

# Original Article

## DNA Analysis of Ancient Skeletal Remains

(ancient DNA / advanced-glycation end-products / amelogenin / STR analysis / settlement at Kněževes)

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**Abstract.** Non-Governmental Organization Archaia (<http://www.archaia.cz>) carried out the rescue archaeological research at Kněževes near Prague in 1998. Most of dating objects in Kněževes come from the period of Late and Final Bronze Age. The approximately 3,000 years old set, which included 11 human remains from three settlement features, was collected for the study. First, gender was determined according to anthropological characteristics. Ancient DNA from bones was extracted by the phenol-chloroform procedure and N-phenacetylthiazolum bromide reagent. Polymerase chain reaction amplification of AMEL XY, part of amelogenin gene, with subsequent polyacrylamide gel electrophoresis and Short Tandem Repeats analysis followed. DNA profiles of skeletal remains were obtained by the fragmentation analysis of autosomal short tandem repeat markers. Genetic profiles showed us whether individuals from Kněževes were in mutual relationship (parent – descendant). The congruence of results in sex determination supported reliability of genetic methods, which are suitable for sex determination of fragmental and subadult skeletal remains.

### Introduction

Ancient DNA (aDNA) can stay preserved in soft or hard tissues that do not yield to decomposition completely owing to taphonomic factors. As sources of aDNA could serve mummified or frozen tissue. The examples of mummification are the human remains in Pompeii, where the sex of 13 individuals was deter-

mined (Cippollaro et al., 1998). The structure of bones protects cells and DNA against exogenous and endogenous degradation factors. In addition, mineral components such as hydroxyapatite stabilize structure of the contained DNA molecules (Lindahl, 1993; Tuross, 1994). DNA is *post mortem* injured especially by hydrolysis and oxidation. Consequently, extracted aDNA is often very fragmented and has mostly low molecular weight. The length of DNA fragments ranges between 40–500 bp; the average fragment size is 100 bp (Pääbo, 1989; Haack et al., 2000).

aDNA analysis of human skeletal material can provide important information to archaeological specialists, such as the sex and DNA profile of individuals, when anthropological methods cannot be applied. The mechanisms of aDNA survival in bone are unexplored; therefore, the results of this study can help solve some of the problems in aDNA analysis. aDNA was isolated from all Kněževes samples, quantified and subjected to different PCR protocols for amplification of nuclear DNA (AMEL XY amplification and MiniFiler kit, which amplifies nine short tandem repeat loci at once). Finally, the amplified aDNA was separated using the polyacrylamide (PAGE) and capillary electrophoresis.

### Material and Methods

The set included skeletal fragments from the period 900–1,300 BC. The bones came from three settlement features and were numbered 823 (A, C, D, H, I), 2545 (A, B) and 2399 (A, B). Anthropological sex determination was carried out during manipulation of skeletal remains before genetic examination (Kubálek, 2007). In genetic analysis, the phases of sampling, extraction and amplification were carried out in separate laboratories.

### Sample pretreatment

A portion of approximately  $0.5 \times 1$  cm was cut from each bone and a layer of 2 mm was removed from the surface by aluminium oxide abrasive jet (Dremel, Racine, WI) followed by submersion in 5% sodium hypochlorite for 5 min and UV light decontamination for 30 min. Each bone sample was mechanically ground

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Abbreviations: aDNA – ancient DNA, AGE – advanced-glycation end-product, PAGE – polyacrylamide gel electrophoresis, PCR – polymerase chain reaction, PTB – N-phenacetylthiazolum bromide, qPCR – quantitative polymerase chain reaction, STR – short tandem repeats.

Table 1. DNA concentration in skeletal samples from Kněževy

Sample (Settlement feature No./Skeletons)	Skeletal fragment	Bone powder [g]	DNA concentration [ng/μl]
K1 (823/A)	Mandible	1.2	34.36
K2 (823/H)	Mandible	1.0	4.91
K3 (823/D)	Cranium	1.0	34.36
K4 (823/I)	Mandible	1.2	0.19
K5 (823/C)	Maxilla	1.2	125.81
K6 (823/C)	Maxilla	0.6	17.96
K7 (823/A)	Mandible	0.8	17.96
K8 (2545/A)	Costa	1.0	2.56
K9 (2545/B)	Scapula	1.0	17.96
K10 (2399/A)	Maxilla	1.2	17.96
K11 (2399/B)	Maxilla	1.2	34.36

into fine powder in a sterile mortar (Freezer Mill, Spex, Metuchen, NJ) under liquid nitrogen and transferred to the DNA extraction laboratory in DNase-free tubes.

#### DNA extraction in the presence of PTB

Bone powder (the amount see in Table 1) was resuspended in 2 ml of digestion buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, pH 8.0 and 2.5 mM PTB (Prime Organics, Woburn, MA) containing 0.1 mg/ml of proteinase K (Sigma-Aldrich, St. Louis, MO) and incubated at 37 °C for 12 h. After digestion, one volume of phenol:chloroform:isoamyl alcohol, 25:24:1, was added to each tube and shaken properly for 1 min, followed by centrifugation at 4000 g for 15 min. The supernatant was then transferred to a newly marked tube and one volume of chloroform:isoamyl alcohol, 24:1, was added, mixed for 1 min and centrifuged at 4000 g for 15 min. The DNA was precipitated with two volumes of absolute ethanol and 3 M CH<sub>3</sub>COONa at -20 °C for 20 min. Ethanol with 3 M CH<sub>3</sub>COONa was removed after centrifugation at 13 000 g for 10 min and the precipitate was air-dried and redissolved in 30 μl TE buffer (10 mM Tris-HCl, 0.2 mM EDTA, pH 7.4).

#### DNA quantification

Extracted aDNA was quantified by qPCR based on the SYBR/Alu system. For amplification of Alu repeats we used primers SP1 (5'-TGGTGGCTCACGCCTGTAA-3') and SP2 (5'-CGATCTCGGCTCACTGCAA-3') (Sifis et al., 2002).

Two μl of extracted DNA was added to a 28 μl reaction mixture containing 15 μl of SYBR Green Supermix (Bio-Rad, Hercules, CA); 100 bpmol/μl of each primer and 50 mM MgCl<sub>2</sub>. The reaction mixture with DNA was subjected in Mastercycler realplex 4 (Eppendorf, Germany) to: initial denaturation at 94 °C for 4 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 45 s and extension at 72 °C for 30 s, then final extension at 72 °C for 7 min.

#### PCR amplification of amelogenin

The quality of DNA extraction was checked by amplification of the human amelogenin gene using primers 5'-CCCTTTGAAGTGGTACCAGAGCA-3' and 5'-GCATGCCTAATATTTTCAGGGAATA-3'. The opti-

mal PCR protocol for obtaining the nuclear segment AMEL XY (X = 80 bp, Y = 83 bp) was multistep PCR enabling specific binding of primers to DNA templates (Evison et al., 1997). Elongation maintains only minimally, while change of denaturation and annealing assures high stringency of the primer to target sequences.

PCR reaction was performed in the MJ Research PTC-220 DNA Engine Hyad Cycler (Bio-Rad) in total volume of 25 μl. Two μl of extracted DNA was added to reaction containing 1× PCR buffer, 2.25 mM MgCl<sub>2</sub>, 0.025 U Taq polymerase (TaKaRa, Otsu, Japan), 0.2 mM dNTPs (Fermentas, Burlington, Canada) and 5 pmol/μl of each primer AMEL. PCR protocol included: initial denaturation at 94 °C for 2 min, followed by 10 cycles of 94 °C/20s and 58 °C/30s, 25 cycles of 94 °C/30s, 56 °C/30s and 72 °C/30s, and 11 cycles 94 °C/30s, 55°/30s and 72 °C/30s. Final extension at 72 °C was kept for 5 min.

PCR products were separated using standard 15% polyacrylamide gel electrophoresis and visualized by ethidium bromide staining with UV illumination.

#### STR analysis

Short Tandem Repeats (STR) analysis was made by AmpFℓSTR® MiniFiler™ PCR Amplification Kit (Applied Biosystems, Foster City, CA), which has the ability to obtain the DNA profile even from degraded aDNA. MiniFiler amplification kit is an eight-locus multiplex comprising the loci D13S317, D7S820, D2S1338, S21S11, D16S539, D18S51, CSF1PO and FGA, as well as AMEL. Multiplex PCR was carried out in thermocycler Mastercycler® ep (Eppendorf, Germany) and PCR program was set according to MiniFiler User's manual (2007).

One μl of a PCR product was combined with 8.7 μl formamide and 0.3 μl LIZ GeneScan 500 size standard (Applied Biosystems) prior to the capillary electrophoresis. PCR products were detected by ABI Prism 310 Genetic Analyzer (Applied Biosystems) and the data analysis was done by GeneMapper ID Software, version 3.2 (Applied Biosystems).

## Results

The Kněževy set of 11 samples was subjected to genetic analysis to confirm or correct the results of anthro-

Table 2. Results of anthropological and genetic method for sex determination

Settlement feature No.	Sample	Anthropological determination	AMEL XY amplification	STR analysis (Minifiler®)
823/A	K1 = K7	unknown gender	male	male
823/H	K2	male	male	male
823/D, 823/C	K3 = K5 = K6	female	female	female
823/I	K4	female	male	male
2545/A	K8	unknown gender	male	male
2545/B	K9	male	male	male
2399/A	K10	female	male	male
2399/B	K11	female	female	female

polo gical sex determination. In genetic analysis aDNA was extracted using the procedure utilizing N-phenacetylthiazolum bromide (PTB). DNA extraction was followed by DNA quantification by qPCR. The sample labelled K5 yielded 125.81 ng/μl DNA, which means that it was 10-fold diluted, while sample K4 (0.19 ng/μl) gave very low DNA concentration. According to Table 1, the concentration of extracted DNA was independent of the preliminary amount of bone powder in particular samples; e.g., sample K5 contained seven times more DNA than sample K6 – both came from same person and same fragment of maxilla.

Genetic sex determination of the Kněževes remains was performed by the amplification of AMEL XY, separated by PAGE and visualized by ethidium bromide and UV illumination. According to the electrophoreograms, capillary electrophoresis evaluated by specific PC software seemed to be more objective than polyacrylamide electrophoresis. The PAGE technique is more widely available, but the interpretation of results may often be subjective. Although advanced versions of PAGE coupled with fluorescence-labelled primers allow detection and quantification of DNA, they often give inaccurate estimation of allele sizes. Capillary electrophoresis, on the other hand, produces accurate and consistent results. Hence, it is necessary that a microsatellite-based genotyping assay to detect and quantify product employs capillary electrophoresis. Determination of gender is shown in Table 2.

STR analysis using MiniFiler kit was carried out with eight autosomal STR markers and one gonosomal STR

marker. The results are summarized in Table 3. Genetic profiles were obtained from all bone samples tested; samples K8 and K9 yielded only partial profiles. We did not succeed in getting results for the FGA locus, except for sample K8 and positive control, sample K<sup>+</sup>. The FGA locus could not be amplified due to a mutation occurring in the primer binding site. It was discovered that sample K3, originally marked skeleton 823/D, belongs to the individual marked as 823/C – K5, K6. The relationship among individuals, parent and descendant, was estimated based on the comparison of genetic profiles. Relationships of father-son were established between samples K2 – K4, K2 – K8, K2 – K9, K4 – K8, K4 – K9, K8 – K9; the relationship of father-daughter or son-mother was established only between samples K10 – K11.

The gender of skeletal remains labelled 823/A and 2545/A belonging to age category ‘infant’ was unknown. Genetic analysis determined male sex in both cases.

The comparison of sex determination methods showed that STR analysis was in 100% accord with amplification of AMEL XY and 75% with anthropometric sex determination (samples 823/A and 2545/A were not included in the classification).

## Discussion

Low concentration of highly fragmented DNA is often a problem during the processing of ancient biological material. aDNA in this study was efficiently extracted in the presence of PTB, which has been reported to

Table 3. Genetic profiles made using MiniFiler kit. The samples K1 = K7 and K3 = K5 = K6 came from two persons

Locus Sample	D13S317	D7S820	D2S1338	D21S11	D16S539	D18S51	CSF1PO	FGA	AMEL
K1	8/11	10	19/25	29/30	12/13	17/18	11/12	-	XY
K2	9	9/10	20/23	27/29	14	15/23	8/12	-	XY
K3	11	7/11	21/25	31.2/32.2	12	15/18	9/11	-	XX
K4	9/11	10	23	27/32.2	9/14	23	8	-	XY
K5	11	7/11	21/25	31.2/32.2	12	15/18	9/11	-	XX
K6	11	7/11	21/25	31.2/32.2	12	15/18	9/11	-	XX
K7	8/11	10	19/25	29/30	12/13	17/18	11/12	-	XY
K8	-	9	20/23	27/32.2	-	23	8/12	20	XY
K9	-	9/10	-	27	9/14	15/23	8	-	XY
K10	11/12	8	18/20	27/33.2	11/12	11/16	10/11	-	XY
K11	10/12	8/10	18/20	27/28	9/11	11/16	10/12	-	XX
K <sup>+</sup>	7	7/11.3	17/20	29/31	13	15.6/19	10	24/26	XX

Legend: (-) non-amplified locus; (grey panel) possible allelic drop-out

help DNA release (Hofreiter et al., 2001). PTB reacts with advanced-glycation end-products (AGEs) and cleaves covalent glucose-derived protein cross-links. AGEs are products of non-enzymatic glycosylation or glycation (Vasan et al., 1996; Thornalley and Minhas, 1999; Rohland et al., 2004). DNA extraction in the presence of PTB was first described by Poinar et al. (1998). The ability of PTB to break AGEs *post mortem* offers a potential approach to acquisition and amplification of more DNA molecules from bones.

According to Table 1, the results of qPCR confirmed that aDNA extraction from costae is less advantageous than from mandible or maxilla. aDNA from costae is hard to be extracted – its compact bone is very thin. aDNA is thus less protected and degrades faster. With regard to that, Faerman et al. (1995) recommended for aDNA analysis taking samples from long bones – femur or humerus.

Comparison of genetic methods for sex determination, amplification of AMEL XY with PAGE and STR analysis, showed 100% accordance in all 11 cases. Amplicons of MiniFiler kit and AMEL XY ranged between 70–283 bp and could be generated from the recommended PCR target DNA input (0.5–0.75 ng). Therefore, they are suitable for aDNA analysis. Genotypes of longer loci can often be lost in ancient samples. Most ancient samples are degraded due to the environmental exposure or contain a highly limited amount of cellular material.

Different results between the anthropological and genetic method of sex determination were discovered in two samples (823/I and 2399/A). This discrepancy can be explained by highly fragmented skeletal remains and the presence of more individuals in the same settlement feature. Genetic methods are also reliable for sex determination of skeletal subadult remains that have undeveloped secondary sex characteristics.

MiniFiler kit, using miniSTR technology, helped in clarification of filiations. It shows a remarkable promise for use in archaeogenetics. Genetic profiles confirmed kinship in seven cases. However, allelic drop-out can affect determination of the relationship (Table 3).

In summary, this study raises the possibility of recovering aDNA from historical skeletal remains and generating STR profiles for assessment of relationship and sex in archaeogenetics. Some samples in this study came from the same person (K1 = K7, K3 = K5 = K6) and we therefore also performed an internal check of the MiniFiler DNA profile accuracy.

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