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Anthropological and epidemiological characterization of Great-Moravian population in connection with the social and economic structure

Genetic Sex Determination of Sub-adult Individuals from the Great Moravian Settlement in Mikulčice (9th Century AD)

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Specimens of human sub-adult bones from the Mikulčice settlement (9th century) were genetically investigated for sex determination, the main goal of this study. DNA sequence length differences in the amelogenin gene locus were chosen for their importance in sexing. A PCR system that focuses on the Y chromosome was applied to confirm the amelogenin sexing results of female individuals. We have shown that a second system for sex determination is essential, because allelic drop-out is very frequent among samples of aDNA amplicons. The reliability of the genetic analysis was verified on a set of 5 adult individuals with morphologically determined sex. The genetic analysis was successful in 37 out of 47 sub-adult individuals (78.7%). The data obtained showed a higher presence of male individuals – 24 males to 13 females. The number of sub-adult individuals studied was too low to statistically confirm the significance of the higher number of male individuals. As a second step in our research, we took information from genetic analysis as a standard, and applied specific morphological methods to estimate its validity. We checked sex by means of two qualitative methods based on the morphological traits of the hip bone and mandible. We determined the sex of 23 out of 37 genetically determined skeletons (62.2%). Likewise, the genetic analysis results showed a uniquely higher presence of male sub-adult skeletons – 19 males to 4 females. The concordance between the genetic and morphological sex determination was remarkably high at 87%. In view of the low sexual morphological dimorphism of immature skeletons, we consider the 87% value of concordance to be a speculative result. Nevertheless, selected morphological methods have proved to be very useful when applied to individuals from the 9th and 10th centuries. Records of grave goods were also included in the final sex evaluation, and this multidisciplinary approach has yielded interesting results. The higher male to female ratio will be further studied.

Key words: Early Medieval population – sub-adult individuals – genetic sex determination – morphologic sex determination

1. Introduction

The Mikulčice settlement is located in south Moravia in a low-lying region where the highest point does not even reach 300 m above sea level. The climatic, pedologic and other natural conditions in this area made it suitable for the development of agriculture. Cremation was the main method used for disposing of the dead between the 6th and 8th centuries. From the turn of the 8th and 9th centuries, inhumation predominated and thus anthropological studies focus on this period of time. From the demographic point of view, burials of immature individuals usually represent more then 40% of all burials (e.g. Stloukal 1989). Current osteological methods allow the relatively precise determination of age at death of sub-adult skeletons, but sex determination is very problematic.

The main aims of our study were to determine the sex of a group of sub-adult individuals

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from the Mikulčice settlement cemetery using genetic methods, and to establish a reliable protocol for aDNA analysis. The sex information in this group of sub-adult individuals would serve as a standard for the development of new morphological methods. Selected morphological methods would be applied to the same group of individuals to validate them. There are several grave goods records from Mikulčice cemetery which would be used for the final sex determination.

1.1 aDNA

This study applied genetics to archaeological finds. Ancient DNA (aDNA), first introduced by Higuchi (Higuchi et al. 1984), has great potential in archaeology. The extraction and analysis of this highly informative molecule provides insights into many fields of research. Anthropologists mainly focus on aDNA research that may answer questions regarding population characteristics. On the level of a human individual, aDNA genetics are able to determine sex, hereditary and infectious disease that may have caused death, and even a relationship between the individual studied and other people.

1.1.1 aDNA molecule status

The nature of aDNA differs from recent DNA molecules. After a long time spent in a burial environment, aDNA is present in very low quantities and fragmented into short sequences. The aDNA bases have been chemically modified to a great extent (Hoss et al. 1996; Lindahl 1993). Those properties have a noticeable effect on the amplifiability of ancient DNA through the use of PCR methods. For aDNA analysis, the aim is to amplify fragments to lengths of around 150bp, because aDNA has been degraded and fragmented into pieces usually shorter than 200bp (Paabo 1989).

Contamination with exogenous DNA and very low amounts of endogenous aDNA are the biggest challenge faced (Handt et al. 1996; Montiel/Malgosa/Francalacci 2001). Strict precautions must be taken to ensure that authentic aDNA is obtained. There are overwhelming numbers of sources of potentially contaminating molecules and, unfortunately, modern DNA copes with genetic analysis more easily than ancient molecules. Cooper and his colleague have published a set of rules to eliminate contamination in aDNA studies (Cooper/Poinar 2000).

1.1.2 Scope of aDNA applications

Regarding the question of sex, two out of 46 chromosomes determine whether a person will be male or female. With exceptions, females are carriers of two X chromosomes, while males carry one X and one Y chromosome. The region shared by both sex chromosomes is very short. In this pseudo-autosomal region there is, among other things, a gene for the protein amelogenin that has minor differences in its DNA sequence between the two sexes. The detection of these differences is the main goal of genetic sexing analysis. Since the DNA fragment for sex determination is present even in ancient biological material, its analysis is becoming established in archaeogenetic laboratories worldwide.

To answer the question about specific hereditary disease one must detect causative aDNA mutation in an appropriate gene. Using aDNA analysis, the delta F508 mutation that causes cystic fibrosis has been successfully detected in a historical specimen (Bramanti et al. 2003).

Several infections affecting the quality of human life were common in the medieval period. Infectious diseases are caused by pathogenic agents such as bacteria, viruses or protozoa. Detection of their DNA indirectly confirms their presence in an ancient human body. Recent history has seen several cases of successful pathogen detection in archaeological specimens. *Yersinia pestis*, for example, was successfully detected in dental pulps of individuals from France, from specimens 400 years old (Drancourt et al. 1998). Among others, we should mention the detection of *Plasmodium falciparum* (Taylor/Rutland/Molleson 1997) and *Mycobacterium leprae* (Rafi et al. 1994) – pathogens causing malaria and leprosy respectively. A study conducted in the

Czech lands proved the presence of leprosy in the bones of human individuals who died before the Crusades, the main source of widespread leprosy in Europe (Likovsky et al. 2006). Even though the syphilis agent has been genetically determined in mummies from Easter Island (Kolman et al. 1999), recent research into syphilis suggests that its DNA cannot be detected due to its rapid and total degradation in an open environment (Bouwman/Brown 2005).

Confirming kinship between individuals excavated in close proximity (suggesting a close relationship) is a big challenge for anthropologists today. The genetic approach to relationship detection works well for forensic laboratories but is not, unfortunately, that successful in aDNA studies. Every individual is a genetic mix of their parents, with each parent giving exactly one half of their genes hidden in the cell nucleus to their offspring. Close examination of the DNA pattern can precisely detect such direct relationships. Genetic approaches focus on analysis of STR loci (short tandem repeat) polymorphism in autosomes, Y chromosomes and on mtDNA (mitochondrial DNA) hyper-variable region examination.

Until today not many results have been publicly known concerning the STR analysis of ancient related individuals. A group of biomolecular archaeologists from Germany has gone furthest in this task, for example (Gerstenberger/Hummel/Herrmann 2002). Their studies show the possibility of information retrieval with many methodological restrictions – underestimation of larger STR alleles, high allelic drop-out frequency, etc. According to their studies, it is possible to use recent data from population genetics and to apply these on a population from ancient times without loss of information.

The mtDNA molecule is strictly transmitted from mother to offspring and there are thousands of copies of mtDNA in a cell, compared to only two copies of nuclear DNA in chromosomes. The biggest problem in mtDNA analysis is the ease of contamination by recent mtDNA and the high mutation rate. The Y chromosome, on the other hand, is transmitted strictly from males to male offspring. Its STRs are analyzed when seeking to detect relationships between male individuals as close male relatives should have the same STR profile.

At the level of groups of human individuals, kinship genetics analysis may be employed together with population origin, migration patterns, the spectrum of infectious diseases, the degree of endo/exogamy, as well as the social behaviour of earlier human societies.

1.2 Genetic approach to sex determination

Molecular genetic analysis of aDNA allows determination in skeletons of sub-adult individuals in cases where morphological methods are restricted due to a lower manifestation of sexual dimorphic indicators. Not only sub-adult bones but also fragmentary osteological material is highly suitable for such an approach.

Many anthropological investigations employ genetic methods to uncover information about sex that can uncover new social and cultural facts about our ancestors (e.g. Faerman et al. 1998; Faerman et al. 1995; Gotherstrom et al. 1997; Stone et al. 1996). In a study by Mays (Mays/Faerman 2001), it was speculated that infanticide occurred during the Roman Britain period because many skeletons were found to be neonates (38-40 weeks of gestational age) when age at death distribution was monitored. aDNA analysis confirmed the sex of 13 neonates (nine males and four females). This result cannot directly prove infanticide in favour of one sex due to the low number of successful aDNA analyses, but it gives rise to many anthropological questions.

1.3 Morphological approach to sex determination

Skeletal morphological differences between the sexes exist from the intra-uterine stage onwards, but they probably do not reach a sufficiently high level for the reliable determination of sex until after the pubertal modifications take place (Scheuer/Black 2000). The mineralization of dentition shows less sexual dimorphism than do the rates of skeletal maturation (Scheuer/Black 2000).

Many morphological methods for sex determination of immature skeletons currently exist. Due to the low expressivity of sexual dimorphism, most of these methods usually report a 60-90% success rate in sexing (e.g. Mittler/Sheridan 1992; Weaver 1980). In addition, the validity and reliability of many of these methods have not been tested. One of the reasons for this is the lack of samples of known biological identity. The essential problem is the different age structure of skeletal samples for which such methods have been proposed.

Most frequently, the following morphological characteristics are utilized for sex diagnosis: general shape of the pelvis, in particular, the greater sciatic notch and the sub-pubic angle (Schutkowski 1993), configuration of the auricular surface of the sacro-iliac joint (Mittler/Sheridan 1992); the morphology of the mandible (Loth/Henneberg 2001); differences in the robusticity index of the humerus and femur (children aged between 0 and 4; (Coussens et al. 2002); the dimensions of teeth (deVito/Saunders 1990) or the shape of the orbit (Molleson/Cruse/Mays 1998).

With regard to the lack of sex dimorphism in the sub-adult skeleton, we cannot utilize the term "sex determination" in sex morphological methods, but rather "sex estimation" (e.g. Lewis 2007; Saunders 2000; Scheuer/Black 2000). In comparison, sex determination in skeletons of adult individuals – skeletons with finished "ontogenesis" and a "definitive" magnitude of sex dimorphism – generally has a success rate of over 95% when based on morphology of the pelvic bones. With regard to the fact that the sex dimorphism of all human bones, with the exception of the pelvis, is population specific, it is appropriate to apply the technique of primary and secondary sex diagnosis (Murail et al. 2005).

2. Materials and methods

2.1 Contamination precautions

Precautions were taken against contamination with intrusive DNA (Poinar 2003). aDNA extraction procedures were conducted in a room where no modern DNA had ever been isolated. This room was separate from the laboratory where PCR and electrophoresis took place. All the laboratory equipment was maintained in such a way as to keep it uncontaminated. We used 5% hypochlorite and household bleach for instruments and surfaces. Plastic gloves were changed frequently. Plastic laboratory ware and glass vessels were autoclaved before use. The isolation of aDNA and PCR mastermix was set up in a laminar flow cabinet and a system of "blind" controls was applied.

2.2 Sample collection

We have selected a group of 47 sub-adult skeletons excavated in the Mikulčice settlement between 1957 and 1988. These human individuals were chosen for the high degree of preservation of specific bones – a high state of preservation of the maxilla, mandible, teeth, pelvic bones and long bones of the upper and lower limbs. The whole group of 47 sub-adults consists of individuals whose age span is from 18 months to 17 years. This collection will serve in the future for other scientific purposes – mainly for the development of new morphological methods for sex determination in sub-adults.

We have taken five adult individuals from the same cemetery. These skeletons have a high expression of morphological traits for sex determination. These individuals will be genetically analyzed for sex using laboratory procedures. The data obtained will be compared with sex-related data determined using morphological methods. Morphological determination of sex of those selected individuals will have high credibility. If molecular biology provides similar results, one can objectively consider such a study to be reliable. We presume that a high degree of data agreement between the two different approaches (95% and more) will confirm the reliability of both methods. High concordance should strongly support the authenticity of the aDNA studied using our laboratory procedure. The morphological sex was unknown to the geneticist.

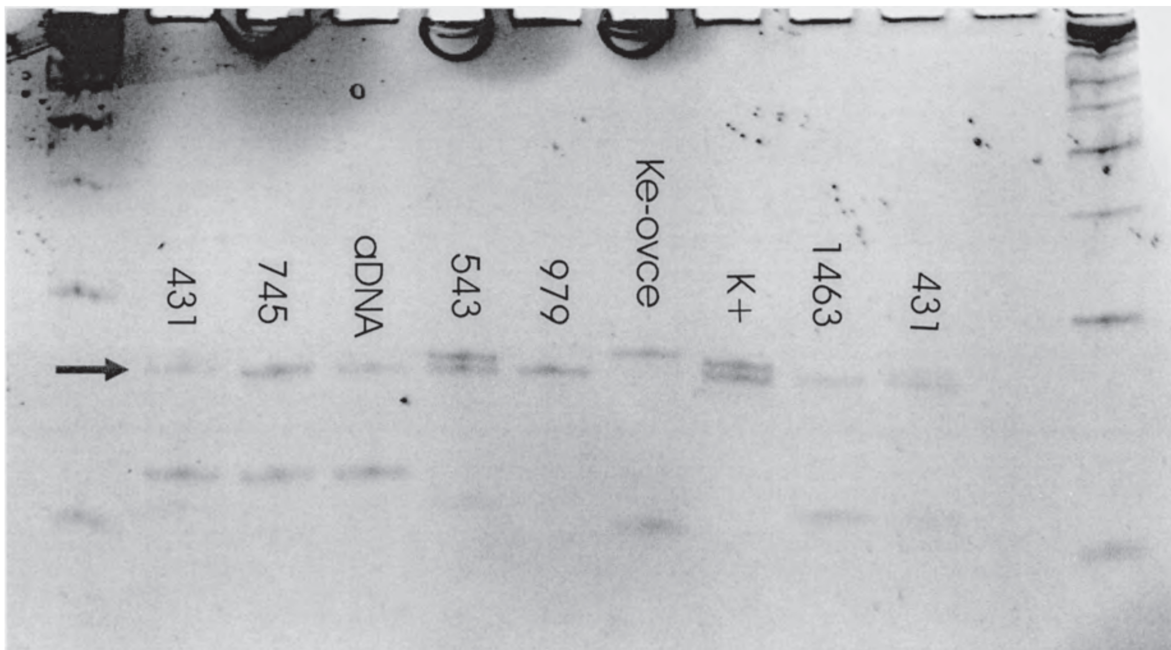

Figure 1. PAGE gel. Number on the figure represents grave number of the specific subadult individual. Positive control – K+ is used for monitoring the success of amplification, its DNA belongs to a man from recent population. aDNA notation represents a historic sample that is not part of this study. Ke-ovce notation represents isolation control – aDNA sample of sheep (amelogenin PCR fragment of different length). The arrow identifies the area for reading the proper length of a PCR fragment Amel 80/83. Double band represents lengths of 80/83bp (male) and single band 80/80bp (female).On the first and the last row of the PAGE gel there is a length marker. Allelic drop-out is apparent for the first sample of individual 431.

2.3 Genetic approach

2.3.1 Extraction of ancient DNA

For genetic analysis two teeth per individual were withdrawn. All such handling took place so that the samples were protected from contamination. Whenever possible, only uncarved teeth were withdrawn. The selected teeth from both groups (sub-adult and adult) had no cavities or visible holes and no openings into the pulp were apparent, according to Richards/Sykes/Hedges 1995. The intact teeth were soaked in 5% hypochlorite to decontaminate the surface. 5 min of UV light exposure (254nm) was applied on all sides. The specimens were ground to a fine powder using a vibrating mill.

For extraction, we used a modification of the original Evison protocol (Evison et al. 1997), with only minor changes applied for our laboratory purposes (Bromová et al. 2003). About 0.5g of bone powder was mixed with 0.5M EDTA (pH 8.0) for two days in a rotary mixer. Proteinase K was then added and the contents were kept in motion for one more day. The extraction procedure then consisted of steps including the addition of 4M GuSCN (pH 7.0) and 20µl silica solution and incubation for two hours in a rotary mixer. The pellet formed by the aDNA and silica suspension was, after a short spin, washed twice in 70% ethanol and once in acetone. The aDNA was eluted in water and stored in a freezer at -20ºC. One to three rounds of extraction from the same bone sample were used to investigate whether or not this could influence the aDNA yield.

2.3.2 Amplification of nuclear aDNA

The PCR reaction was performed in the Techne cycler (Techgene) in a total volume of 25µl. 7µl of aDNA extract was added to the reaction consisting of 7pmol of primers Amel 80/83 (Haas-Rochholz/Weiler 1997) and Fermentas chemicals: 1x buffer, 0.2mM dNTPs,

Table 1. The confrontation of results of morphological and molecular genetic sex determination of the five adult individuals from Mikulčice.

		Primary sex diagnosis			Secondary sex diagnosis [41]		Genetical analysis
Sample No.	Grave No.	pF	pM	Visual	SD1 F	SD1 M	Teeth
1	1588	0.999	0.001	F	0.997	0.003	F
2	1648	0.936	0.064	F	0.705	0.295	F
3	1784	0.001	0.999	M	0.373	0.627	M
4	1821	0.007	0.993	M	-	.	M
5	1835	0.971	0.029	F	0.874	0.126	F

name – inventory number of the skeleton
pF – probability of female sex by primary diagnosis (metric approach)
pM – probability of male sex by primary diagnosis (metric approach)
visual – result of visual approach of primary diagnosis
SD1 F – female posterior probability of discriminant function based on the cranial measurements
SD1 M – male posterior probability of discriminant function based on the cranial measurements
F – female, M – male individual

2.25mM MgCl2, 40ug BSA and 0.625U Taq polymerase. Another amplification system for sex determination was applied – DYZ1 – Y repetitive segment 102bp long (Pfitzinger/Ludes/Mangin 1993). This system securely identifies male individuals in cases where there is a female appearance at the amelogenin locus caused by allelic drop-out of the Y-amelogenin locus. The amplification reaction was also performed in 25µl of $MgCl_2$ at a concentration of 3.0mM. Both systems were employed several times to rule out the possibility of allelic drop-out and unsuccessful amplifications.

2.3.3 Analysis of amplified products

A volume of 8µl of amplified product was analyzed for its length first on 2% agarose gel electrophoresis. If a product was detected with ethidium bromide staining, then the particular sample was placed on 14% polyacrylamide gel electrophoresis (PAGE) for detection of potential 3bp length-difference in male individuals (see figure 1). The PCR product of DYZ1 was analyzed only on 2% agarose gel.

2.4 Morphological approach

The sexing of immature skeletal remains was determined on the basis of two morphoscopic evaluations of certain morphological characteristics of the pelvic bone and mandible. The first method, proposed by Schutkowski (Schutkowski 1990, 1993), evaluates both mandibular and pelvic traits: protrusion of the chin region, the shape of the anterior dental arcade and eversion of the gonion region on the mandible, the angle and depth of the greater sciatic notch, and "arch" criterion and curvature of the iliac crest on the iliac bone. This method was derived from an ancient sample of immature skeletons of known sex and age from Spitalfields.

The second method applied was taken from Molleson and her colleagues (Molleson/Cruse/Mays 1998). Determination of sex is based on the character of the mandibular angle, mentum and the shape of the orbit. The shape of the orbit was not evaluated in this study. The method was proposed on non-adult (juvenile) skeletons of known sex and age from Christ Church Spitalfields.

In total, we evaluated seven qualitative traits – the iliac bone and the mandible separately. For the mandible we recorded four visual scopic characteristics applying the aforementioned methods. Final sex determination was carried out as a summary of all the evaluated morphoscopic traits.

Table 2. Sex of subadults – the comparison of genetical, morphological and archaeological evaluations.

Grave No.	Age at death	Genetic approach	Morphological approach	Grave equipments
170	infans I (8 y.)	M	M ?	gold gombic
207	infans II (10-11 y.)	F	?	
260	infans I (2 y.)	F	F ?	
315	infans I (3-4 y.)	F	? (F ?)	
424	infans I (7-8 y.)	M	M	silver earring, silver gombic
431	infans II (8-9 y.)	M	M ?	silver gombic, knife
460	infans I (2-3 y.)	M	M ?	
462	infans I (4-5 y.)	M	? (F ?)	
477	juvenis (14-16 y.)	M	?	knife, calcar, bronz earing
489	infans II (8-9 y.)	M	M	knife
491	infans I (2-3 y.)	F	M ?	2x silver gombic
513	infans I (1,5-2 y.)	M	?	
539	infans I (2-3 y.)	F	M ?	
543	infans II (8-9 y.)	M	M ?	
597	infans I (4-5 y.)	M	M ?	
619	infans II (8-9 y.)	M	M	
621	infans II (8-9 y.)	M	M ?	
623	infans I (2-3 y.)	F	? (M ?)	
638	infans I (2-3 y.)	M	M	
644	infans II (10-12 y.)	M	M ?	knife
658	infans I (6-7 y.)	M	M ?	
745	infans II (9-10 y.)	F	?	
979	infans I (6-7 y.)	F	F ?	
1124	infans II (10-11 y.)	F	F	
1127	infans II (7-8 y.)	M	? (M ?)	
1158	infans I (4-5 y.)	M	? (M ?)	
1235	infans II (12-15 y.)	M	M ?	
1463 a	infans I (4 y.)	F	? (M ?)	
1509	infans II (7-8 y.)	M	? (M ?)	
33/IV	infans II (12-15 y.)	M	? (M ?)	
39/IV	infans II (12-15 y.)	F	? (M ?)	knife
41/IV	infans I (5-6 y.)	F	M	
64/VI	infans II (12-15 y.)	M	M ?	calcar, strap-end
121/VI	infans II (7 y.)	M	M	calcar, little knife (iron)
140/VI	infans II (8-9 y.)	M	?	
160/VI	infans I (6-7 y.)	F	F ?	4x earring (gold), 2x earring (silver), gombic (silver), bucket, little knife
167/VI	infans I (1-2 y.)	M	M ?	

? – non determinable, ? (M ?) or ? (F ?) – non determinable-ambiguous sex, M – male, M ? – more likely a male, F – female, F ? – more likely a female, y – years

The sex of five adult skeletons was evaluated on the basis of the morphoscopy (Bruzek 2002) and metric characteristics of pelvic bones (so-called primary sex diagnosis) (Murail/Bruzek/Braga 1999; Murail et al. 2005). Besides that, sex was also determined with the help of discriminating functions based on cranial measurements (secondary sex diagnosis). These functions were derived from the Great Moravian population (Brůžek/Velemínský 2006).

3. Results

The results of the primary and secondary morphological analysis as well as the molecular genetic analysis of the five adult skeletons are presented in Table 1. In all five adult samples, the sex data of the genetic and morphological approaches matched precisely (100% concordance). This result allowed us to apply the same genetic procedure to the group of 47 sub-adult individuals.

In 37 out of 47 ancient teeth samples (78.7%), the extraction aDNA was successful (it yielded amplifiable aDNA). The sex of each individual is shown in Table 2. We obtained results for 24 males and 13 females (a male to female ratio of 1.85:1). When there was no PCR product after the first amplification, the use of a smaller amount of DNA in the PCR reaction sometimes helped. The second round of extractions in particular yielded detectable amounts of aDNA compared to no product detection in the first round.

On the basis of the morphological characteristics of the iliac bone and mandible, we determined the sex for 23 out of 37 genetically sex-determined skeletons (62.2%). For the remaining 37.8% of the sub-adults, the morphological sex traits were equivocal (see Table 2). In nine cases we did not determine the sex affinity, even though one of the sexes is more probable according to the morphological characteristics (e.g. ? (M ?) or ? (F ?)). The sex determination of some individuals was also limited by skeletal preservation. Likewise, in the case of genetic analysis, the results showed a uniquely higher presence of male sub-adult skeletons. We recorded the presence of 19 males and four females.

When the sex of an individual was genetically determined, it was compared with the morphological sex estimate (Table 2). The concordance between genetic and morphological determination of sex was high (87%). There was disagreement in only three out of 23 skeletons (graves No 491, No 539 and No 41/IV). However, when the morphological approach was based on the iliac bone and mandible separately, a different concordance was achieved (83.3% and 73.9% respectively). Although the reliability of morphological methods is low due to the low degree of sexual dimorphism in immature skeletons, we recorded an unexpectedly high concordance between both applied approaches. We consider the value of the coincidence (87%) as a rather random result. The concordance obtained on the basis of separate morphologic approaches (iliac bone and mandible) is approximately 60%-90% (e.g. Schutkowski 1993; Weaver 1980), which appears more realistic (Lewis 2007).

In some sub-adult graves from the Mikulčice cemeteries included in this study, grave goods attributed to females or males was found (e.g. Hrubý 1955; Stloukal 1970). Only a few records of grave goods exist for our 23 morphologically studied samples. Particular grave goods are also listed in Table 2.

4. Discussion

4.1 Genetic approach

To test the reliability of the method for genetic sex analysis that we applied, we first worked with a test group of five adult individuals with distinct morphological traits. Amel80/83bp and DYZ1/102bp PCR systems provided sufficient information about genetic sex for all of the studied subjects. A comparison of the genetic data and morphological data revealed 100% concordance. Although the number of studied adult individuals is very low. We presume that the very high concordance obtained is not a result of random

matching . This observation of the five adult individuals proved the reliability of the applied genetic procedure and the authenticity of the aDNA studied.

Subsequently, we genetically analyzed 47 sub-adult individuals from the 9th century cemetery. All the samples underwent an identical procedure to the five samples of adult individuals. We were able to record the sex of 37 out of 47 examined sub-adults. In those 37 samples it was possible to isolate aDNA and to apply an optimized PCR protocol to obtain DNA fragments containing sex information. The successful analysis rate of 78.72% is lower than in our prior study (94.7% in (Bromová et al. 2003)), but higher than in several other studies (Faerman et al. 1998; Gotherstrom et al. 1997; Mays/Faerman 2001) – 28.5%, 44% and 42% respectively.

There are several possible explanations for the unsuccessful aDNA isolation rate of over 20% (10/47). Most probably, the aDNA material was degraded to such an extent that no fragments of corresponding length could be amplified. Secondly, the process of isolation could have been successful, but substances that inhibit the elongation step during PCR amplification may have been present as a co-extract (Kemp/Monroe/Smith 2006). Different amounts of aDNA template in a PCR reaction sometimes help overcome this problem. Finally, specimen-specific attributes (for example sequence mutation at the site of primer annealing) could have blocked the correct amplification procedure.

In previous years, some scientists have developed methods that could ascertain whether a specific specimen is suitable for aDNA analysis. The extent of amino acid racemization is one such tool (Poinar et al. 1996). This method is very labour-intensive, expensive and time consuming and some authors suggest that it may not be accurate since amino acid racemization and DNA degradation could have different kinetics in bones (Collins/Waite/van Duin 1999). The histological preservation of bones seems to be a better method, although it also has some drawbacks. Even bone samples with outstanding histological parameters are not ideal for aDNA analysis (Haynes et al. 2002). However, some authors have obtained 100% concordance between histological preservation and success of DNA amplification (Colson et al. 1997). This process is also very labour-intensive and, according to some studies, not as accurate. When sub-adult bone specimens were analyzed for concordance between specific histological preservation and success of aDNA amplification (Colson et al. 1997), it was shown that one bone specimen from the group with less preserved histological parameters was also very weak at aDNA amplification. An important point is that this was one from a total of five successful amplifications out of seven specimens, and even low histological preservation allowed aDNA amplification. The grouping of bones for aDNA analysis according to histological examination could, for that reason, be very inappropriate.

As to aDNA isolation, a second round of extraction from the same bone powder usually increased the aDNA yield to a detectable level. We presume that in the second and following isolation, the number of protein and other co-extracts (Hagelberg/Clegg 1991) decreases, and amplification is thus not inhibited. Similar results have been obtained by Kemp (Kemp et al. 2006). We have experienced how difficult it may be to establish an optimized PCR protocol for the detection of low amounts of degraded aDNA. When the aDNA concentration was diluted, this sometimes helped us to obtain amplification products. Inhibitory substances were probably present in many specimens.

Allelic drop-out was frequent among our specimens. When the Y-amelogenin locus did not amplify, the resulting band of the X-amelogenin locus appeared to originate from a female individual (see figure 1). We also amplified the aDNA on DYZ1. This system was better optimized, gave results for more specimens than Amel80/83bp and determined all male individuals. Similar results were obtained in a work by Ovchinnikov (Ovchinnikov et al. 1998). In their

study they used the amelogenin sexing system Amel106/112bp (Sullivan et al. 1993), which was successful in only 25% of samples, as well as the DYZ1 system, which was better optimized. Since Amel80/83bp focuses on shorter sequences than Amel106/112bp, it is advisable to use it for aDNA sexing analysis.

It was apparent that a higher number of PCR cycles was needed when aDNA was amplified. Recent studies have shown that the amount of aDNA starts to rise after the 25th cycle (Kefi et al. 2003). Our amplification profile consists of 35 to 40 cycles on average.

The protocol developed for our study seems to be a very useful tool for sex analysis. It is easily recognizable from primer-dimer formation, which was always up to 70bp long.

4.2 Morphological approach

Using skeletal morphology it was possible to estimate the sex of 23 sub-adults (19 male and four female), approximately 60% of genetically determined individuals. Among the remaining 14 sub-adults, sexual morphological traits were ambiguous. Nevertheless, in nine cases (9/37), we could not precisely determine sex, although one of the sexes was, according to morphological characteristics, more probable (e.g. ? (M ?) /? (F ?) – in Table 2). The sex determination from specific morphological markers of some bone remains was also limited by skeletal preservation. In seven individuals the mandible was not preserved, while the iliac bone was not present in two individuals. If we compare the two applied morphological methods (Molleson/Cruse/Mays 1998; Schutkowski 1990, 1993), the concordance in sex determination was 100% (N=17). Whilst the Schutkowski method is based on signs on the mandible and iliac bone, using the method of Molleson /Cruse/Mays (1998) we only applied the signs on the mandible. This concordance is thus not surprising.

A significantly different situation arises if we compare the results of sex determination based on the mandible and iliac bone. Sex was determined concordantly in the case of 11 children (78.6%), while antagonistic sex determination occurred in three cases (i.e. 21.4%). The determined concordance/divergence cannot be generalized due to the small number of cases involved.

It appears that sex determination based on the structure of the iliac bone has a greater predicative value. This, though, may be affected by the worse preservation of the mandible in the studied group. On the other hand, sex determination using the mandible is based on multiple signs, meaning that the subjective view of the researcher plays a relatively smaller role. With regard to the size of our group and the number of children in our study, neither the bones nor any of the methods applied may be considered ideal for sex determination.

The reliability or accuracy of sex morphological methods in sub-adults mostly varies from approx. 60% to 90% (e.g. Mittler/Sheridan 1992; Schutkowski 1993; Weaver 1980). Moreover, a different accuracy of determination was reported between males and females (e.g. Mittler/Sheridan 1992). The age of a child's skeleton may also play an important role – it follows that the age used to draw up the relevant methods may be an important factor (see Lewis 2007).

4.3 Comparison of genetic and morphological approaches

The sex information from the genetic analysis was taken as a standard. The results of such determination were not known prior to the qualitative evaluation of the morphological traits. This criterion helped us to avoid the subjective factor from interfering with the results of the morphological approach. We were able to record the sex of sub-adults using morphological methods in 23 out of 37 genetically characterized individuals. We monitored the concordance of the obtained data for the 20 identified sub-adults. Surprisingly, there was 87% concordance (20/23). There was disagreement as to the recorded sex for only one sub-adult.

The morphological method itself was not given high credibility by the anthropologist. However, these results provided very interesting evidence for the appropriate application of the Schutkowski (Schutkowski 1990, 1993) and Molleson (Molleson/Cruse/Mays 1998) methods on sub-adult bones for sex determination. The probability of a random match among all 21 individuals is very low.

If we compare the determination on the basis of individual bones with genetically determined sex, in the case of the iliac bone, sex was determined concordantly in 83.3% of the children (20/24), and on the mandible, the sexual signs corresponded to the genetic determination in 69.6% of cases (11/14; method by Schutkowski), in 73.9% of the children (16/23; method by Molleson) respectively.

It thus appears that sex determination based on the structure of the iliac bone has a greater predicative value.

The very low number of samples studied may interfere with this high (87%) success rate. The data for morphologically identified sub-adults was recorded as a summary of all the evaluated morphological traits on the pelvic bone and mandible. The value of concordance of both approaches obtained on the basis of the two morphological methods separately reached a value of approximately 65% to 90%. This value of concordance resembles the standard success rate (e.g. Lewis 2007).

Several earlier studies also focused on the issue of comparing sexing results obtained using genetic and morphological approaches (Ovchinnikov et al. 1998; Waldron/Taylor/Rudling 1999) etc. Different morphological methods were applied and so it would be inappropriate to compare the success rate of genetic versus morphological data. However, these studies imply the necessity of a multidisciplinary approach.

All the morphological sex determination techniques have been based on the study of contemporary populations. For correct application, the characteristics analyzed should be calibrated to the population examined (Vernesi et al. 1999). In our group from the 9th century, it is apparent that it was possible to apply morphological techniques without losing correct information. This is possible to deduct (with some exaggeration) from high sex determination agreement of different methods of evaluation.

The higher number of male individuals in our sample is surprising. We uncovered almost twice as many males as females – 24M:13F in the genetic and 19M:4F in the morphological approach. This ratio could have been influenced to a certain extent by the selection of the children evaluated. Most (two thirds) of the evaluated children were buried on the grounds of the Mikulčice castle itself (N=28), and only one third came from the surrounding settlement. If we were to speculate that individuals of male sex in the Early Middle Ages held a more important social position, then in the castle we would expect to find a greater proportion of graves containing the remains of boys rather than girls. If we look at the current paleo-demographic studies of the Mikulčice cemetery, then it is clear that males predominate within the grounds of the castle, while women predominate in the surrounding settlement. As to the age structure, older individuals were buried mainly in the castle, while the graves of non-adult individuals had a higher representation in the settlement. For example, the so-called "index of representation of non-adult persons" is 75.2 in the castle and 84.3 in the settlement (Stloukal 1989; Velemínský et al. 2005). On the social ladder, small children were probably not considered to be "fully fledged" individuals. But we must take into consideration the unresolved paleo-demographic issue – namely the absence of children under two years of age. The demographic conclusions, though, do not definitely exclude the presumed more frequent burial of boys in the castle. We may speculate that if a child were buried in the castle, it would probably have been a boy.

On other hand, we can also speculate that boys had a lower chance of survival during childhood. The demographic mortality curve of children from Mikulčice castle and estates, though, follows a similar course, with no significant fluctuations,

and it begins falling steeply only after the tenth year of life. This reflects the natural mortality of the population of children. Finally, the higher proportion of boys may also be explained by the different state of preservation and conservation of the mandible and pelvic bone in both sexes.

The suggestion that sub-adult male skeletons may be better protected from environmental factors due to the higher inorganic fraction in their bones (DNA seems to bind to hydroxyapatite structures (Burger et al. 1999) is refuted by the fact that some older individuals have no amplifiable aDNA (g.n. 481,643) when compared to very young ones (g.n. 513,167/VI), whose inorganic fraction is remarkably lower. In this manner, more male specimens could have been classified as sub-adults.

In a study by Lassen (Lassen/Hummel/Herrmann 2000), individuals found in Aegerten in Switzerland also presented a disproportion between male and female individuals. In this work, only stillborn and neonatal individuals were studied and the disproportion was explained by the higher mortality of males from the last months of pregnancy until six months after birth (Eiben et al. 1990). In our study, the sub-adult individuals had a life span from birth to 12 years of age.

The possible socio-cultural reason for the higher representation of male individuals will be studied further. However, random sampling of sub-adults from different regions of the Mikulčice cemetery tends to exclude any socio-cultural reason. This subset of sub-adult individuals is unfortunately too small for the use of statistical methods.

Limited archaeological records of grave goods have also been included in Table 2. Records are available for 10 of the 23 morphologically determined individuals, although their low number precludes systematic evaluation. Identical genetic, morphological and "archaeological" determination of sex was found in the case of three graves (graves No. 64/VI, 121/VI and 160/VI), if we accept the fact that specific grave goods are attributed strictly to individuals of certain sex (Hrubý 1955). Agreement between multidisciplinary approaches favours the usefulness and correctness of the methods applied.

Our extracts were free of exogenous contaminating DNA, as we can deduce from the system of blind controls and the high percentage of sex determination agreement between morphologic and genetic data among adult individuals. According to some findings (Kolman/Tuross 2000), blank controls without product amplification do not signify that contamination was absent in other tubes.

5. Conclusion

This study focused on developing a protocol for aDNA sex analysis from sub-adult archaeological bone remains. aDNA was successfully analyzed in 37 out of 47 sub-adult individuals. The laboratory-developed protocol proved to be a very powerful tool for sub-adult sex determination. This approach was very successful for sex determination in a majority of samples. The results obtained served as a standard for comparison with the results of specific morphological approaches, similarly to grave goods records. The Schutkowski and Molleson morphological methods proved to be applicable, but provided data for a significantly lower number of samples. Their advantage lies in the low cost of analysis. The study group in question will serve as a standard for ongoing research focusing on the development of new morphological methods for the sex determination of sub-adult skeletons. Our contribution shows the value of the simultaneous application of different approaches as the best tool for objective evaluation.

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