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

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)
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 Kněževs 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 1x1cm 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 procedures developed by Sambrook et al. (1989). The isolation by Qiagen kit cannot be unambiguously evaluated because all samples showed zero result after the

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′-GGTGGCTACGCTGTAA-3′) and SP2 (5′-CGATCTCGGCTACGTGCAA-3′) were used (Sifis et al. 2002).

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 isolation by Qiagen kit cannot be unambiguously evaluated because all samples showed zero result after the
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 Kněževs 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 Kněževs 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 Kněževs samples.

Genetic sex determination of the Kněževs remains was performed by the amplification of AMEL X/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 determine the gender. Genetic analysis established male sex in these both cases.

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

<table>
<thead>
<tr>
<th>Extraction methods [ng/µl]</th>
<th>Phenol-chloroform protocol</th>
<th>Dextran Blue extraction</th>
<th>DNA IQ System Kit</th>
<th>QIAamp DNA Blood Mini Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>F1</td>
<td>F2</td>
<td>F3</td>
<td>F4</td>
</tr>
<tr>
<td></td>
<td>33.56</td>
<td>50.34</td>
<td>18.83</td>
<td>26.90</td>
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<tr>
<td></td>
<td>3.56</td>
<td>8.62</td>
<td>1.30</td>
<td>0.31</td>
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<td>19.13</td>
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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 Kněževs.

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 Kněževse 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 Kněževse. 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.

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