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A study of mtDNA from human fossils dating from the Bronze Age found in Romania

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Abstract

*The study of mtDNA polymorphisms from old human populations from the Bronze Age, found in Romania, has been used to show the degree of their genetic relationships with other old and modern European populations and their contribution to foundation of the modern Romanian genetic pool. Our study focuses on HVRI region (**h**yper**v**ariable **r**egion I) from mitochondrial D-loop.*

In order to optimize an old DNA extraction method we applied the DNA extraction methods with phenol/chloroform, as described by Hummel [1] and the extraction method with guanidine tiocianat and silica particles, as described by Hoss&Paabo [2], which were adapted to the degraded state of human fossil bones. We found that silica particles didn't inhibit the DNA-Taq- polymerase activity in the PCR reactions, on the contrary, they helped us to obtain better results than when we used DNA elution.

The amplified mtDNA sequences differ from the human modern mtDNA with to 4 substitutions. In order to make a statistical interpretation about the genetic kinships of these old populations with other old or modern human populations, we need mtDNA sequences from more individuals belonging to these old populations.

Keywords: old DNA, genetic variability, mtDNA, HVRI.

Introduction

Molecular paleogenetics is a modern and topical field of human genetics and studies paleo-DNA extracted from fossil tissues belonging to some individuals from old human populations. Up to now, the paleo-DNA studies have led to better understanding of the human genom's evolution and to a better estimation of the genetic distances between several old and modern populations, confirming or infirming some anthropological data.

Paleogenetic research uses several genetic molecular methods (PCR reactions, DNA sequencing, etc), but it needs to adapt methodologies to the degradation status of biological material, represented by different fossil tissues (such as bones and teeth, or some soft tissues from mummy bodies).

Because of the age of biological material and its preservation under unfit conditions in and/or after its taking up from the archaeological sites, the recovery of some DNA fragments suitable for genetic study is difficult.

The DNA polymerase inhibitors are a significant problem concerning paleogenetic research; they originate from the archaeological sites (i.e. hummic acids from soil) or from nucleic acid degradation (i.e. hidantoins from the nucleobase oxidative processes).

In order to prevent any contamination, especially with human modern DNA, it's necessary to handle the biological material into a sterile environment or even into a special laboratory (in which no modern DNA is worked) and to carefully prepare the biological samples for removing any previous contaminants [1].

The validation of the data in paleogenetics research must be done by reproducing the results by a second experiment and in a second laboratory, and by using the negative controls for detecting any contamination during the DNA extraction or the PCR amplification (**p**olymerase **c**hain **r**eaction).

The mitochondrial DNA markers are frequently used in paleogenetic research because of some particularly traits of the mitochondrial genome, such as: its presence in multiple copies per cell (1000-10000), it is only maternally inherited, it does not recombine, and its mutation rate is about 10 times faster than the average nuclear genes.

So far, the highest sequence polymorphism shows the D-loop region of mtDNA, an uncoding sequence of about 900 bp in the human genome and it contains the replication origin of the H strand, the transcription promoters of the two strands and the binding sites of two transcription factors [3]. This region mainly consists of HVR I and HVR II, the highly valuable DNA markers for the population genetics or phylogenetic studies. The HVRs are suitable for identifying at population and family lineage levels.

In human mitochondrial genome, the HVR I consists of 341 bp and the HVR II consists of 267 bp and they reveal about 3% variability between two individuals unrelated [1].

Our study focuses on the analysis of HVR I and HVRII polymorphisms on the old Thracian populations dating from the Bronze Age (of about 3500 years old) from Romanian territory, in order to show their genetic relationships with other old and modern European populations and their contribution to foundation of the modern Romanian genetic pool.

We have performed this study together with Human Biology Institute from Hamburg, Germany and Romanian National Institute of Tracology Bucharest.

This paper reports on the preliminary results of our study, namely the finding of a suitable method for old DNA extraction, adapted to the degradation state of the biological material.

Material and Methods

The samples have been human fossil bones of individuals from old Thracian populations of about 3500 years old, dating from the Bronze Age and found in some archaeological sites in the SE of Romania: Zimnicea, Smeieni, Cioinagi-Balintesti and Candesti.

To prevent any contamination, we have used sterile equipment and instruments and handled the samples on the sterile premises (bench with a laminar air-flow, a special laboratory for old DNA).

Preparing of the biological material for DNA extraction

To remove any contaminants, especially the modern DNA of the past handlers, the fossils were washed with bidistilated water and on each side, were UV irradiated for at least 5 min. The bone fragments remained after removing the outer layer with a sterile scalpel were

washed with absolute ethanol, then with ethanol 70% and with bidistilated water again, were dried at 30°C in the oven, over night and after that, the fossils bones were powdered with liquid nitrogen.

The old DNA extraction

To optimize an extraction method in agreement with the degradation status of the fossil biological material, we have applied several methods and performed more variants for each of them:

I. *the DNA extraction method using the extraction kit Wizard Genomic DNA Purification (Promega)* with and without proteinase K;

II. the DNA extraction method with phenol/chloroform as described by Hummel [1] and some of its modified variants (each modification represents one distinguished variant): without the decalcification step; cell lysis performed by incubation with lysis buffer with EDTA, SDS and proteinase K at 56°C for 2 hours, 3 hours, 4 hours and respectively, over night; DNA precipitation by the incubation with absolute ethanol, without silica particles, at 4^0C, over night, followed by centrifugation 1 hour at 14,000 rpm, at 4^0C;

III. the extraction method with guanidine tiocianate and silica particles as described by Hoss and Paabo [2] and some of its modified variants, i.e. using the lysis buffer with proteinase K from the previous method, with and without DNA elution step.

For each old DNA extraction we used a negative control to detect any contamination from the reagents.

Spectrophotometry analyses were performed to verify the results of the DNA extractions, and the absorbance was read in the UV light, at λ between 200 and 350 nm, by means of Specord 40 spectrophotometer and WinAspect 1.6.3.0.program.

The electrophoresis analyses for the old DNA extractions were done on agarose gels 1% and 2% in TBE 1X (Tris, boric acid, EDTA), at a constant voltage of 8 V/cm, 60min and visualized in UV light at 254 nm (after staining with ethidium bromide).

The mtDNA amplification

Mitochondrial HVRI region was amplified by PCR reactions (single and duplex), in two fragments (207 bp and 194 bp), for each of them we designed different sets of primers by means of PRIMER 3 program:

-for the 207 bp fragment : 1. TGA CTC ACC CAT CAA CAA CC
2. GTG GCT TTG GAG TTG CAG TT

-for the 194bp fragment : 3.AACTGCAACTCCAAAGCCAC
4. CGG GAT ATT GAT TTC ACG GA .

The reaction mixture consisted of: 15µl DNA extract, 5µl of PCR buffer (10x), 1,5µl of $MgCl_2$ (25 mM), 1µl of dNTP (10mM), 0,5µl of each primer (10 µM), 0,3µl of Taq-polymerase (5 U/µl), 3µl of BSA (Bovine Serum Albumin) (10 mg/ml), 7.5 to15µl of DNA extract or controls and sterile distillated water up to a final volume of 50 µl.

The PCR reaction profile consisted of an initial step of 3 min at 94^0C, followed by 35 cycles (45 cycles for blank controls) of 94^0C for 1 min, 55^0C for1 min and 72 ^{0}C for 1 min; the final step was performed at 72^0C for 7 min.

The test for inhibitors consisted on amplification of the control DNA (DNA K_{562}) in the presence of variable amounts of old DNA extracts.

The PCR reactions were carried out in a Perkin Elmer GeneAmp PCR System 9600 Thermal Cycler.

In order to prove the human origin of the amplified old mtDNA, as described by Cattaneo [4], the duplex PCR reactions were developed. Thus, a sequence of 120 bp from the control region V toghether with one of the two HVR regions of human mtDNA were

amplified. The control region V is the control region of cytochrome c oxidase subunit II gene (Col II/ $tRNA^{Lis}$).

The presence of PCR products was demonstrated by UV visualization by electophoresis on agarose gels 2% and the amplicons were compared with a molecular weight marker (100 bp leader). The gels were photographed with a digital camera.

The mtDNA products sequencing d at MWG by Sanger method, after cleaning them with the MBS Spin PCRapace Kit(50) Invitek kit.

To compare the old DNA with the modern reference European sequence, CRS (**C**ambridge **R**eference **S**equence, Anderson)[5], with the control DNA (DNA K_{562}) and with the modern DNA of the individuals who handled the samples into the laboratory, the alignement of DNA sequences was realised using Bioedit Program.

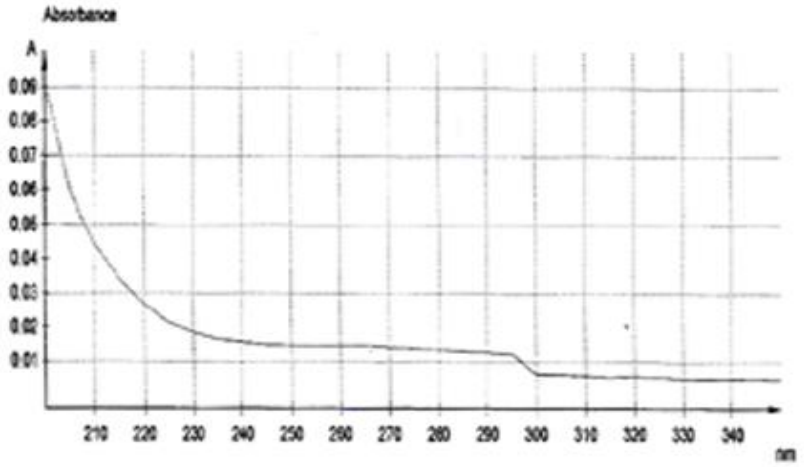
Results and Discussions

Our preliminary study focuses on selecting an optimal method for the DNA extraction from degraded human fossil bones, dating back to the Bronze Age (of about 3500 years old), found in Romania.

Not all the methods we used have led to positive results.

To examine the outcomes of the DNA extractions, we performed analyses in electrophoresis and UV spectrophotometry only for few DNA extractions in the beginning of our research, because the both methods required too much biological material, unavailable in this study.

The extraction kit Wizard Genomic DNA Purification (Promega*)* has been recommended by the producer for DNA extraction from blood and other fresh tissues. We have tried to adapt the use of this kit to the old DNA extraction by adding proteinase K in cell lyses and removal protein steps. We have obtained only small amounts of DNA in the extracts (calculated by means of OD_{260}), which were contaminated with proteins and polysaccharides (**Figure 1**).



A_{230}=0.0186 ; A_{260}=0.0149 ; $A_{260/}$ A_{280}=1.1119 ;
A_{280}=0.0134 ; A_{300}=0.0061 ; $A_{260/}$ A_{230}= 0.8010 ;
DNA concentration =4.4ng/µl

Figure 1. UV spectrophotometry of an old DNA extract by the extraction method with kit Wizard Genomic DNA Purification (Promega) and the proteinase K (dilution factor 1/10).

The DNA amplification by PCR reactions from these extracts has failed because of the presence of Taq-polymerase inhibitors, as the positive inhibitor tests showed. When smaller

amounts of DNA extracts were added (and so the DNA inhibitors were diluted) the DNA amplification was noticed.

The two original DNA extraction methods described by Hummel [1] and Hoss & Paabo [2] weren't suitable for our study because of the degradation state of the biological material. For this reason we have made some changes on the methods mentioned above and we have obtained DNA amplification from some of the DNA extracts.

Three of all variants of DNA extraction methods used in our study provided better results (although the mtDNA differed by amount and quality), these are:

a. a variant of the DNA extraction method with phenol/chloroform as described by Hummel[1] without decalcification step and the cell lyses step performed by incubation with lyses buffer over night (**Figure 2**);

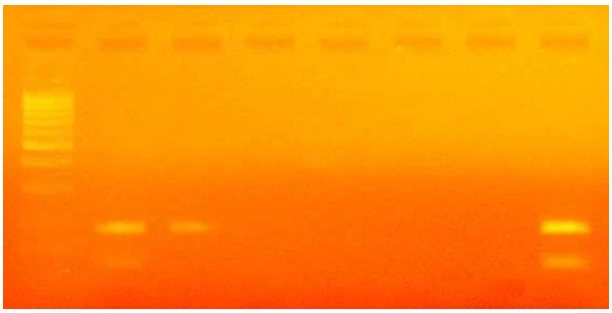


Figure 2. The results of the duplex PCR reactions for the amplification of the 197 bp fragment of HVR I and the 120 bp fragment of the control region V of mtDNA from DNA extracts obtained by the variant of the DNA extraction method described by Hummel (2003) without decalcification step and using the lysis buffer over night;
line 1: weight molecular marker 100bp DNA;
line 2-4:diferrent DNA extracts (DNA amplification in lines 2 and 3, no DNA amplification in line 4);
line 5,6: negative controls of DNA extractions by the same variant of extraction method (no DNA amplification)
line 7: positive control of PCR reaction:
line 8: negative control of PCR reaction.

b. two variants of the extraction method with guanidine tiocianate and silica particles as described by Hoss and Paabo[2],one using the original extraction buffer and the second using the lysis buffer with proteinase K from the previous method; both were done without the DNA elution step. The DNA extracts were used with silica particles in PCR reactions (**Figure 3**).

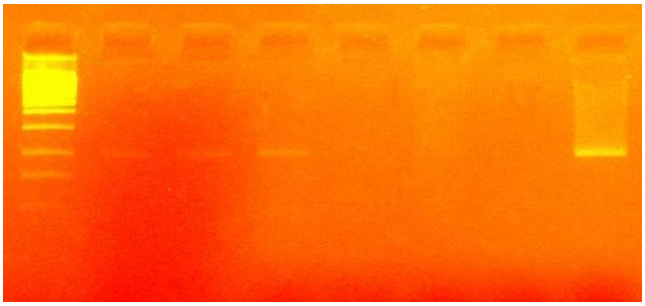


Figure 3. The results of the PCR amplification of the HVR I mithocondrial region-the 207 bp fragment- from different old DNA extracts obtained by two variants of the extraction method with guanidine tiocianate and silica particles described by Hoss & Paabo (1993):
line 1: weight molecular marker 100bp DNA;
lines 2,3: different DNA extracts obtained with the original extraction buffer and without the DNA elution step (DNA amplifications on both lines);
line 4-6: different DNA extracts obtained with the lyses buffer with proteinase K and without DNA eluting step (DNA amplification on line 4, no amplification on line 5 and a weak DNA band and 'smear' on line 6);
line 7: : positive control of PCR reaction:
line 8: negative control of PCR reaction.

We noticed that the silica particles didn't inhibit the Taq-polimerase activity in the PCR reactions, on the contrary they helped to yield better DNA amplification than when the DNA elution step was not done. For this reason we supposed that significant amounts of DNA fragments have been adsorbed on the silica particles surface. These DNA fragments couldn't be recovered by eluting at 65ºC during the extractions, but only at higher temperatures, of about 90 ºC, during the DNA denaturation step of PCR reactions. Performing the DNA eluting at such temperatures without PCR (in which the DNA fragments are used as template immediately after eluting) it could determine their destruction.

The mtDNA sequences amplified from the old DNA extracts have been compared with the reference European sequence CRS [5], the DNA sequences of the individuals who handled the samples into the laboratory (G.C., A.K., S.V. and K.T. samples) and the DNA used as a positive control in PCR reactions, DNA K_{562}.

The mtDNA sequences from the human fossils bones have differed from the CRS with 4 point mutations (substitutions):

- in the 194 bp fragment two substitutions at positions 16299 A→T (transversion) and 16311 T→C (transition) (**Figure 4**);

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                      pb 16 299      pb 16 311      pb 16 324      pb16 337
                          ↓              ↓              ↓              ↓
                                10             20             30             40
                     ....|....|....|....|....|....|....|....|
CRS          1   taacagtacatagtacataaagccatttaccgtacatagc 40
G.C.         1   ........................................ 40
A.K.         1   ........................................ 40
S.V.         1   ........................................ 40
K.T.         1   ........................................ 40
ADN K562     1   .T.......................C.............. 40
proba 5      1   .T...........C........................... 40

                                50             60             70             80
                     ....|....|....|....|....|....|....|....|
CRS         41   acattacagtcaaatcccttctcgtccccatggatgaccc 80
G.C.        41   ..................C.....C............... 80
A.K.        41   ........................................ 80
S.V.        41   ........................................ 80
K.T.        41   ........................................ 80
ADN K562    41   ........................................ 80
proba 5     41   ........................................ 80

                                90            100            110
                     ....|....|....|....|....|....|
CRS         81   ccctcagataggggtcccttgaccaccatc 110
G.C.        81   ............................... 110
A.K.        81   ............A.................. 110
S.V.        81   ....................A......c.. 110
K.T.        81   ............................... 110
ADN K562    81   ............................C. 110
proba 5     81   ............................... 110
                      ↑                              ↑
                  pb 16 377                      pb 16 407
```

Figure 4. Old and modern mtDNA sequences (HVRI,119 pb from the 194pb fragment).
CRS= Cambridge Reference Sequence (Anderson et al. 1981, revised de Andrew et al. 1999)G.C., A.K., S.V., K.T.samples = mtDNA sequences of the individuals from the laboratory ;
Proba 5= old DNA sample.

- in the 207 bp fragment two substitutions (transitions) at positions 16186 C→T and 16189 T→C (**Figure 5**).

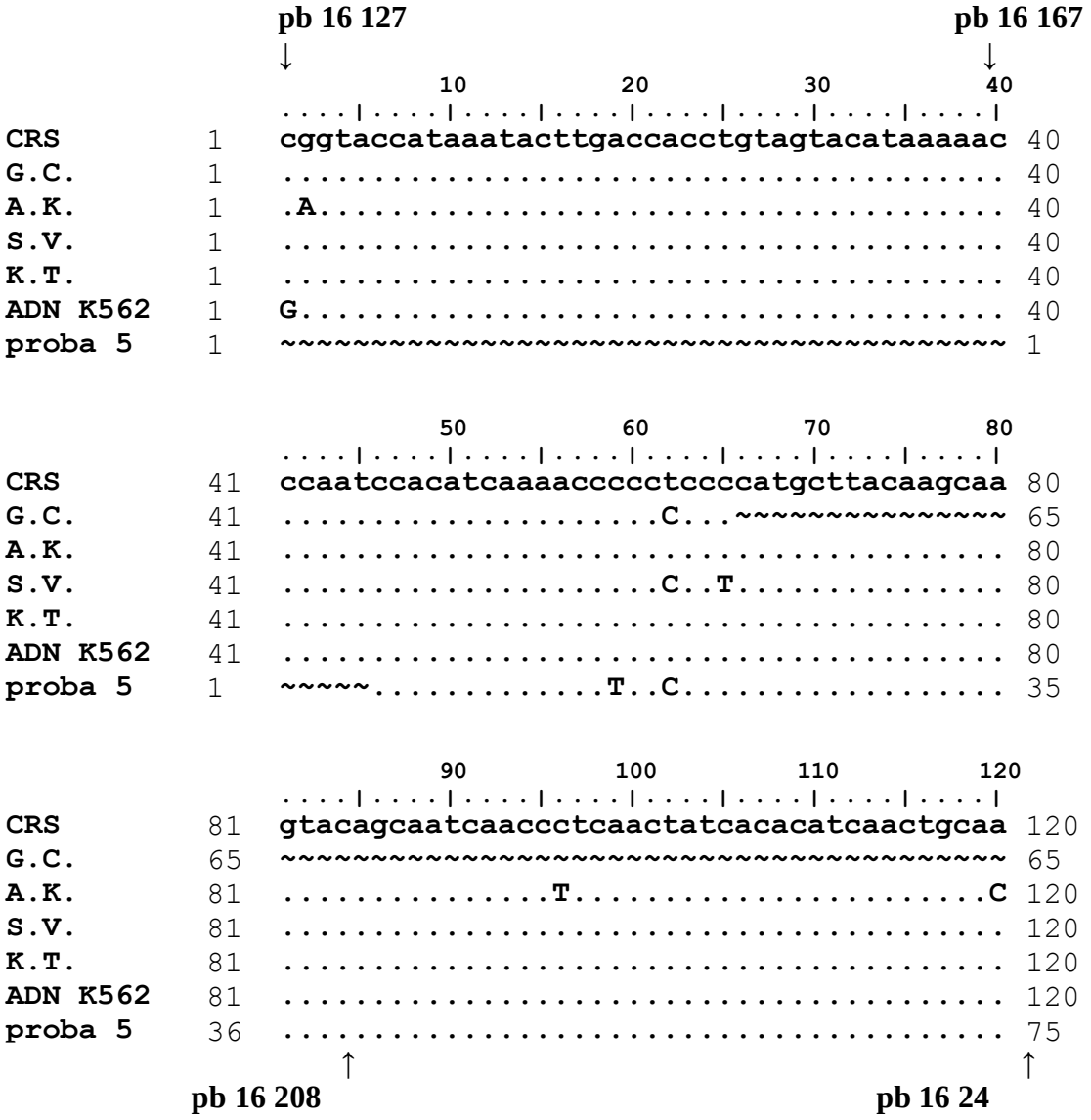

Fig.5. Old and modern mtDNA sequences (HVRI,79 pb from the 207pb fragment). CRS= Cambridge Reference Sequence (Anderson et al. 1981revised by Andrew et al. 1999) G.C., A.K., S.V., K.T.samples = mtDNA sequences of the individuals from the laboratory ;
Proba 5= old DNA sample.

The old mtDNA sequences have been different from all modern DNA sequences wicth they were compared by 1 to 4 point mutations (substitutions) as well (fig.4 and fig.5).

Estimates are that any two copies of the human nuclear haploid genome differ from one another at 1/100 to 1/500 of all nucleotide positions [6]. The mutation rate of nuclear genome is estimated at 10^{-5}-10^{-6}/locus/generation [6]. The mutation rate in mtDNA is about 10 times faster than the one in nuclear DNA and the mitochondrial genome is maternally inherited and it does not recombine[1].

Krings et al. (1997) compared the same mtDNA sequence of 379 bp taken from 994 different modern human individuals and found that humans differ from each other in (on average) 8 +/- 3.1 nucleotide positions (the '3.1' represents one standard deviation), that

means about 2.1% +/- 0.9% [7]. In another study, Krings et al. studied the differences between two modern human individuals on a 340 bp mtDNA sequence and found on average 10.9 +/- 5.1 differences (range 1-35), that means about 3.2 +/- 1.5% [8].

Hummel (2003) has shown that mitochondrial HVRs reveal about 3% variability between two randomly selected individuals who are not related. We know also that within the HVRs the polymorphic sites are not distributed uniformly, but cluster in some hotspots [1].

Emphazing that the old mtDNA sequences analyzed in this preliminary study have been different from all modern DNA sequences wich they were compared by 1 to 4 point mutations (substitutions), however we can't draw a conclusion yet about an whole population starting from the mtDNA sequences. We need mtDNA sequences from more than one Tracian individuals in order to correctly develop a statistical interpretation.

Conclusions and Prospects

In order to extract old mtDNA, we applied some DNA extraction methods from literature. The best results were obtained by the DNA extraction methods with phenol/cloroform described by Hummel [1] and guanidine tiocianat and silica particles described by Hoss & Paabo [2] with small modifications for adapting them to the degradation state of human fossils bones. These variants of the two methods described in literature will be used further in this study.

So far, we have obtained mtDNA sequences from a Tracian individual wich differs from the corresponding modern mtDNA sequence with 1 to 4 point mutations (substitutions). To elucidate the degree of the genetic relationships of old Tracian populations from Romania with other old and modern European populations and their contribution to the foundation of the modern genetic pool, we need to extract mtDNA sequences from human fossil bones from at least 20 Tracian individuals and perform analyses by means of modern statistical methods.

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