ANTHROPOLOGIE • XLVII/1–2 • pp. 69–72 • 2009

IRENA ELIÁŠOVÁ, PAVEL KUBÁLEK

COMPARISON OF GENETIC AND ANTHROPOLOGICAL ANALYSES IN SEX DETERMINATION OF SKELETAL REMAINS FROM KNĚŽEVES (CZECH REPUBLIC)

ABSTRACT: The settlement at Kněževes is situated near Prague, and the non-profit company Archaia carried out a rescue archaeological research there in 1998. A set of skeletal remains was collected from three settlement features dated in the Late Bronze Age. The skeletons were subjected to anthropological and genetic analyses for sex determination. First, in the genetic analysis recent DNA from the femur was used to compare isolation methods. We tested four different DNA extraction protocols: phenol-chloroform method, Dextran Blue mediated extraction method, and two isolation protocols using the DNA IQ™ System, and the QIAamp DNA Blood Mini Kit, respectively. Skeletal remains from Kněževes were subjected to the selected extraction method and subsequently to PCR (Polymerase Chain Reaction) reaction for sex determination. The amplified aDNA (ancient DNA) was separated using the polyacrylamide electrophoresis. After comparison of genetic and anthropological methods, aDNA analysis confirmed that it is reliable for sex determination of subadult skeletal remains with undeveloped secondary sex characteristics.

KEY WORDS: Ancient DNA – Sex determination – PCR (Polymerase Chain Reaction) – Amelogenin – Settlement at Kněževes (Czech Republic)

INTRODUCTION

Sex determination of archaeological human remains is essential for exploration of gender differences in past populations. Traditional morphometric analyses fail to identify the gender of incomplete skeletal remains and that of immature individuals. Investigation of gender differences plays an important role in the reconstruction of social structure of past societies. Gender has been traditionally determined through the identification of grave goods and the bone morphometric analysis. However, for fragmentary adult skeletons or those of children and infants conventional anthropometric methods are unreliable (Faerman *et al.* 1995). Analyses of DNA sequences specific to the X and Y chromosomes may provide an ideal solution.

The specific human amelogenin gene sequenced by Nakahori *et al*. (1991a, b) can be used to determine the sex of the donor of a sample because it resides on both the X and Y chromosomes in humans. The human AMELX gene has a size of 2872 bp and is located on the p22 region of the X chromosome, while the human AMELY gene has a size of 3272 bp and is located on the 11p12.2 region of the Y chromosome (Bailey *et al*. 1992). Several PCR primer sets have been developed to use this gene as a sex determinant. The most commonly used amelogenin PCR-based sex test is the one described by Sullivan *et al*. (1993), in which primer results in 106 and 112 bp PCR products from the X and Y chromosomes respectively. However, *post mortem* DNA begins to degrade immediately (especially by hydrolysis and oxidation), so that ancient DNA is very fragmented and has

mostly low molar weight. The lengths of DNA fragments range between 40–500 bp, average fragment size is 100 bp (Pääbo 1989, Haack *et al.* 2000). Hence, for degraded aDNA the AMEL primer system is available resulting in 80 bp and 83 bp PCR products from the X and Y chromosomes (Haas-Rochholz, Weiler 1997).

In our study, recent DNA was extracted from the femur by four different DNA isolation protocols and quantified. We chose one extraction method with the highest human nuclear DNA yield and used it to the aDNA analysis of the Kněževes set. Followed amplification of AMELX/Y, part of amelogenin gene, and PCR products were separated by the polyacrylamide electrophoresis (PAGE). The congruence of results in sex determination supported the reliability of genetic methods which are suitable for sex determination of fragmental and subadult skeletal remains.

MATERIAL AND METHODS

The bone material included: (1) a femur, which was provided to the Department of Anthropology and Human Genetics at the Charles University, for the optimisation of four different DNA extraction protocols; (2) 11 samples of skeletal fragments, labelled K1-K11, coming from three settlement features.

Anthropological analysis

Anthropological sex determination of skeletal remains was carried out during handling with bones (Kubálek, in print).

Sample pre-treatment

The bones were scraped with a brush to remove the outer layer and the surface contamination. Further, skeletal remains were cut to approximately 1×1cm portions, and subsequently treated with 5% sodium hypochloride (commercial bleach) and exposed to UV filter for 30 min at 20 cm distance. Lastly, each bone sample was mechanically ground into a fine powder in a sterile mortar (Freezer mill, Spex) under liquid nitrogen and transferred to the DNA extraction laboratory in sterile tubes.

DNA extraction

Four extraction methods were used and modified to optimize the extraction DNA yield. We applied 4 samples of femoral diaphysis (every sample consisted approximately of 1 g bone powder) for each isolation protocol. The first method applied, the phenol-chloroform extraction, was an organic procedure developed by Sambrook *et al.* (1989). The protocol by Kalmár *et al.* (2000) with addition of Dextran Blue was used to the second DNA isolation. The last two methods were carried out by means of QIAamp DNA Blood Mini Kit (Qiagen) and DNA IQ^{TM} System (Promega).

Skeletal remains from Kněževes were finally extracted with the help of Sambrook *et al*. (1989) phenol-chloroform extraction with the presence of 2.5 mM PTB (N phenacetylthiazolum bromide) reagent which was added to digestion buffer.

DNA quantification

Extracted aDNA was quantified by qPCR (quantitative PCR) based on the SYBR/*Alu* system. For the amplification of *Alu* repeat sequences the primers SP1 (5´-TGGTGGCTCACGCCTGTAA-3´) and SP2 (5´-CGATCTCGGCTCACTGCAA-3´) were used (Sifis *et al.* 2002).

2 µl of extracted DNA were added to a 28 µl reaction mixture containing 15 µl of SYBR Green Supermix (Bio-Rad, USA), 100 pmol/µl of each primer and 50 mM $MgCl_2$. The reaction mixture with DNA was subjected to Mastercycler realplex 4 (Eppendorf, Germany): initial denaturation at 94°C for 4 min, followed by 40 cycles of denaturation at 94°C for 30 s, ramp down to annealing at 55°C for 45 s and ramp up to extension at 72°C for 30 s, then final extension at 72°C for 7 min.

PCR amplification of amelogenin

In order to amplify the highly degraded aDNA, which is usually found in archaeological specimens, we chosen the primers 5´-CCCTTTGAAGTGGTACCAGAGCA-3´ and 5´-GCATGCCTAATATTTTCAGGGAATA-3´ spanning short DNA fragments from the sequence of amelogenin gene. The 80 bp and 83 bp PCR products from X and Y chromosomes were separated by PAGE.

Optimal conditions for the PCR reaction were established using recent DNA. NTC (no template control) obtaining sterile water in space of the DNA template was used during each series of bone samples.

PCR was performed on the MJ Research PTC-220 DNA Engine Hyad Cycler in total volume of 25 µl. 2 µl of extracted DNA was added to reaction mixture containing 1× PCR buffer, 2.25 mM $MgCl_2$, 0.025 U *Taq* polymerase (Ta-Ka-Ra, Japan), 0.2 mM dNTPs (Fermentas, Canada) and 5 pmol/µl of each primer AMEL. PCR protocol includes: initial denaturation at 94°C for 2 min, followed by 10 cycles of 94°C/20 s and 58°C/30°C, 25 cycles of 94°C/30 s, 56°C/30 s and 72°C/30 s, and 11 cycles 94°C/30 s, 55°C/30 s and 72°C/30 s. Final extension at 72°C was kept for 5 min.

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

RESULTS

Anthropological analysis was carried out during the archaeological treatment of skeletal remains (Kubálek, in print).

First, we extracted 16 samples from the femoral diaphysis by different isolation protocols (always four samples were extracted by the same method) and quantified them by qPCR which established concentration of specific human DNA.

The protocol of extraction by Kalmár *et al.* (2000) seemed to be very simple in terms of its realisation but provided only a minimum amount of specific human DNA. The isolation by Qiagen kit cannot be unambiguously evaluated because all samples showed zero result after the

TABLE 1. DNA concentration from different extraction protocols obtained by qPCR [ng/µl].

Extraction methods [ng/µl] / **Sample**	**Phenol-chloroform protocol**	**Dextran Blue extraction**	**DNA IQ System Kit**	**QIAamp DNA Blood Mini Kit**
F1	33.56	3.56	19.13	-
F2	50.34	8.62	10.00	-
F3	18.83	1.30	70.06	-
F4	26.90	0.31	36.61	-

DNA quantification. The extractions by DNA IQ System kit and phenol-chloroform method demonstrated detection of human DNA with good results (*Table 1*). Phenol-chloroform showed a little more DNA yield than DNA IQ kit. This fact was decisive for its being applied to DNA extraction of the Kněževes samples. Further, according to *Table 1* we confirm that DNA concentration was independent on the amount of bone powder available (values of DNA yield were distinguished whereas the amounts of femoral bone powder were approximately 1 g).

For the aDNA analysis of Kněževes samples we selected phenol-chloroform extraction and added PTB reagent to digestion buffer. An aDNA extraction with the presence of PTB was first described by Poinar *et al.* (1998) who compared two series of aDNA samples – with added PTB reagent and without PTB. In all extractions with added PTB, PCR products were observed, whereas no PCR products were detected in the absence of PTB. Hence, in selecting a suitable aDNA extraction we based ourselves on the study by Poinar *et al.* (1998). aDNA manage to extracted from all Kněževes samples.

Genetic sex determination of the Kněževes remains was performed by the amplification of AMELX/Y and separated by PAGE. The results are shown in *Table 2*. The comparison of sex determination methods showed that genetic analysis differed in 25% from the anthropometric sex determination. Samples K1=K7 and K8 were not included in the classification because they belonged to the age category *infans.* This age group has fully undeveloped secondary sex characteristics so that the anthropological analysis could not unambiguously determinate the gender. Genetic analysis established male sex in these both cases.

TABLE 2. Comparison of genetic and anthropological analyses for sex determination.

Sample	**Anthropological determination**	**AMEL XY amplification**
K1 = K7	unknown gender	male
K2	male	male
K3 = K5 = K6	female	female
K4	female	male
K8	unknown gender	male
K9	male	male
K10	female	male
K11	female	female

Several samples in this study came from the same person (K1=K7, K3=K5=K6), and thus we also performed an internal check of DNA analysis correctness where we confirmed identical gender.

CONCLUSIONS

This study demonstrates the applicability of genetic method for sex determination in skeletal remains. A successful retrieval of amplifiable DNA does not relate to the period of hard tissue but to composition of soil, physical and chemical factors which may influence the structure of bones. Another question is which suitable kind of hard tissue should be used for the DNA analysis. Faerman *et al*. (1995) recommend long bones such as the femur or humerus, cranial bones, and teeth providing sufficiently preserved DNA. Hence, of the whole skeleton it was the femur that we chose for a comparison of extraction methods.

Moreover, exogenous and endogenous environmental factors may affect *post mortem* each part of a bone in a different way (Kaiser *et al.* 2008). Our results confirmed this supposition. We used approximately 1 g of bone powder from one femoral diaphysis, and the DNA yield varied in all samples (e.g., sample F1 contained approximately twice as much DNA yield as sample F3 by using the same isolation protocol). After comparing the four different isolation protocols applied, the phenol-chloroform DNA extraction exhibited the highest DNA yields, and that is why we used it to aDNA analysis of the 3,000-year-old set from Kněževes.

Further, for the selection of a suitable isolation protocol we based ourselves on the study by Poinar *et al.* (1998) declaring the importance of PTB reagent in the aDNA extraction. PTB breaks AGEs (advanced-glycation end-products) *post mortem*, which can inhibit the aDNA analysis, and thus offers a potential approach for elicitation and amplification of more DNA molecules from bones.

According to Haack *et al.* (2000), extracted aDNA shows average fragment size 100 bp. Therefore, we optimised the PCR amplification suitable for the system of AMELX/Y primers (80/83 bp). We chose the PCR protocol by Evison *et al*. (1997), in which elongation maintains only minimally while the changes of denaturation and annealing assure high stringency of primers to target sequences, and which assures specific binding of primers to DNA templates. This protocol of selective amplification of X and Y AMEL alleles proved to be very sensitive and reliable for genetic analysis.

Discrepancy between the results of DNA and anthropological analyses might be attached possibilities of allelic drop-out (amplification of one allele totally outweighs the amplification of second allele during PCR, and thus the heterozygote appears like homozygote) or confusion of skeletal remains from different individuals (Eliášová 2007). Skeletal remains may get confused during the pre-laboratory phase of sample treatment because any unified protocol for collecting the biological material is not yet available. Another problem associated with the settlement at Kněževes were the burials of skeletons in settlement features. Settlement features could not be clearly distinguished in topsoil level, and thus bones could be mixed together (Smejtek 2001).

Furthermore, we have successfully determined the sex of all skeletal remains from Kněževes. Thus, genetic method provides an important tool for sex identification in archaeogenetics, and for examination of gender differences in past societies where anthropological analyses are no longer sufficient.

ACKNOWLEDGEMENTS

We would like to thank PhDr. Ing. Lubor Smejtek for archaeological comments on the manuscript and RNDr. Pavel Čapek, PhD. for genetic advices on DNA optimisation. We are especially indebted to Mgr. Jana Hájková and RNDr. Daniel Vaněk, PhD. for the providing of qPCR. We also thank the Department of Anthropology and Human Genetics, Faculty of Science at the Charles University in Prague for enabling us to use their laboratory equipment.

REFERENCES

BAILEY D. M., AFFARA N. A., FERGUSON-SMITH M. A., 1992: The X-Y homologous amelogenin maps to the short arms of both the X and Y chromosomes and is highly conserved in primates. *Genomics* 14: 203–205.

DOBISÍKOVÁ M., 1999: Určování pohlaví. In: M. Stloukal (Ed.): *Antropologie, příručka pro studium kostry.* Pp. 168–234. Národní muzeum, Praha.

ELIÁŠOVÁ I., 2007: *Optimalizace některých parametrů DNA isolace historického kosterního materiálu.* Diploma thesis. Department of Anthropology and Human Genetics, Faculty of Science, Charles University, Prague.

FAERMAN M., FILON B., KAHILA G., GREENBLATT C. L., SMITH P., OPPENHEIM A., 1995: Sex determination of archaeological human remains based on amplification of the X and Y amelogenin alleles. *Gene* 167: 327–332.

HAACK K., HUMMEL S., HUMMEL B., 2000: Ancient DNA fragments longer than 300 bp. *Anthropologischer Anzeiger* 58, 1: 51–56.

HAAS-ROCHHOLZ H., WEILER G., 1997: Additional primer sets for an amelogenin gene PCR-based DNA-sex test. *International J. of Legal Medicine* 110: 312–315.

KAISER C., BACHMEIER B., CONRAD C., NERLICH A., BRATZKE H., EISENMENGER W., PESCHEL O., 2008: Molecular study of time dependent changes in DNA stability in soil buried skeletal residues. *Forensic Science International* 177, 1: 32–36.

KALMÁR T., BACHRATI C. Z., MARCSIK A., RASKÓ I., 2000: A simple and efficient method for PCR amplifiable DNA extraction from ancient bones. *Nucleic Acids Research* 28: e67.

KUBÁLEK P., in print: Základní antropologické zpracování lidských kosterních nálezů z Kněževsi. In: L. Smejtek (Ed.): *Osídlení z doby bronzové v Kněževsi u Prahy*. Praha.

NAKAHORI Y., HAMANO K., IWAYA M., NAGAGOME Y., 1991a: Sex identification by the polymerase chain reaction using X-Y homologous primers. *American J. of Medical Genetics* 39: 472–473.

NAKAHORI Y., TAKENAKA O., NAKAGOME Y., 1991b: A human X-Y homologous region encodes amelogenin. *Genomics* 9: 264–269.

PÄÄBO S., 1989: Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. *Proc. Natl. Acad. Sci. U.S.A.* 86: 1939–1943.

POINAR H. N., HOFREITER W., SPALDING G., MARTIN P. S., STANKIEWITZ B. A., BLAND H., EVERSHED R. P., POSSNERT G., PÄÄBO S., 1998: Molecular coproscopy: Dung and diet of the extinct ground sloth *Nothrotheriops shastensis. Science* 281: 402–406.

SAMBROOK J., FRITSCH E. F., MANIATIS T., 1989: *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, New York.

SIFIS M. E., BOTH K., BURGOYNE L. A., 2002: A more sensitive method for the quantitation of genomic DNA by Alu amplification. *J. of Forensic Sciences* 47, 3: 589–592.

SMEJTEK L., 2001: Únětické pohřebiště a sídliště v Kněževsi u Prahy. *Archeologie ve středních Čechách* 5: 209–278.

SULLIVAN K. M., MANNUCCI A., KIMPTON C. P., GILL P., 1993: A rapid and quantitative DNA sex test: Fluorescence-based PCR analysis of X-Y homologous gene amelogenin. *Biotechniques* 15: 636–641.

Irena Eliášová
Institute of Biology and Medical Genetics
2nd School of Medicine, Charles University
and University Hospital Motol
V Úvalu 84
150 06 Praha 5, Czech Republic
E-mail: i.eliasova@seznam.cz

Pavel Kubálek
Labrys o.p.s.
Mezi Školami 2321/95
158 00 Praha 13, Czech Republic
E-mail: kubalek@labrys.cz