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PCR AMPLIFICATION OF SEVEN SINGLE COPY NUCLEAR GENES FROM THE BELGRADE MUMMY

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Abstract - Here we report a DNA analysis of a human mummy dated approximately 2300 years BC. We extracted the DNA and used it for further PCR amplification of the following genes: c-fms proto-oncogene for CSF-1 receptor gene (CSF1PO); coagulation factor XIII a subunit gene (F13A01); c-fes/fps proto-oncogene (FESFPS); tyrosine hydroxylase gene (TH01); thyroid peroxidase gene (TPOX), von Willebrand factor gene (vWA) and Y chromosome specific sequence. The alleles from Belgrade mummy do not differ from the most frequent alleles of all present human populations. The exception is FESFPS gene. Belgrade mummy is homozygous for this locus which contains alleles with 7 motif repeats while such allele is found in less than 0.38% of living humans.

INTRODUCTION

As stressed by Ikram *et al.* (1998), DNA testing of mummies is still in its infancy, and its full potential has yet to be explored. So far only limited paleogenetical research has been conducted on Egyptian mummies, *i.e.* only minute sections of genetic information encoded in the DNA were examined. However, as emphasized by Germer (1997), and Nielsen *et al.* (1998) different perspectives promise new areas for future work. Aside from identifying gender, DNA testing of Egyptian mummies can also be applied in questions of genealogy, disease and population studies.

Difficulties in analysis are often encountered in samples from the mummified human remains, since resin and other materials used in embalming process usually impregnated and contaminated both hard and soft tissues. For that reason DNA analyses of some mummies, such as one acquired by the University of Illinois, have not yielded satisfactory results (S. Wissan, personal communication). Consequently, the current procedures for DNA extraction and purification need to be optimized. Successful aDNA and aRNA extractions were carried out, for the first time, in 1980 (Hunan Medical College 1980) from a rib of the corpse preserved for almost 2000 years. The first successful amplification of human ancient DNA (aDNA) was the cloning of 3.4 Kb DNA from 2400 years old Egyptian mummies (Paabo 1985). The invention of PCR in mid 1980s led into a boom in this research area but despite of the potential of this method aDNA analysis has been limited to sequences that are found in multiple copies within the cell: mitochondrial DNA (Hanni *et al.* 1990; Stoneking *et al.* 1991) and nuclear repeated sequences (*Alu* or Y chromosome specific sequences, usually used in sex determination: Hagelberg *et al.* 1991). Rare exceptions of successful amplification of single copy nuclear genes published so far include the analyses of class I Major Histocompatibility Complex (Lawlor *et al.* 1991), beta-globin gene (Beraud-Colomb *et al.* 1995), several microsatellite loci (de Pancorbo *et al.* 1995) and amelogenin gene (Stone *et al.* 1996). DNA content of an organism degrades rapidly after death giving short fragments of several hundred base pairs. Thus, size reduction is setting limits to the size of fragment that can be amplified.

We extracted DNA using a modified protocol for nucleic acid extraction from the cotton leaves (Hughes *et al.* 1988). This protocol was chosen because other published protocols for aDNA extraction (Herrmann *et al.* 1992) did not work well enough: DNA would be visible on a gel but it would not be PCR

amplifiable possibly because of the presence of some inhibitors of *Taq* polymerase.

We used Promega Gene Print STR System for DNA typing. This kit amplifies 6 genes each of which have 7 to 14 different alleles. We amplified the following human genes:

c-fos proto-oncogene for CSF-1 receptor gene (CSF1PO); coagulation factor XIII a subunit gene (F13A01); c-fes/fps proto-oncogene (FESFPS); tyrosine hydroxylase gene (TH01); thyroid peroxidase gene (TPOX) and von Willebrand factor gene (vWA).

The expected lengths of the amplified fragments were: for CSF1PO gene 295 bp to 327 bp; for F13A01 283 bp to 331 bp; for FESFPS 222 bp to 250 bp; for THO1 179 bp to 203 bp; for TPOX gene 224 bp to 252 bp and for vWA gene from 139 bp to 167 bp. Alleles of these loci differ in the number of copies of the repeat sequence contained within the amplified region and are distinguished from one another using silver stain detection following electrophoretic separation. This typing is more tolerant to the use of degraded DNA templates than others because the amplification products are less than 331 bp long.

MATERIAL AND METHODS

The same experimenter conducted all experiments. All buffers and water were autoclaved or filter sterilized. A large amount of PCR premix containing all the necessary components, except DNA and *Taq* polymerase, was prepared and frozen in small sealed aliquots at -20 °C. A "quality control" PCR without added DNA but only *Taq* polymerase was performed on random aliquots.

The preparation of bone samples and the DNA extractions were performed in one room and stored in a freezer. On arrival in the laboratory, the specimens were cleaned and UV irradiated (254 nm) for 60 min. Specimens were subsequently handled under sterile conditions. Small samples of bone were extensively cleaned by cutting off approximately 2 *mm* of the entire bone surface with scalpel blades. Bones were then placed in disposable sterile tubes further used for DNA extraction. Preparation of buffers and PCR set-up were performed in a dedicated sterile hood under constant UV illumination (254 nm). Dedicated pipettes with aerosol resistant plugged tips were used throughout. PCR experiments as well as the analyses of PCR products were performed in a separate room. For each set of PCR experiments reaction blanks were conducted.

The extractions of DNA were performed with 1-3 *g* powdered bone. One half of patella and fragment of vertebra were used. The bones were ground with a mechanical grinder. Powdered bone was washed with chloroform 3 times (in order to remove resinous matter which was left over after physical scraping and which was inside the bones), chloroform was removed and pellet left to dry in PCR hood under UV light. The pellet was then mixed with lysing buffer in the ratio: 1 *g* of powdered bone and 2 mL of lysing buffer (200 mM Tris-HCl pH 8.3; 10 mM EDTA; 1.5% lithium dodecylsulphate; 300 mM LiCl, 1% sodium deoxycholate; 1% NP-40). The samples were incubated at 55 °C for 48 *h* with gentle shaking, followed by phenol/chloroform extraction and isopropanol precipitation. The resulting pellets were washed two times in 70% ethanol, dried and resuspended in 200 µL deionized water. Mummy's genomic DNA was analyzed on 1% agarose gel (Fig. 1). PCR amplification of CFS1PO, F13A01, FESFPS, TH01, TPOX and vWA genes was performed using Promega Gene Print STR System for DNA typing. Conditions were as recommended by the supplier, except for BSA that was added in all reactions in final concentration of 0.15 *mg*/mL.

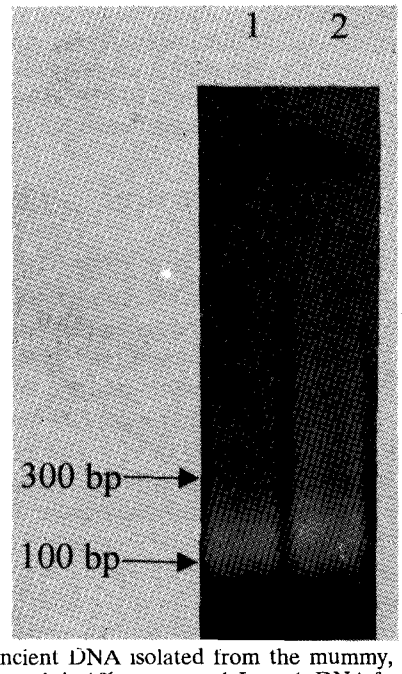

Fig. 1. Ancient DNA isolated from the mummy, analyzed by electrophoresis in 1% agarose gel. Lane 1: DNA from mummy's patella; Lane 2: DNA from mummy's vertebra.

Amplification of Y chromosome fragment was carried out using primers: p20/21 B2-1: 5' ATC TCA GGA CCC AAC ACC CG 3' and p20/21 B2-2: 5' GAT ATG TCC CAG TGT CCC CAT 3' (Iida *et al.* 1993). PCR reaction was performed in 50 µL volume containing 1x PCR buffer (Pharmacia LKB), 200 µM dNTPs each, 1.5 mM $MgCl_2$, 50 ng of each primer, 1.5 U *Taq* polymerase (Pharmacia LKB), BSA 0.15 *mg*/mL (final

concentration) and 10 μL of aDNA. Cycling conditions were as follows: 30 cycles of 1min at 94 °C, 1min at 65 °C and 1.5 min at 72 °C, and a final 10-min extension at 72 °C. PCR products were analyzed on ethidium bromide stained 2% agarose gel.

Alleles of CFS1PO, F13A01, FESFPS, TH01, TPOX and vWA loci were distinguished from one another using silver stain detection following electrophoretic separation. Five μL of each reaction were mixed with 2.5 μL of loading buffer (from Promega kit) and heat-denatured at 95 °C for 2 min prior to loading on to 6% polyacrylamide-urea denaturing gel (as recommended by onto the supplier) along with allelic markers supplied in the Promega Gene Print STR System for DNA typing kit.

RESULTS AND DISCUSSION

Ancient DNA isolated from patella and vertebra were analyzed on 1% agarose gel (Fig. 1). DNA was degraded as expected but we did not see any traces of fulvic and humic acids, which could be seen as fluorescent blue color under UV light. Fulvic and humic acids are apparently the main contaminants of aDNA and inhibitors of enzymatic reactions. It is important to mention that washing of the powdered bones in chloroform removed most of the resin. If this step was omitted the yield of aDNA was very poor if any.

The DNA profile of Belgrade mummy is given in Table 1 and Fig. 2. For vWA gene the mummy is homozygous giving PCR products of 151 bp/151 bp in length; for THO1 gene alleles were 195 bp/198 bp long; for FESFPS gene the mummy was homozygous with alleles 222 bp/222 bp long; for TPOX gene alleles were 236 bp/244 bp long and for CSF1PO gene the mummy was also homozygous with identical 307 bp long alleles. Since the length of alleles for CSF1PO gene may vary between 295 bp and 327 bp it is possible that the mummy is heterozygous for this locus but contains a larger allele (*e.g.* 327 bp long) which could not be ampli-

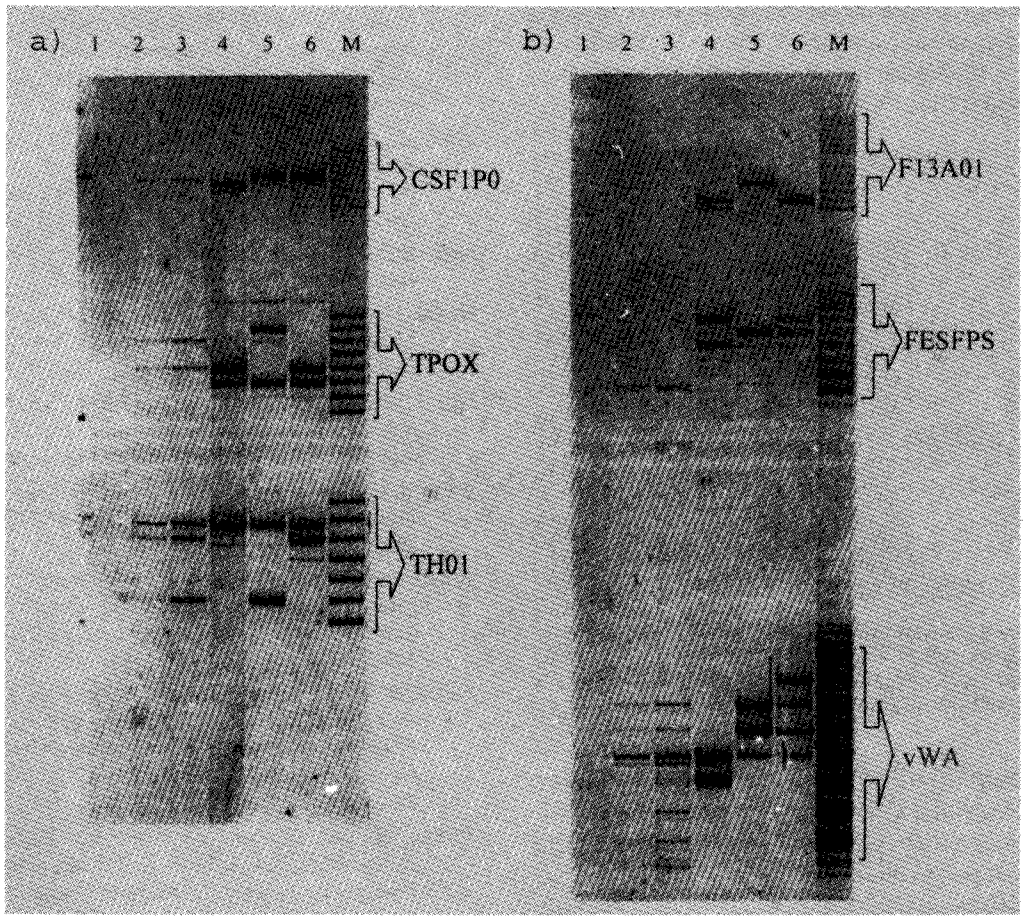

Fig. 2. Products of PCR amplification of mummy's and modern human DNA of a) CSF1PO, TPOX and THO1 and b) F13A01, FESFPS and vWA. Lanes 1-3: mummy's DNA 30,35 and 40 PCR cycles. Lanes 4-6: modern human DNA from different individuals. Lane M: allelic ladder. Products were separated by 6% denaturing PAGE.

fied since aDNA is degraded. The longest amplified allele from the mummy is 307 bp long and it may well be that it is the upper limit for amplification from this aDNA template.

Table 1. DNA profile of the Belgrade mummy.

Gene:	No. of repeated motif	The length of PCR products
vWA	16/16	= 151 bp/151 bp
---THO1	9/9.3	= 195 bp/198 bp
FESFPS	7/7	= 222 bp/222 bp
TPOX	9/11	= 236 bp/244 bp
CSF1PO	10/10	= 307 bp/307 bp

In three different experiments using the same aDNA preparation we never managed to amplify F13A01 gene. For F13A01 gene we expect to obtain fragments ranging from 283 bp up to 331 bp. This mummy might have long alleles for F13A01 locus and thus they could not be amplified. In all three experiments we repeatedly amplified vWA, FESFPS and CSF1PO loci obtaining the same alleles but only once successfully amplified TH01 and TPOX loci.

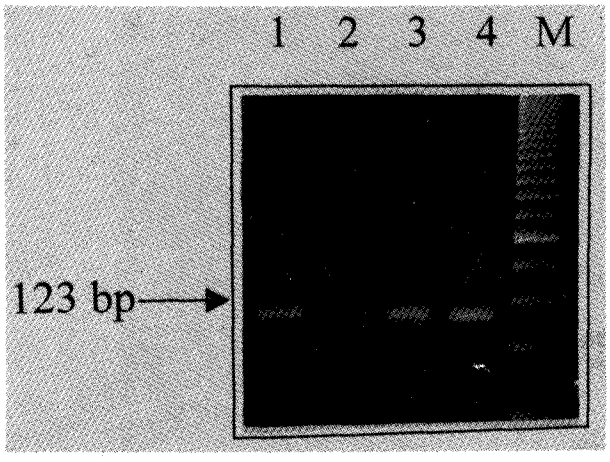

Fig. 3. Agarose gel analysis of PCR products of Y chromosome specific sequence from: Lane 1: present day (male) DNA; Lane 2: present day (female) DNA; 3: mummy's DNA (vertebra); Lane 4: mummy's DNA (patella); Lane 5: 50 bp ladder.

PCR amplification of Y chromosome specific sequence gave specific 123 bp long product (Fig. 3) which is in accordance with X-ray analysis showing that the mummy belonged to a male.

According to published studies for present human population the results of DNA typing of Belgrade mummy show that except for FESFPS locus other found for the mummy represent also the most frequent alleles in present human population (Lins *et al.* 1998).

The main problem in this type of experiments is a possible contamination with contemporary DNA. We performed all possible precautions to avoid contamination and run several controls (water instead of aDNA in the PCR mix, water treated as powdered bone going through entire procedure of aDNA isolation *etc.*) which were all negative.

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PCR АМПЛИФИКАЦИЈА СЕДАМ НУКЛЕАРНИХ ГЕНА БЕОГРАДСКЕ МУМИЈЕ

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У овом раду дати су резултати анализа молекула DNK изолованих из узорка мумије човека старе 2300 година. Геномска DNK је изолована и коришћена за PCR амплификацију следећих гена: c-fms протоонкоген за CSF-1 рецептор ген (CSF1PO), ген за *a* субјединицу хуманог фактора коагулације XIII (F13A01), c-fes/fps протоонкоген (FESFPS), ген за тирозин хидроксилазу (TH01), ген за тироидну пероксидазу (TPOX), ген за вон Willebrand-ov фактор (vWA) и специфичне секвенце за Y хромозом. Алели београдске мумије се не разликују од најучесталијих алела садашње хумане популације. Изузетак је FESFPS ген. Београдска мумија је хомозиготна за овај локус и има алеле са мотивом од 7 поновака. Учесталост овог алела у данашњој хуманој популацији је мања од 0.38%.