.1 g of powder was suspended in 500 µl extraction buffer and proteinase K and incubated overnight at 56° C. DNA extraction was performed as described for bone marrow.

DNA extraction from muscle tissue

Ground samples of muscle tissue were treated in the same way as bone samples.

DNA extraction from blood samples

After freezing and thawing the nuclei were pelleted from EDTA-treated blood samples by centrifugation (5 min at 8000 g). After discarding the supernatant the pellet was washed twice with 1 × SSC buffer (15 mM sodium citrate, 150 mM NaCl) and centrifuged again (5 min at 8000 g). The nuclei were resuspended in 700 µl bidistilled water containing 50 µl 3 M NaAc pH 7.0, 50 µl 10% SDS and 20 µl proteinase K (20 mg/ml) and incubated overnight at 56° C. Purification and extraction of DNA was carried out as described for bone marrow.

DNA amplification of genomic DNA

Amplification of extracted DNA was carried out using 5–10 ng DNA. For amplification and typing conditions the recommendations given in the manufacturer's protocols were followed. The PCR kits used for typing were HLADQa [18, 19], D1S80 [20] (Perkin Elmer Corporation) and HumTHO1 [21], HumVWA [22], HumFES [23], HumCD4 [24] (Serac GmbH, Bad Homburg). DNA sex tests were performed by amplification of the X-Y homologous gene amelogenin [25, 26].

mtDNA sequencing

PCR amplification using the primer pair PA/PrevA yielded a fragment of approximately 1200 bp which contains the entire mitochondrial control region (Fig. 2). The products were separated from residual primers and directly sequenced by cycle-sequencing. The L-strand was sequenced using fluorescence-labeled dideoxy nucleotides and primers PA or P1–P4, the H-strand was sequenced with the aid of the fluorescence-labeled primers Prev.1, Prev.2 or Prev.3. The 373A DNA Sequencer (Applied Biosystems) was used

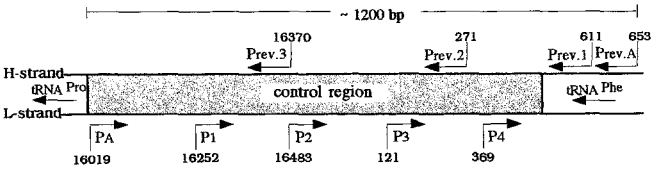

Fig. 2 Schematic representation of the human mitochondrial control region. Positions (according to Anderson et al. [6]) and orientations of the primers used in this study are indicated by arrows

Table 1 Sequence of primers used in mitochondrial amplification

Primer	Nucleotide sequence
PA	5′ CTCTGTTCTTTCATGGGGAAGCAGATTTGG 3′
Prev.A	5′ CCTATTTGTTTATGGGGTGATGTGAGCCCG 3′
P1	5′ AAAGCCACCCCTCACCCACTA 3′
P2	5′ GTGAACTGTATCCGACATCTGG 3′
P3	5′ GCAGTATCTGTCTTTGATTCC 3′
P4	5′ CCCTAACACCAGCCTAACCAG 3′
Prev.1	5′ CAGGACAGTGTATTGCTTTGAGG 3′
Prev.2	5′ CAGGAGTGGAAAGTGGCTGTGC 3′
Prev.3	5′ CAGGACCATGGGGACGAGAAGGG 3′

for separation of the fluorescence-labeled chain termination products. The primers used in mitochondrial amplification are indicated in Table 1.

Results

Case 1

A summary of the PCR typing results using genomic DNA extracted from tissue samples of the charred body (VP1), the blood samples of the parents (VP2 mother, VP3 father) and a brother (VP4) of the missing person is given in Table 2.

Sequencing of mtDNA

Sequencing was performed on both the H- and the L-strand of the mitochondrial control region of the charred body as well as the parents and a brother of the missing person. The Anderson sequence [6] was used as reference sequence and all nucleotide deviations were recorded. We sequenced the entire control region because we found that there is another hypervariable region between positions 440 and 560 (according to Anderson et al. [6]) which we called HVIII. In addition two positions were found in the central portion of the control region which showed high frequencies of polymorphism.

Due to the maternal inheritance of the mitochondrial genome it was expected that the sequences of the charred body, the mother and the brother of the missing person would be identical if the charred body was the missing person. On the other hand, the mitochondrial sequence of the father of the missing person should differ from these sequences and was therefore used as control sample.

Table 2 Results of the PCR typing systems using genomic DNA

Sample	HLA DQa	D1S80	Hum TH01	Hum VWA	Hum FES	Hum CD4	XY
VP1	4 /4	18/31	7/8	15/17	11 /13	6/10	f
VP2	3 /4	18/29	6/8	15/17	10a/11	6/10	–
VP3	1.1/4	24/31	7/9	15/16	11 /13	5/10	–
VP4	–	18/24	6/9	15/16	11 /11	5/10	–

XY: amplification of the X-Y homologous gene amelogenin as sex test; *f* female; *VP1* comparative sample 1, charred body; *VP2* mother of the missing person; *VP3* father of the missing person; *VP4* brother of the missing person; –: no tests performed

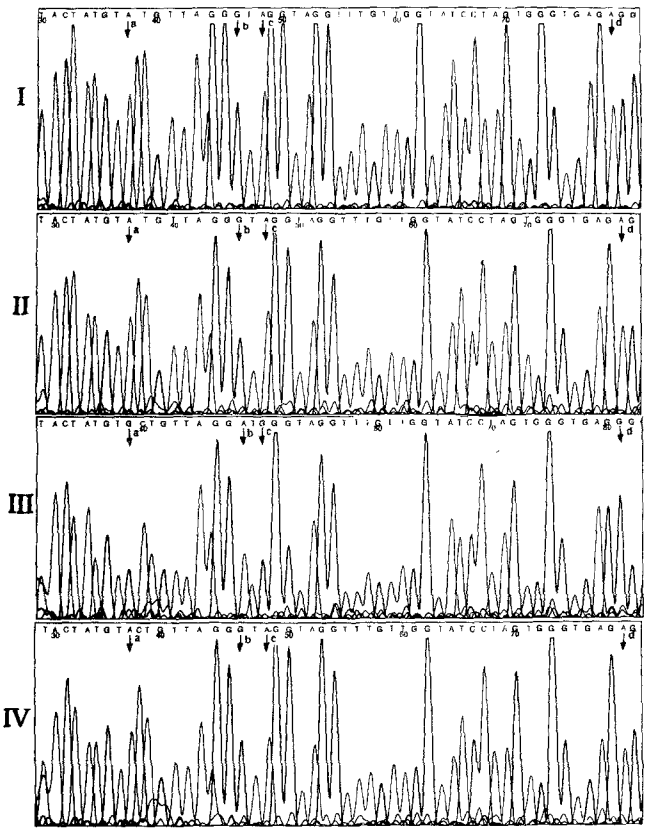

Fig. 3 DNA sequence plot of part of the H-strand in the control region of mitochondrial DNA with 4 different base variations as compared to the Anderson sequence (a–d). (a corresponds to position 16304 according to Anderson et al. [6]). I: sequence plot for the charred body (VP 1); II: sequence plot for the putative mother (VP2); III: sequence plot for the putative father (VP3); IV: sequence plot for the putative brother (VP4)

Figure 3 shows part of the sequence of the H-strand of the mitochondrial control region of the charred body, the parents and the brother of the missing person. Compared to the Anderson sequence, the sequences of the bodily remains, the putative mother and the putative brother contained 19 identical base variations. In contrast the sequence of the putative father shared six variable positions with the other sequences, and differed by two other base variations (Table 3). The remaining positions were identical with the Anderson sequence.

At position 16222 the sequences of the body parts, the putative mother and the putative brother showed a mixture of both A and G. The sequence of the putative father corresponded to the reference sequence at this position.

Case 2

In this case human DNA could not be detected using the ACES 2.0⁺ Human DNA Quantitation System and typing

Table 3 Results of mtDNA sequencing in case 1

Position	And.	VP1	VP2	VP3	VP4
16069	G	A	A	G	A
16126	A	G	G	G	G
16145	C	T	T	C	T
16172	A	G	G	A	G
16222	G	A/G	A/G	G	A/G
16261	G	A	A	G	A
16292	G	A	A	G	A
16294	G	G	G	A	G
16304	A	A	A	G	A
16519	A	G	G	G	G
73	T	C	C	C	C
175	T	G	G	G	G
242	G	A	A	G	A
263	T	C	C	C	C
295	G	A	A	G	A
309a	–	G	G	–	G
315a	–	G	G	G	G
462	G	A	A	G	A
489	A	G	G	A	G

Position: Position according to Anderson et al. [6]; And.: Sequence according to Anderson (H-strand); the numbering system of Anderson et al. has been adopted with the exception of 309a and 315a. These notations beside the dashed (–) indicate additional nucleotide positions between 309 and 310 and 315 and 316 in sequence VP1, VP2, VP4 and VP1–VP4, respectively. VP1: charred body; bone marrow sample; VP2: mother of the missing person; VP3: father of the missing person; VP4: brother of the missing person; A/G: sequence plot shows two peaks

Table 4 Results of mtDNA sequencing in case 2

Position	And.	1	2	3	4	5
16057	G	G	G	G	G	A
16120	T	T	T	T	T	C
16162	T	C	C	C	C	T
16239	G	G	G	G	G	A
16519	A	G	G	G	G	G
16527	G	G	G	G	G	C
73	T	C	C	C	C	C
137	T	T	T	T	T	C
164	G	G	G	G	G	C
263	T	C	C	C	C	C
309a	–	–	–	–	–	G
315a	–	G	G	G	G	G

Position: position according to Anderson et al. [6]; And.: sequence according to Anderson (H-strand); the numbering system of Anderson et al. has been adopted with the exception of 309a and 315a. These notations beside the dashed (–) indicate additional nucleotide position between 309 and 310 and 315 and 316 in sequence 5 and 1–5, respectively. 1: right arm, muscle-tissue sample; 2: trunk, muscle-tissue sample; 3: left arm, bone sample; 4: left arm, muscle-tissue sample; 5: operator's sequence

of the body parts by PCR methods for genomic DNA did not produce any useful results.

Therefore, the method of sequencing mitochondrial DNA was applied. Both strands of the entire control region of the various body parts were sequenced. The Anderson sequence was selected as reference sequence and all base variations were recorded.

Due to the fact that a human being is usually homoplasmic with respect to the mitochondrial genome population, sequences derived from different body parts should be completely identical, provided that these parts belong to one and the same person.

Table 4 shows the differences recorded in the sequenced control region from the various parts of the body found in the river and the control DNA of the operator as compared to the Anderson sequence.

Discussion

One advantage of mitochondrial DNA compared to genomic DNA is the much higher copy number per cell which makes amplification of mtDNA easier if material is extremely limited. An alternative method that can be used for human identification when genomic DNA systems are unsuccessful is sequencing of the only major non-coding region of mitochondrial DNA.

The characteristic pattern found in the genomic DNA of the charred body in case 1 matched the respective chromosomal DNA profiles of the putative parents of the missing person in each of the conventional PCR systems applied. This finding suggested that the charred body was indeed that of a natural daughter of the parents examined. The calculation of the probability that the hypothesis "natural daughter" (versus unrelated person) is true yielded a value of W = 99.99%. The results of the DNA sequencing confirmed that the charred body was related to the putative mother and the putative brother. From all the results of the police and the forensic investigations it became clear that the body must be that of the missing person.

A comparison of the results of both PCR methods, i.e. analysis of genomic DNA and sequencing of mtDNA, showed convincingly that data obtained from mtDNA analyses are reliable and reproducible.

Because of the maternal inheritance of mitochondrial only one allele normally exists in an individual and thus no sequence ambiguities are to be expected from the presence of more than one allele [9]. But at position 16222 two different nucleotides were detected in three out of the four samples. Several sequencing reactions were done with the products of two different DNA extractions and with two different amplifications. The L and H strand were sequenced for each sample and the same results were observed in all experiments. This procedure reduces the possibility of contamination or a sequencing artefact.

Another explanation for the two different nucleotides is the presence of two alleles of the mitochondrial DNA which differ only at position 16222 in the analysed region. This may be explained by the occurrence of a de novo mutation in the germ track of one of the female ancestors of the missing person's mother. As shown by Koehler et al. [27], a de novo mutation in the mtDNA non-coding region appeared to become fixed in a single generation of a Holstein cattle maternal lineage.

The heteroplasmy found could also be explained by assuming a partly paternal inheritance of mitochondrial DNA however this implies that the mtDNA of that person in the analysed region differed from the sequence of the woman only in one base. According to our experience such a coincidence is possible but unlikely. Irrespective of how the heteroplasmy developed, its presence in a maternal line is powerful evidence supporting identification.

In case 2 typing of the body parts by genomic DNA analysis was not possible. The nuclear DNA seemed to be so degraded or was present in such small amounts that even short repeat sequences (200–300 bp) could not be amplified. Amplification of mitochondrial DNA, however, resulted in a 1200 bp PCR product. DNA sequencing revealed that the DNA extracted from the various body parts exhibited identical sequence variations compared to the Anderson sequence, i.e. that they were identical. Therefore, it was assumed that they belong to one and the same person.

A detailed statistical evaluation of the variations, as usually carried out for genomic sequences, is not yet possible for mtDNA. Due to the lack of a data base, no calculation of base variation frequencies and sequence patterns can be carried out at present. However, the sequences presented here were compared with more than 270 sequenced control regions. These sequence data were extracted partly from published sources [14, 28–30] and partly determined in this institute where the mitochondrial control region from over 100 unrelated people was amplified and directly sequenced. Only those publications were considered which contained the entire control region of the mitochondrial genome and no single sequence was found to be identical with the sequences presented here. The pattern of variations determined for the two cases presented did not occur twice in more than 270 sequence samples. Our laboratory is currently working on establishing a data base covering the mitochondrial control region sequences of people from southern Germany. We hope to provide a basis for the biostatistical evaluation of mitochondrial sequences in the near future.

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References

1. Gill P, Ivanov PL, Kimpton C, Piercy R, Benson N, Tully G, Evett I, Hagelberg E, Sullivan K (1994) Identification of the remains of the Romanov family by DNA analysis. Nat Genet 6:130–135
2. Ginther C, Iseel-Tarver L, King M-C (1992) Identifying individuals by sequencing mitochondrial DNA from teeth. Nat Genet 2: 135–138
3. Holland MM, Fisher DL, Mitchell LG, William MD, Rodriquez WC, Canik JJ, Merril CR, Weedn VW (1993) Mitochondrial DNA sequence analysis of human skeletal remains: identification of remains from the Vietnam war. J Forensic Sci 38:542–553
4. Sullivan KM, Hopgood R, Gill P (1992) Identification of human remains by amplification and automated sequencing of mitochondrial DNA. Int J Legal Med 105:83–86
5. Robin ED, Wong R (1988) Mitochondrial DNA molecules and virtual number of mitochondria per cell in Mammalian cells. J Cell Physiol 136:507-513
6. Anderson S, Bankier AT, Barrell BG, de Bruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG (1981) Sequence and organization of the human mitochondrial genome. Nature 290:457–464
7. Giles RE, Blanc H, Cann HM, Wallace DC (1980) Maternal inheritance of human mitochondrial DNA. Proc Natl Acad Sci USA 77:6715–6719
8. De Francesco L, Attardi G, Croce CM (1980) Uniparental propagation of mitochondrial DNA in mouse-human cell hybrids. Proc Natl Acad Sci USA 77:4079–4083
9. Monnat RJ, Reay D (1986) Nucleotide sequence identity of mitochondrial DNA from different human tissues. Gene 43:205–211
10. Cann RL, Brown WM, Wilson AC (1984) Polymorphic sites and the mechanism of evolution in human mitochondrial DNA. Genetics 106:479–499
11. Brown WM, George MJ, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. Proc Natl Acad Sci 76:1967–1971
12. Brown WM, Prager EM, Wam A, Wilson AC (1982) Mitochondrial DNA sequence in primates: tempo and mode of evolution. J Mol Evol 18:225–239
13. Cann RL, Wilson AC (1983) Length mutation in human mitochondrial DNA. Genetics 104:699–711
14. Aquadro CF, Greenberg BD (1983) Human mitochondrial DNA variation and evolution: analysis of nucleotide sequences from seven individuals. Genetics 103:287–312
15. Horai S, Hayasaka K (1990) Intraspecific nucleotide sequence differences in the major noncoding region of human mitochondrial DNA. Am J Genet 46:828–842
16. Cantatore P, Attardi G (1980) Mapping of nascent light and heavy strand transcripts on the physical map of HeLa cell mitochondrial DNA. Nucleic Acids Res 8:2605–2625
17. Walsh PS, Varlaro J, Reynolds R (1992) A rapid chemiluminescent method for quantitation of human DNA. Nucleic Acids Res 19: 5061–5065
18. Gyllensten UB, Erlich HA (1988) Generation of a single stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLADQa locus. Proc Natl Acad Sci USA 85: 7652–7656
19. Saiki RK, Walsh, Levenson CH, Erlich HA (1989) Genetic analysis of amplified DNA with immobilized sequence specific oligonucleotide probes. Proc Natl Acad Sci USA 86:6230–6234
20. Kasai K, Nakamura Y, White R (1990) Amplification of a variable number of tandem repeat (VNTR) locus (pMCT1118) by the polymerase chain reaction (PCR) and its application to forensic science. J Forensic Sci 35:1196–1200
21. Edwards A, Hammond HA, Jin L, Caskey CT, Chakraborty R (1992) Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. Genomics 12:241–253
22. Kimpton CP, Walton A, Gill P (1992) A further tetranucleotide repeat polymorphism in the vWF gene. Hum Mol Genet 1:287
23. Polymeropoulos MH, Rath DS, Xiao H, Merril CR (1991) Tetranucleotide repeat polymorphism at the human c-fes/fps proto-oncogene (FES). Nucleic Acids Res 19:4018
24. Edwards MC, Clemens PR, Tristan M, Pizutti A, Gibbs RA (1991) Pentanucleotide repeat length polymorphism at the human CD4 locus. Nucleic Acids Res 19:4791
25. Casarino L, De Stefano F, Mannucci A, Canale M (1994) HLA-DQA1 and amelogenin coamplification: a handy tool for identification. J Forensic Sci 40:456–458
26. Mannucci A, Sullivan KM, Ivanov PL, Gill P (1994) Forensic application of a rapid and quantitative DNA sex test by amplification of the X-Y homolgous gene amelogenin. Int J Legal Med 106: 190–193
27. Koehler CM, Lindberg GL, Brown DR, Beitz DC, Freeman AE, Mayfield DR, Myers AM (1991) Replacement of bovine mitochondrial DNA by a sequence variant within one generation. Genetics 129:247–255
28. Piercy R, Sullivan KM, Benson N, Gill P (1993) The application of mitochondrial DNA typing to the study of white Caucasian genetic identification. Int J Legal Med 106:85–90
29. Stoneking M, Hedgecock D, Higuchi RG, Vigilant L, Erlich HA (1991) Population variation of human mt DNA control region sequences detected by enzymatic amplification and sequence-specific oligonucleotide probes. Am J Hum Genet 48:370–382
30. Vigilant L, Pennington R, Harpending H, Kocher T, Wilson AC (1989) Mitochondrial DNA sequence in single hairs from a southern African population. Proc Natl Acad Sci USA 86:9350–9354