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INITIAL ANALYSIS OF DNA FROM MUMMIFIED AND NEOLITHIC HUMAN REMAINS

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Abstract: *Depending on thermal, chemical and microbial circumstances DNA fragments in archaeological remains are able to survive up to 100,000 years for the longest, although almost complete degradation within just a few months can also occur during the time. Even DNA of Neanderthal bones can be analysed. The prehistoric data of very early, 7,000–5,000 years old Neolithic remains can provide crucial information on the origin of modern Europeans.*
The two main alterations of ancient DNA molecules with time are fragmentation and base modification. Following an adequate DNA extraction from human remains, the low copy number authentic DNA templates can be amplified via PCR and analysed further. To assess the authentic DNA template quality, the degree of fragmentation of human mtDNA was estimated using overlapping primer design, with different amplicon sizes ranging from 60 to 600 bp. Samples of markedly different age, as well as the 7,000 years old Neolithic remain from Vörs-Máriaasszony sziget, Hungary has been chosen and 150 years old naturally mummified body from the Dominican Crypt of Vác, Hungary. Their ancient DNA characterisation can be the first step of our future aim: to search for polymorph sequence pattern in samples from more remains in order to investigate population structure.
Keywords: *Ancient DNA; Bone samples; PCR; Genetic polymorphism.*

Introduction

In 1984 Russ Higuchi and colleagues at Berkeley published a finding that traces of deoxyribonucleic acid (DNA) from a museum specimen of the Quagga, Equus quagga, (an Equid believed to have gone extinct in the late 19th century) not only remained in the specimen over 150 years after death of the individual, but could be extracted and sequenced (Higuchi et al. 1984). The development of the Polymerase Chain Reaction (PCR; Mullis and Faloona 1987, Saiki et al. 1988) and the recent advances in the methodology of ancient DNA (aDNA) had an evolutionary impact on biological anthropology. After the first published experiments of the 1980s, the widely appreciated publication of Neanderthal mtDNA sequence fragment by Krings et al. (1997) showed a way to follow to everyone who tries to analyse authentic ancient DNA.

The traditional approach in physical anthropology is based on craniometry. On the long term we are aiming at examining how the craniometric and genetic data correspond. The sample of Neolithic human remains found in Hungary can serve a base for this purpose. In an earlier craniometrical study, we assumed the survival of the early Neolithic Körös (autochthonous) population to have been the determinative factor with which the southern genetic impulses may have become integrated. The interconnection system of regional, cultural and chronological differences of thorough metric analysis of cranial samples referred to an elimination of former (early Neolithic) anthropological

differences between Körös and Linear Band Keramik (LBK) people (Guba et al. 1997). This hypothesis can be tested via analysis of genetic polymorphism gained from ancient mitochondrial DNA. Haak et al. (2005) suggested in an aDNA study involved Hungarian Neolithic bone samples, that their maternal lineage showed that the present Europeans may have not been the direct descendant of the early Neolithic people lived in Central Europe. Further data can be gained from the aDNA analysis of early Neolithic human remains of Hungary and the multivariate analysis of genetic and craniometric data. We also have preliminary data from the survival rhythm from the Neolithic times (Guba et al. 2001).

Following an adequate DNA extraction from human remains, the size of low copy number authentic DNA templates can be analysed via a PCR based approach. A simple method to estimate DNA fragment sizes in a sample is through gel electrophoresis followed by visualisation of fragments. To assess DNA quality one can observe the decrease in PCR amplification signal from PCR targets of increasing size (e.g. Poinar et al. 2003), as the ability to recover large fragments via PCR indicates a relatively low level of DNA damage. To assess the authentic DNA template quality in the samples to be analysed further, the degree of fragmentation of human mtDNA was estimated via a PCR based approach using overlapping primer design, with different amplicon sizes ranging from 60 to 600 bp.

Subjects and Methods

The quality and quantity of authentic DNA molecules depends on many physical and chemical factors, which are not easy to predict even if the taphonomic history of a sample is known. A newly set up aDNA laboratory, which is in accordance with the strict criteria required for representing authentic ancient DNA results (see Cooper and Poinar 2000), started in the Hungarian Natural History Museum in 2006, with two projects aiming at characterising aDNA of two groups of findings of different age: 5–7,000 years old Neolithic human bone samples and teeth of 150–100 years old naturally mummified human remains (Table 1).

Table 1. Amplicons analysed and their abbreviations.

Primer pairs	Amplicon size	Abbreviation
LAF-LAR	185 bp	B1
LAF-LBR	280 bp	B2
LAF-LCR	393 bp	B3
LAF-LDR	455 bp	B4
LBF-LAR	64 bp	B5
LBF-LBR	160 bp	B6
LBF-LCR	270 bp	B7
LBF-LDR	333 bp	B8
LCF-LBR	66 bp	B9
LCF-LCR	178 bp	B10
LCF-LDR	240 bp	B11
LDF-LCR	100 bp	B12
LDF-LDR	162 bp	B13

B: DNA fragment

Long bone fragments of the cc. 7,000 years old Neolithic human findings of Vörs-Máriaasszonysziget object No. 52, Hungary (Kalicz et al. 2002) and tooth samples of an 100–150 years old naturally mummified human remains of the Dominician Crypt in Vác, Hungary (Pap et al. 1997) have been primarily used in the experiments published here. Previous studies have shown that significant anthropological differences exist among early Neolithic human specimens. Two other Neolithic human remains Szarvas 8 grave No 1, Polgár-Ferencihát grave No 521 and Vörs-Máriaasszonysziget object No 52, respectively differ markedly in anthropometry, and we are to reveal their sequence polymorphism, whether it shows correlation with each other or the contrary. Gilbert (2003) made a schematic representation of typical DNA extraction, amplification and sequencing protocols, which applies for our work set up as well (Fig. 1).

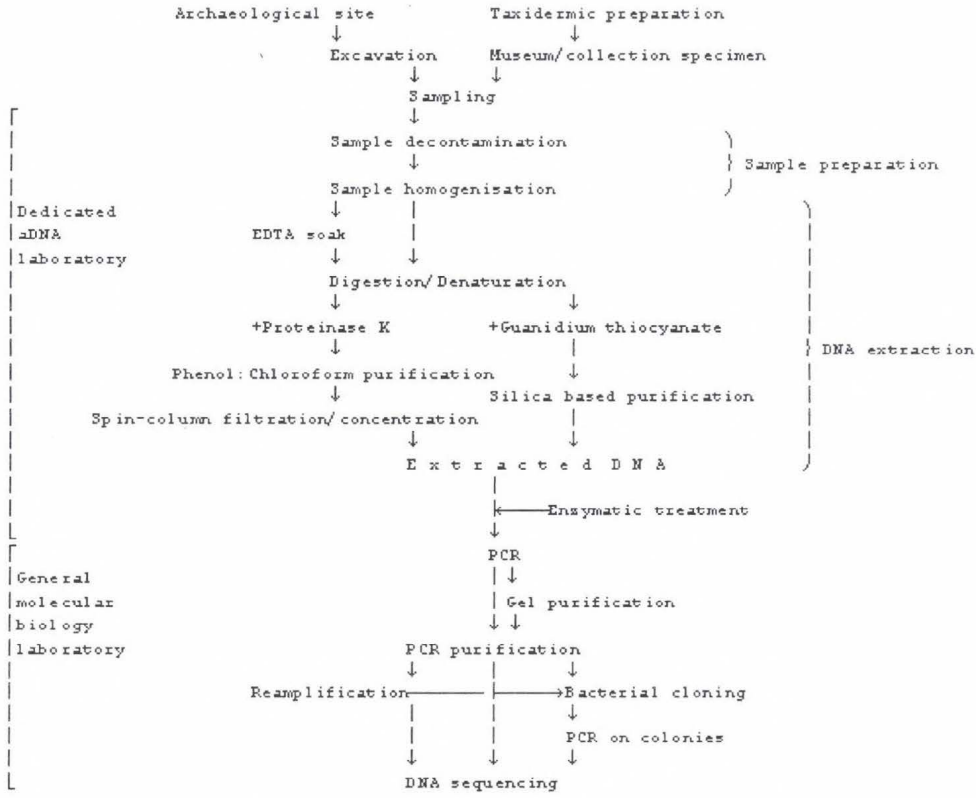


Figure 1: Schema of ancient DNA extraction (after Gilbert 2003).

All prePCR modification including sampling are carried out in spatial and temporal separation from the postPCR modification in the laboratory of the Hungarian Natural History Museum, Budapest, which is dedicated to human aDNA work. The laboratory rooms are fitted with a positive air pressure, overnight UV exposure and the laboratory workers use suitable protective clothing and equipment to avoid modern DNA contamination.

To avoid modern DNA contamination standard aDNA method will be used:

- Sampling, prePCR, postPCR modification to be carried out in different isolated places.
- Use of dedicated room (no previous human DNA work), dedicated tools and pipettes, filter tips, protective clothes (full body suits, hairnets, filter-containing facemasks, and gloves are used).
- Frequent surface cleaning: with detergent followed by 10% NaClO, overnight UV irradiation of surfaces and tools.
- Reagents are to prepare in small aliquots, UV irradiation if possible.

The teeth are soaked in bleach then they as well as bone fragments are pretreated by scraping their outer surfaces. After removing external contaminants (surface removing and soaking in sodium hypochlorit followed by UV exposure), bone and teeth samples are pulverised in a spherical mineralogy mill. Pulverised samples are incubated in extraction buffer (0.5 M EDTA, ph 8.5; 0.5% N-lauryl sarcosine; 20 mg/μL proteinase K) at 37°C for 12–36 hours. DNA will be extracted using a phenol-chloroform method (Burger et al. 2004), aqueous phase to be washed and concentrated on a 30kDA Centricon following the manufacturer's instruction.

Usually, aDNA investigations are based on the amplification of mtDNA. This small genome is more prone to give positive amplification, since it has a cell copy number higher than nuclear genes. The amplification of the HVR I region of the human mitochondrial DNA was performed according to Figure 1. With their combined use, PCR products ranging from 64 base pairs to 455 base pairs can be amplified. The cycle conditions consisted of an initial denaturation at 94°C for 3 min, 40 cycles of 94°C for 35 sec, 53°C for 35 sec, 72°C for 35 sec, followed by a final extension at 60°C for 30 min.

PCR products were evaluated on 2% agarose gel electrophoresis and extracted by QIAEX II kit.

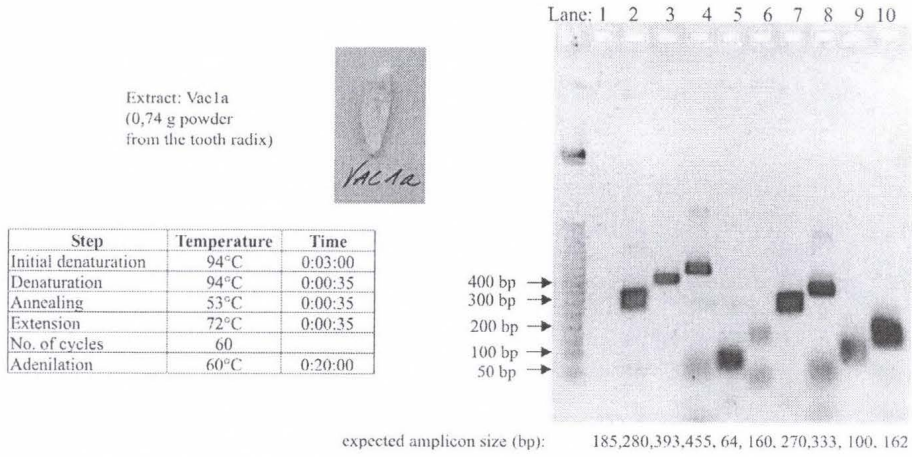
Results

The first extraction experiments have been carried out in collaboration at the laboratory of Palaeogenetics Group, Johannes Gutenberg University, Mainz, Germany which have shown the presence of endogenous DNA in our archaeological samples (data not shown).

Then an extract made from 0.74 g powder from the tooth radix of an 150 years old naturally mummified body of the Vác sample have been characterised for authentic DNA fragment size. Nine out of the 13 primer pairs resulted in positive amplification product. Interestingly, primer LCF (abbreviation B9, B10, B11, cf. Table 1) gave no positive amplicon, however amplicon from other reaction contains the sequence corresponding the LCF primer sequence.

Primer pair LAF-LAR also misses positive amplification result, however in other combination both the forward LAF (B2, B3, B4) as well as the reverse LAR (B5) primers showed ability to get amplified. In all other cases positive results can be gained repeatedly (Fig. 2), which suggests that the DNA damage in this remain is low, and authentic DNA fragment sizes ranging from 64 bp up to even 455 bp are present in the extract.

Except the B1 combination all primer pairs performed well from the mummified tooth remain, unlike Neolithic bones, it is an easy tool for analysing polymorpism of mtDNA.



Step	Temperature	Time
Initial denaturation	94°C	0:03:00
Denaturation	94°C	0:00:35
Annealing	53°C	0:00:35
Extension	72°C	0:00:35
No. of cycles	60	
Adenilation	60°C	0:20:00

Figure 2: Checking Vac1a sample extraction for DNA template integrity.

Long bone samples of Neolithic human remains differ markedly in anthropometry have also been subjected to DNA extraction. Using the primer pair L16287-H16410 (expected amplicon size: 123 bp; Haak et al. 2005) two of them gave a faint but positive result, the sample of Szarvas 8/ grave 1 and that of Vörs-Máriaasszonysziget obj. No 52. The latter one has been chosen to assess DNA template quality via our PCR based approach using overlapping primer design, with different amplicon sizes. Figure 3 shows the result of positive amplification and it is clear that is much more ambiguous then for the mummy's teeth sample. Nevertheless DNA fragment of 160 bp seems to be amplifiable and this should be appropriate for mtDNA haplotype and sequence based analysis via overlapping primer pairs for polymorphism.

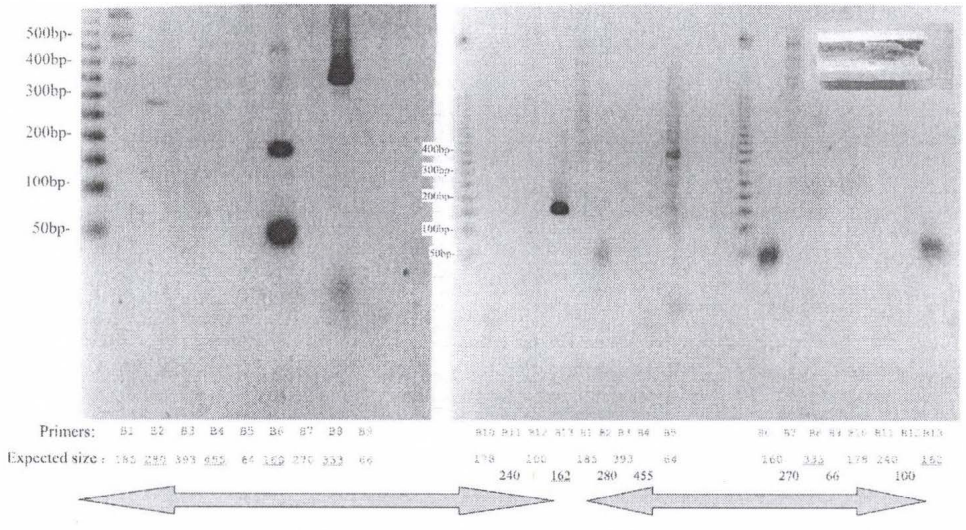


Figure 3: PCR analysis of paralel DNA extraction from tooth and bone sample.

Discussion

DNA degradation presents several problems for the aDNA field (Burger et al. 1999). The quantities of amplifiable DNA is limited and due to DNA fragmentation and cross-linking, researchers are rarely able to amplify templates over a few hundred bp in size (c.f. Paabo 1989, Cooper and Poinar 2000, Hofreiter et al. 2001). Depending on thermal, chemical and microbial circumstances DNA fragments in archaeological remains are able to survive up to 100,000 years for the longest, although almost complete degradation with in just a few months can also occur during the time.

Generally, from an old archaeological biological remain, only small amounts of DNA, which is invariably highly damaged can be extracted, since after death the body's metabolisms stops and DNA strand breakage rapidly begins to occur as a result of endogenous endonuclease activity and spontaneous depurination (Lindahl 1993), which results in the two main alteration of aDNA molecules with time: fragmentation and base modification. Depending on the ambient conditions further breaks, oxidative damage and molecular crosslinks accumulate (Paabo 1989, Höss et al. 1996, Mitchell et al. 2005).

The most serious for the credibility of the field is that of sample contamination. The low abundance of DNA extracted from a degraded specimen can easily be "swamped" by modern DNA from external contaminants of the same or similar DNA sequence. Without proper treatment to remove the environmental DNA, both sources of DNA can co-amplify. The biggest challenge of aDNA studies is avoiding contamination with recent DNA. Due to the multi-step processes that are employed to successfully extract the low concentrations of DNA associated with ancient remains, contaminants can enter the system at many stages. A list of suggested criteria has thus been published to help limit the effect of sample contamination. This list has been originally suggested by Handt et al. (1994), and later updated by Cooper and Poinar (2000) and Paabo et al. (2004).

In our PCR analysis of tooth and bone samples we successfully amplified different DNA fragments from mummified and Neolithic remains. According to the expected conservation of DNA the 150-year-old sample contains longer surviving DNA strains as the much older Neolithic samples. Even 455 bp DNA templates can be repeatedly extracted and amplified from the naturally mummified remains, which underlines the excellent DNA preservation in them. The 7,000-year-old human bones also produce amplifiable DNA with lower template size. Recently, on the basis of revealing polymorphism via sequencing produced very interesting result on the first farmers in Europe dated back to the Neolithic times (Haak et al. 2005).

The mummified remains are reported to have authentic pathogen microbial DNA (Fletcher et al. 2003). We are interested in further studies to gain information on the relevance on the presence of this and other microbial remains. The same subject which is the Körös and LBK population is analysed not only by our laboratory, but also by the Palaegenetics Group of the Johannes Gutenberg University, Germany in collaboration. Their result (Haak et al. 2005) showed the polymorphic pattern difference between the present day European and Neolithic population with an overwhelming frequency of a nowadays rare haplotype in the Neolithic LBK population which means either Palaeolithic population roots or later immigration into Europe. Even the analysis of the Neanderthal DNA of the well appreciated results of Paabo's group (Höss et al. 1997) showed very interesting polymorphic differences between the old and recent DNA samples of human.

With greater template integrity, mummified remains are more easy to analyse for their mitochondrial aDNA polymorphism and presence of bacterial strains. However, for a more thorough polymorphic pattern in Neolithic remains, more of which are expected to describe the population structure, we launch a sequencing based project. For this it would be useful to extend our study on more long bone, teeth and also cranial samples of different age within the limitation of sample number.

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