DNA ANALYSIS OF LATE BRONZE AGE FUNERARY CONTEXT FROM EASTERN ROMANIA

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Abstract: The aim of this study was to identify the corresponding haplogroup for the analyzed sample, presenting also the first results obtained on ancient DNA isolated from a Late Bronze Age funerary context bone remains samples, discovered in Eastern Romania. Also, to identify an efficient and reliable protocol for ancient DNA extraction and to test whether the protocol is efficient and capable of yielding good quality DNA, the phenol:chloroform and DNA IQ protocols have been compared, evaluating the level of PCR inhibitors and the DNA viability.

INTRODUCTION

In the last decade, advances in genomic sequencing have started to provide insight into the complex narrative of human ancestry embedded in human DNA, particularly through genetic variation. Genetic variants are sequences of DNA base pairs that differ from more common ancestral sequences and can be traced to specific human populations, either present or past by ancient DNA (aDNA). The genetic analysis of human ancestry is a search for variants in an individual's genetic code and determine how related that person is to specific ethnic and geographic populations. For males, if the samples are very good preserved, the Y chromosome is sequenced since this allele is always passed down from father to son withal for females, mitochondrial DNA is sequenced, since a daughter inherits the DNA from her mother. Genetic variants found in these sequencing data can be used to construct a paternal or maternal evolutionary tree, showing how certain human populations connect to each other.

The aim of this study is to introduce the first DNA results obtained on a funerary context assigned to a Late Bronze Age archaeological site from Eastern Romania which is located on the Eastern side of the Carpathians, in a hilly region of the Moldavian Plateau integrated in the Berheci River basin (Figure 1).

Figure 1 The location of the Late Bronze Age funerary discovery

The funerary discovery was located in the Tarnița village (Oncești commune, Bacău County) and was largely attributed to the Noua culture (Antonescu 1976) which is usually depicted as being an eastern intrusion of the Sabatinovka culture. The Noua culture reaches a significant area, limited eastward by the Dniester river, westward by the Apuseni Mt., to the north by the Northern Carpathians and the southern extent is represented by the Siret-Prut confluence (Vulpe 2001). The Late Bronze Age Noua communities located North and North-Western area of the Black Sea are considered to be characterized by a highly nomadic way of life sustained and by a stock-breeding economy. Their main burial practices are represented by inhumation in a supine position, with few vessels, and sometimes with jewelry and tools or weapons as grave goods (Dascălu, 2007).

The funerary context from Tarnița (Figure 2) was discovered in 1972 and consists in a pit containing a human skeleton in a left supine position. The left hand was flexed and sustain the skull while the right hand was slightly lodged on the pelvis and the legs were strongly bent on the left side. The osteological remains were in a poor state of preservation. The burial contains two vessels as grave goods which were attributed based on their typological characteristics to the Noua culture (Antonescu, 1976).

Figure 2 The funerary discovery from Tarnița (drawing by Antonescu 1976)

MATERIALS AND METHODS

Samples preparation

Samples are represented by 1 humerus (Figure 3A) and 1 clavicle (Figure 3B) from an entire skeleton located in the deposits of "Iulian Antonescu" Museum Complex (Bacău County, Romania).

Sample contamination is the major problem in ancient DNA (aDNA) studies and preventing its occurrence is always a priority for researchers. External contamination must be eliminated and several methods have been reported with this aim in the literature: abrasion, treatment with hypochlorite, irradiation with UV, etc. (O´Rourke et al., 2000, Kaestle and Horsburgh 2002, Kemp and Glenn Smith 2005). Extreme measures are usually reported during the procedures of extraction and amplification. The working area must be exclusively employed for ancient specimens and separated from the one where modern DNA is manipulated to avoid that any trace may contaminate samples. UV irradiation of the working area was also used, as well as the sterile and RNA-se/DNA-se free laboratory materials and regents. Masks, gloves and labs coat were used during the samples manipulations. Amplification inhibitors may also be co-purified in the extracts obtained and its presence produces negative results. Two different aDNA isolation protocols have been tested to eliminate substances that may act as inhibitors, so this situation must always be considered and essayed when negative results are systematically obtained (Kemp et al., 2006).

A Humerus   B Clavicle

Figure 3. T72 (Tarnița 1972) osteological samples

        The aDNA extraction from bone remains involve two main activities: samples preparation and extraction protocol. The bones were cleaned from debris and washed with distillated water, followed by a drying step. Further, the bones were washed with 10% NaOCl, dried and exposed to UV light for 15 minutes on each side. Finally, with a sterile bone drill a small area of the bone surface was removed and then bone powder was sampled, using a reduced rotation speed, to avoid the temperature increase followed by an accelerate aDNA degradation. Every bone was prepared using the required standard protocols for aDNA analysis (facemask, gloves, and disposable lab coat), sterile and disposable tools in a controlled environment.

DNA isolation

        For a higher confidence degree, two extraction protocols have been tested: phenol-chlorophorm-isoamyl alcohol (PCI) and DNA IQ (Promega, USA). For both DNA extractions protocols a total demineralization step was used. Around 200 mg of bone powder was used for PCI protocol and incubated at 56°C overnight with 1 ml of bone incubation buffer (*Tris HCl 50mM pH=8, SDS 10%, EDTA 0.5mM* and *Proteinase K 1mg/ml*), followed in the next day by the classic extraction steps. The DNA IQ extraction was made from 40 mg of bone powder according to the manufacture protocol. For both extraction protocols blank control samples were used to assess the potential reagents or lab contamination.

        In order to identify any possible contamination that might have occurred in the different stages of the samples preparation and mainly in the aDNA isolation, at least two extraction blank controls and multiple PCR non-template controls were included in each amplification reaction. The rate of contamination for this analysis was less than 0,4%.

aDNA isolation protocols evaluation by Real-Time PCR

        DNA quality and inhibition degree were evaluated using Real-Time qPCR with GoTaq 1-Step RT-qPCR System (Promega, USA), using the HV1 first part specific primers.

PCR and sequencing

        The mitochondrial hyper variable region 1 (HV1) was amplified via PCR method using four pairs of primers (Table 1). The PCR was performed in a 25 µL reaction volume using GoTaq® Hot Start Polymerase (Promega, USA). The amplicons were purified using the Agencourt AMPure XP (Beckman Coulter, USA) and direct sequenced using the Genome Lab DTCS Quick Start Kit (Beckman Coulter, USA) in the CEQ 8000 Genetic Analysis System (Beckman Coulter). The sequence analysis was performed using the CEQ8000 instrument software. Multiple sequences have been analyzed and low frequency mutations were considered artefacts resulting from post-mortem aDNA damage.

Table 1 HV1 primers sets

Locus	Pimers	Amplicon Size (bp)	Reference
MPS1A	F 5'-CCC AAA GCT AAG ATT CTA AT-3 R 5'-TAC TAC AGG TGG TCA AGT AT-3'	170	Gabriel et al., 2001
MPS1B	F 5'-CAC CAT GAA TAT TGT ACG GT-3 R 5'-TGT GTG ATA GTT GAG GGT TG-3'	126	Gabriel et al., 2001
MPS2A	F 5'-CCC CAT GCT TAC AAG CAA GT-3 R 5'-TGG CTT TAT GTA CTA TGT AC-3'	133	Gabriel et al., 2001
MPS2B	F 5'-CAC TAG GAT ACC AAC AAA CC-3' R 5'-GAG GAT GGT GGT CAA GGG AC-3'	143	Gabriel et al., 2001

RESULTS AND DISCUSSIONS

aDNA quantification and inhibition

The first stage of current experiment was the evaluation of the ability to remove the PCR inhibitors and the aDNA recovery efficiency from bone remains by two widely used extraction protocols. In order to assess the aDNA recovery, the extracted DNA was firstly subject to spectrophotometric quantification. By comparing the DNA concentration in both extraction assays, the DNA quantity extracted with PCI protocol was greater, suggesting that the PCI extraction protocol had a better efficiency. Even if, the DNA quantity it's double in PCI case in comparison with DNA IQ (Table 2), for both protocols the amount of aDNA recovered from ~1.8 kya bones is quite low.

Table 2 The amount of aDNA recovered from ~1.8 kya bones using two extraction protocols

Sample ID	PCI aDNA Concentration (ng/ul)	DNA IQ aDNA Concentration (ng/ul)
Bk*	0.1	0.0
R**	0.0	0.0
T72	1.5	0.7

Bk* - blank probe
R** - reagents control, distillated water instead of incubation buffer
All the samples were read in 2 µl volume

Several previous studies shown a dependency between the aDNA extraction protocol and the degree of inhibition (Rohland and Hofreiter, 2007; Jakubowska et al., 2012). Therefore, a RT-qPCR approach was used to evaluate the co-extraction of PCR inhibition. For both protocols the same amount of aDNA was used, 0.6 ng/. Furthermore, an absolute quantification of DNA copies per reaction was made, using a standard quantification curve ranging between 2 and $2x10^5$ copies per reaction. The cycle threshold (Ct) value shown from the real-time PCR is obvious much lower for the DNA IQ protocol (Figure 1) which indicates a higher aDNA template concentration and a correlated low number of cycles for the amplification to start. Also, the RT-qPCR data shown that the DNA IQ protocol it's able to extract about 565 inhibitors free DNA copies/µl while the PCI protocol extract just 13.5 copies/ µl. Also, the RT-PCR amplicons melting curve analysis which is frequently used as a diagnostic tool for assessing qPCR amplicon length with intercalating dye qPCR assays, indicate the presence of just one single product.

Finally, regarding the aDNA quantity and quality we can conclude that the DNA IQ protocol offer better results with a high aDNA purity and viability, compared to the classic PCI protocol, due to a higher amount of PCR inhibitors co-extracted by PCI protocol.
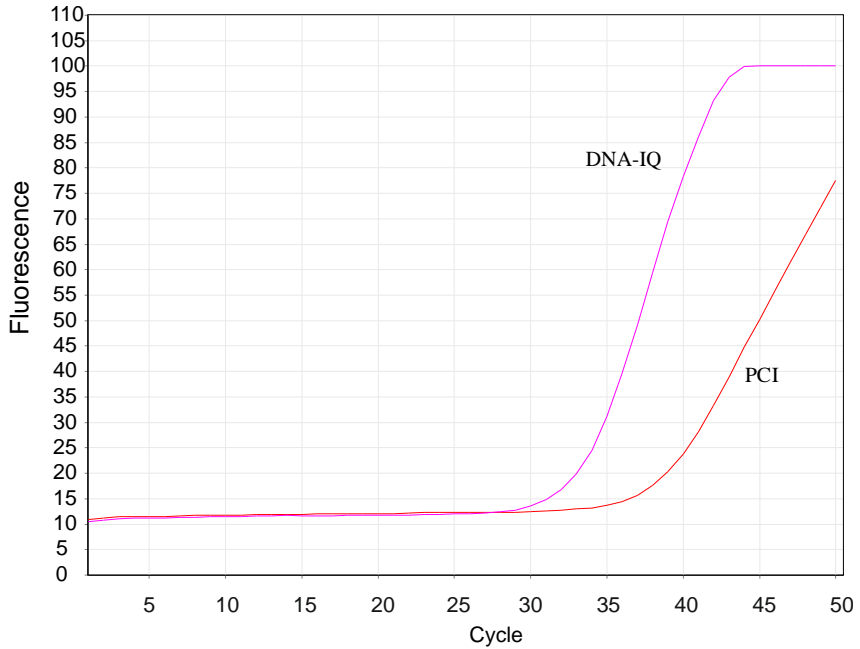
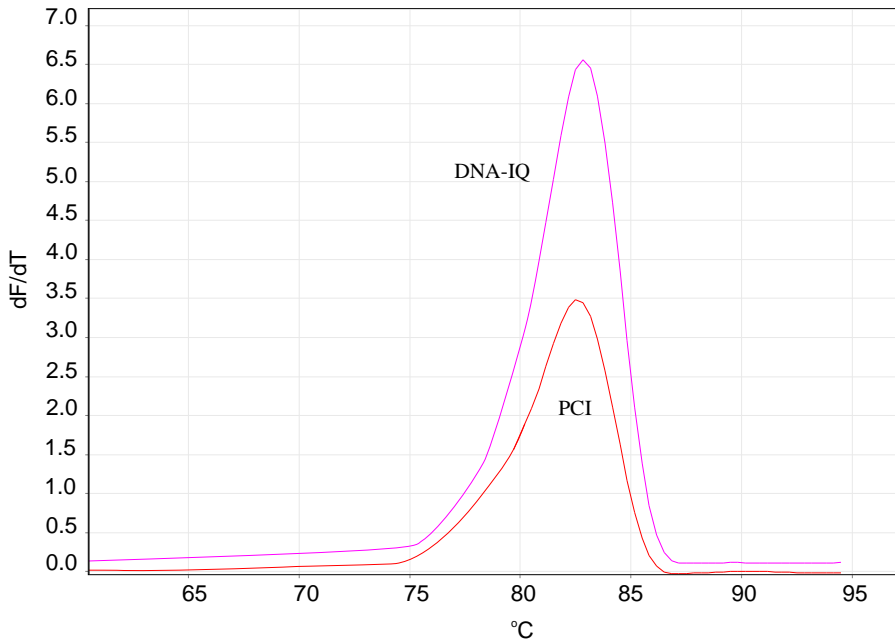Figure 4A The RT-PCR T72 bone aDNA sample analysis for both tested protocols PCI and DNA-IQ: Ct values

Figure 4B T72 bone aDNA sample melting curve analysis for both tested protocols PCI and DNA-IQ: Ct values

Genetic and cultural context

The sequences were subjected to Nucleotide BLAST (Basic Local Alignment Search Tool, Altschul et al., 1990) to identify the similarities with the previous sequences from data base and haplogroup assignation (Figure 5 and Table 3). Also, the T72 sample shown an identity of 97% with HV1 sequences from a previous study conducted by Lippold et al., 2014.

According to Eupedia database, Haplogroup HV is the most successful maternal lineage in Europe and the Near East with a distribution of over half of the European population and between 25% and 40% of the Near Eastern population. Most Europeans belonging to the HV lineage descend from a branch that was renamed haplogroup H. Another small but substantial European branch was called haplogroup V. The major subclades of HV haplogropup are HV0 and V, HV1, HV2, HV4, HV5, HV12, HV13 and H.
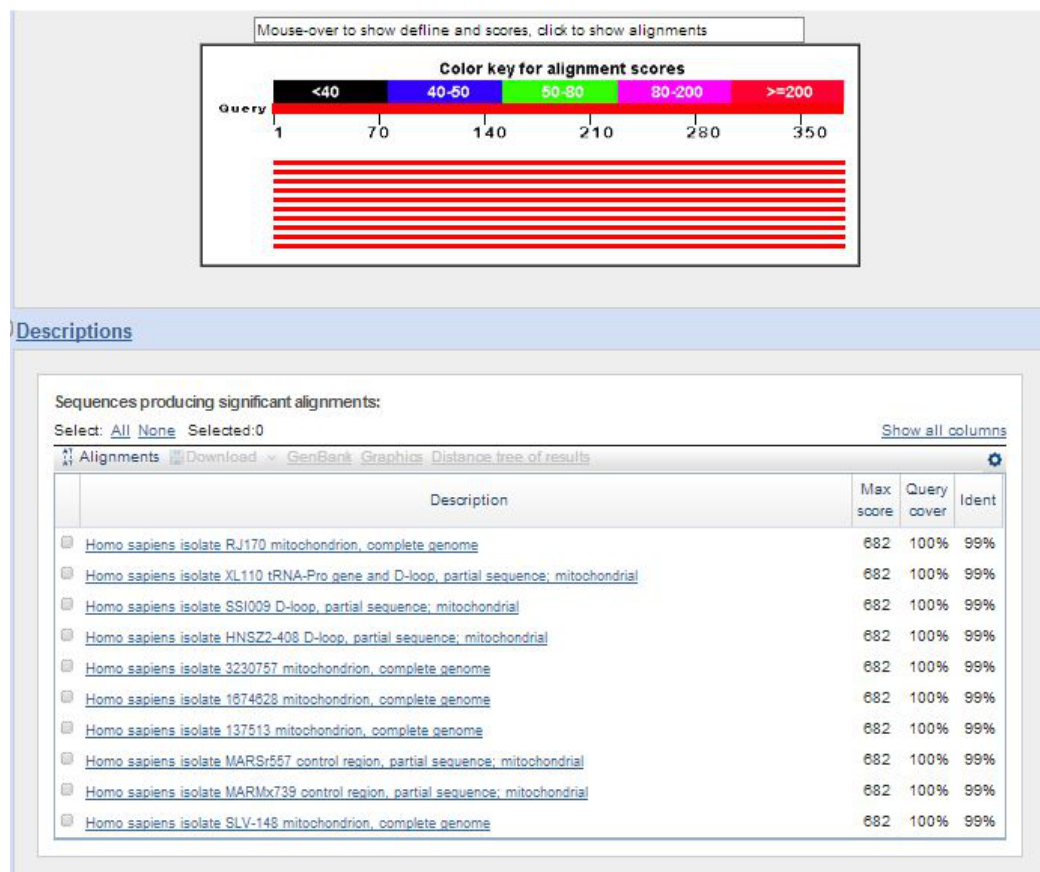

Figure 5 Blast report from NCBI (National Center for Biotechnology Information) indicate a 100% similarity with HV2 haplotype

Table 3 Sample haplogroup prediction based on Alignment Summary: rCRS track view
(http://mitomaster.mitomap.org/)

Sequence	Predicted Haplogroup	Total Variants	Variants
T72	HV2	6	T16126C, T16217C, C16218T, T16229d, C16232CT, T16304C

The T72 sequence was aligned with haplogroup HV and HV2 NCBI sequences and used to construct a ML tree, in order to confirm the haplogroup assignation.
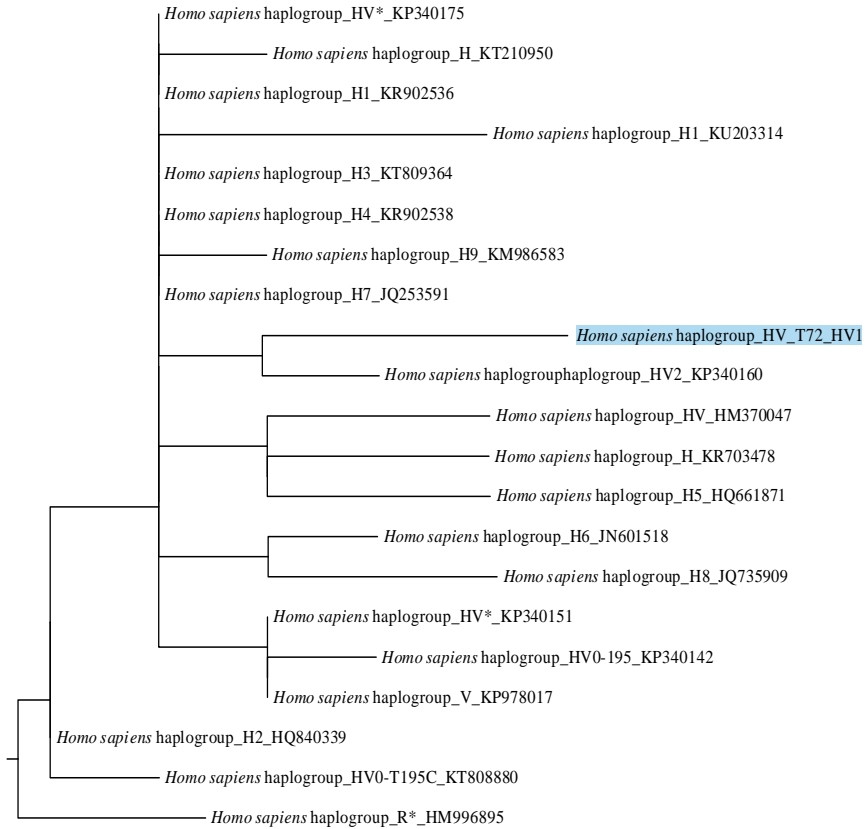

0.003

Figure 6 Molecular Phylogenetic analysis by Maximum Likelihood method

Homo sapiens haplogroup_HV*_KP340175
Homo sapiens haplogroup_H_KT210950
Homo sapiens haplogroup_H1_KR902536
Homo sapiens haplogroup_H1_KU203314
Homo sapiens haplogroup_H3_KT809364
Homo sapiens haplogroup_H4_KR902538
Homo sapiens haplogroup_H9_KM986583
Homo sapiens haplogroup_H7_JQ253591
Homo sapiens haplogroup_HV_T72_HV1
Homo sapiens haplogroup_HV2_KP340160
Homo sapiens haplogroup_HV_HM370047
Homo sapiens haplogroup_H_KR703478
Homo sapiens haplogroup_H5_HQ661871
Homo sapiens haplogroup_H6_JN601518
Homo sapiens haplogroup_H8_JQ735909
Homo sapiens haplogroup_HV*_KP340151
Homo sapiens haplogroup_HV0-195_KP340142
Homo sapiens haplogroup_V_KP978017
Homo sapiens haplogroup_H2_HQ840339
Homo sapiens haplogroup_HV0-T195C_KT808880
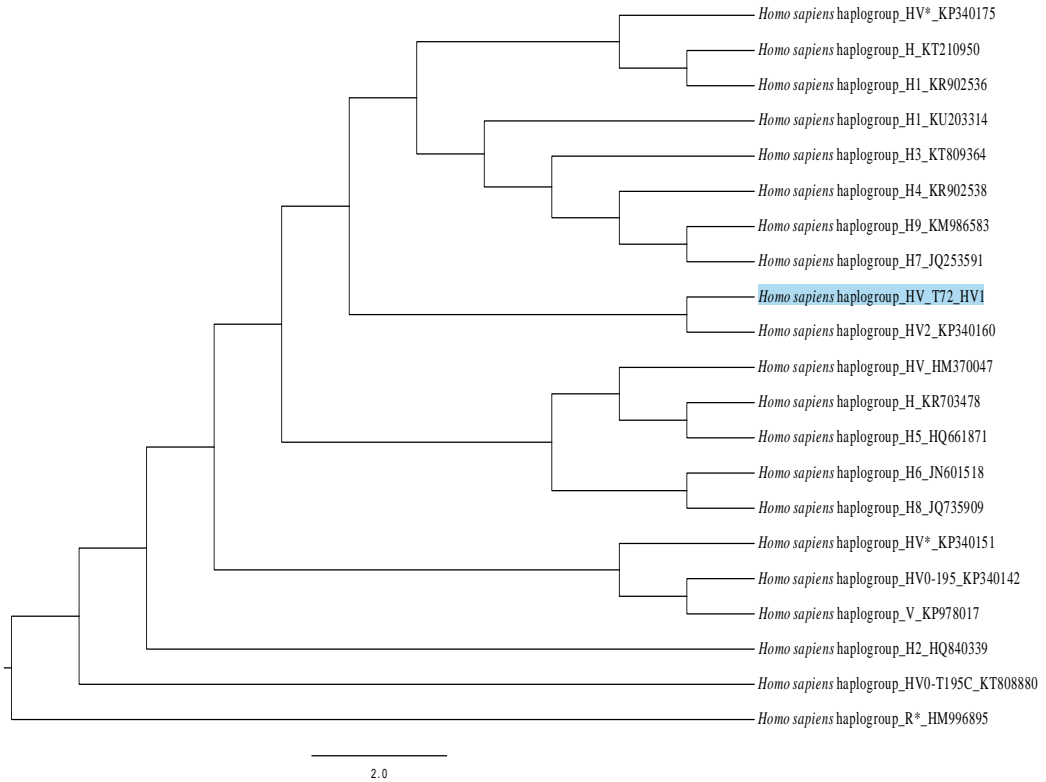*Homo sapiens* haplogroup_R*_HM996895

2.0

Figure 7 The Cladogram of ML tree

The evolutionary history was inferred by using the Maximum Likelihood (Figure 7) method based on the General Time Reversible model (Nei and Kumar, 2000). The tree with the highest log likelihood (-691.0766) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (4 categories (+G, parameter = 0.1000)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.0000% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 21 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 381 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

CONCLUSIONS

The mtDNA is relatively easy to work with, as it has a reduced size, coupled with the conserved arrangement of genes, which means that many pairs of universal primers will amplify regions of the mitochondria in a wide variety of vertebrates and invertebrates.

Even if the arrangement of genes is conserved in the mitochondrial genome, the overall mutation rate is high, may be due partly to the by-products of metabolic respiration and also to less-stringent repair mechanisms compared with those acting on nuclear DNA. The high mutation rates mean that mtDNA generally shows relatively high levels of polymorphism and therefore will often reveal multiple genetic lineages both within and among populations.

mtDNA is effectively a single haplotype that is transmitted from mothers to their offspring, which means that mitochondrial lineages can be identified in a much more straightforward manner than nuclear lineages, which, in sexually reproducing species, are continuously pooling genes from two individuals and undergoing recombination.

The ancient DNA analysis indicate with a maximum confidence, that the T72 sample belongs to HV2 haplogroup.

Genetic information for sample T72 obtained from the amtDNA analysis are in agreement with previously analyzed LBA samples from Romania (Hervella et al. 2015; Bolohan et al. 2016) which were attributed to the Noua culture based on the associated grave goods. The HV clade as a whole has a high occurrence between Western and Central Asia region. Most of the lineages within the HV haplogroup were spread into Europe during the Late Glacial Maximum (27-16 kya). In particular, high HV occurrences are observed in Southern Europe which could have spread into Europe with the amelioration of the climatic conditions (De Fanti et al. 2015).

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