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Sex determination of skeletal remains of 4000 year old children and juveniles from Hoštice 1 za Hanou (Czech Republic) by ancient DNA analysis

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ABSTRACT The aim of this study was to determine the sex by means of modern molecular genetic methods of children and immature individuals from the 4000 years old Eneolithic burial site "Hoštice 1 za Hanou" of the Bell-Beaker people, in central Moravia (Czech Republic). While the anthropological approach was in this case limited either by the state of preservation of the skeletal remains or simply by absence of definite morphological traits in the children, analysis of aDNA (SRY, amelogenin) yielded results consistent with archeological grave findings and body imposition. The burial rites of the investigated culture facilitated the analysis because the gender specific imposition of adults has previously been described (man left-side, head northwards, woman right-side, head southwards) However, this approach is often limited in case of children burials. This study showed high concordance between archeological sex-determination and genetic sex, but also revealed several exceptions in children burial rite of Bell Beaker culture.

KEY WORDS: amelogenin, SRY, aDNA, Bell-Beaker-culture, Hoštice-1-za-Hanou

The determination of sex is one of the most important assessments in the investigation of skeletal remains and also one of the principal anthropological characteristics. Many subsequent analyses, such as facial reconstruction or body sizing, fail if sex is not determined correctly. Male and female attributes range over a continuum of morphologic traits and measurable sizes in the skeleton. More-over, in children and juvenile individuals the sex-specific characters are not developed and thus skeletally based identification is often impossible. In some cases analysis of body imposition or grave inventory is helpful, but these often do not provide a reliable basis for correct determination of gender (LOTH AND IŞCAN 2000). In these cases sex can be determined using a genetic approach when sex-specific sequen-

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ces of DNA preserved in bone fragments are amplified by polymerase chain reaction (PCR) and visualized.

At present, the usual markers commonly used for sex determination are specific sequences in amelogenin and SRY genes. The amelogenin gene lies on homologous parts of X and Y chromosomes and bears female specific deletion of six base pairs in its first intron, allowing discrimination between male and female alleles (FAERMAN *et al*. 1995). Amelogenin was termed by EASTOE in 1965. It is a major constituent unique to the developing enamel. The SRY gene codes for transcription factors localized on Y chromosome triggering development of male specific characters. SRY is an intron-less gene that spans 3.8 kb. The method developed in 1998 by SANTOS *et al*., allows direct identification of male individuals, since it is carried out on a short 93 bp fragment in the SRY gene, which is recognized as the testis determining factor. The study of CUNHA *et al*. (2000) showed that the 93 bp fragment of the SRY gene seems to be easier to amplify than the amelogenin region. However, amplification of divergent X and Y alleles of the amelogenin gene offers the advantage of an internal positive control (HUMMEL and HERMANN 1991).

The Eneolithic burial site, pertaining to the culture of the Bell-Beaker people, is dated to 4000 BP and is located near Hoštice za Hanou, a village in central Moravia (Czech Republic). The burial site was uncovered by rescue research during the D1 highway construction in 2002; it is considered to be one of the greatest burial sites of the Bell-Beaker people discovered so far, with more than 150 identified skeletal graves. This culture performed typical burial rites based on specific imposition of the deceased body into a grave accompanied by various gifts (beakers and other ceramics, leg plates, bone pendants, daggers, needles, bodkins or ear-rings). In almost all cases, men were placed facing leftwards and women rightwards (HAVEL 1978). However, for children or immature individuals, it is unknown if this burial rite was strictly followed, and thus the gender of an individual can be misidentified.

To address the question of accuracy of archeological sex determination in the case of children and juvenile individuals buried at the site, we performed a genetic analysis of aDNA extracted from bone samples by polymerase chain reaction (PCR) of selected regions of sex specific markers i.e. amelogenin and SRY. Our results extend the possibilities of genetic analysis of ancient findings and validate the archeological characteristics observed on this material.

Materials and methods

Samples of ancient bones and teeth were obtained from 55 children and juvenile individuals from Eneolithic burial site of the Bell-Beaker culture "Hoštice 1 za Hanou" in Czech Republic. Each individual grave was archeologically assessed and archeological sex was determined according to grave inventory and body imposition. Since contamination by recent DNA represents a critical problem in genetic analysis, the bone material was unearthed using sterile equipment (dedicated, autoclaved and bleach-treated sets of instruments), and further processed under conditions so as to decrease risk of contamination (separated laboratory, protective wear, laminar hood). In 2000, the work of COOPER and POINAR, which has

been taken into consideration in this study, summarized the putative sources of recent DNA introduced to aDNA analyses and offered molecular approaches to detect and reduce contamination.

To avoid contamination of PCR reactions by recent DNA or cross-contamination by previous amplicons, isolation, the PCR and visualization steps were separated and a special regimen in all laboratory rooms was introduced. Prior to DNA extraction the surfaces of the bones or teeth were treated with household bleach and UV decontaminated. The samples were then grounded to a powder using a bone mill or mortar and pestle under sterile conditions. The bone powder was twice decalcified in sterile 0,5M EDTA (ethylene diamine tetraacetic acid) /4°C/24 hours on a rotator and centrifuged (5 min/4000 rpm). The supernatant was discarded and the sample processed again in 0,5M EDTA/4°C/week. The supernatant was stored in a sterile tube. DNA was extracted from the supernatant using QiaAmp DNA Mini Kit according to manufacturer instructions.

A part of the SRY locus was amplified by PCR according to the protocol introduced by CUNHA *et al*. (2000). The X-Y homologous region of amelogenin gene was amplified using the HUMMEL (2003) protocol, with modification in cycling temperatures, where the pre-amplification step of 20 cycles (93°C/60°C/72°C) was followed by 30 cycles (93°C/66°C/72°C). The PCR products of SRY or amelogenin amplification were resolved by electrophoresis in 3% agarose or 5% MetaPhor gels respectively, and visualized by ethidium bromide. To avoid cross-contamination by PCR amplicons, electrophoresis and visualization was performed in a separate room. To avoid false-positive or false-negative results, each sample was processed three times, including isolation and PCR. Each analysis was separated by the period of at least three months.

Results and discussion

Fifty-five samples of child skeletal remains were excavated from burial site and investigated archeologically. Two samples were excluded from the study owing to unsatisfactory preservation of the skeletal material. Thus fifty-three samples were included in the study. After the isolation step, the concentration and purity of aDNA was assessed and served as confirmation of success of the isolation step. Agarose electrophoresis of an aliquot of isolated DNA was performed to assure the presence of a continuum of short DNA fragments (less than 500bp) and not longer molecules, which can indicate contamination of recent origin.

During both the isolation and PCR steps negative control reactions were performed to determine possible contamination by recent DNA. Only the results of genetic determination in concordance with the SRY and amelogenin markers were considered as valid. Affirmative results of both markers were obtained in 21 samples (~40%); in 5 samples the analysis yielded no markers, and in 27 cases only either amelogenin or SRY was revealed. In both cases, these data were excluded from the overall evaluation. Verified results (consistent results from all independent isolations) of the genetic analysis were compared with the archeological determination and are summarized in Table 1.

Concordance between the genetic and archeological determinations occurred in 13 cases out of 21 (~62%). Sex ratios

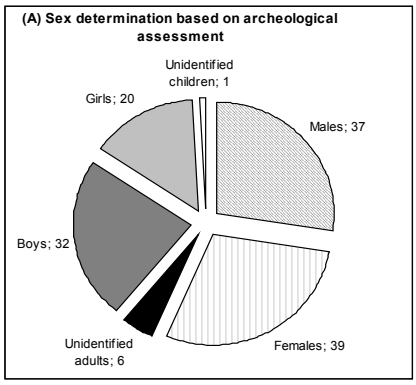
Table 1. Gender status obtained either by archeological or genetic analysis (only valid data shown). Non-correspondence of archeological and genetic assessments indicated in bold

Case #	Archeological sex determination	Genetic sex determination
H801	M	M
H813a	M	M
H814	**F**	**M**
H823	**F**	**M**
H826	F	F
H829	**F**	**M**
H834	**M**	**F**
H837	M	M
H841	M	M
H855	**F**	**M**
H879	Unidentified	F
H886	M	M
H888	M	M
H897	M	M
H905	M	M
H912	M	M
H923	M	M
H924	**F**	**M**
H942	**F**	**M**
H946	M	M
H950	M	M

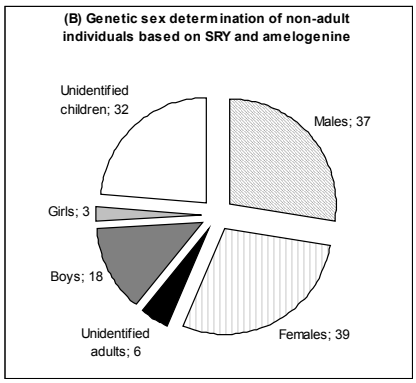
based on archeological (A), genetic (B) or combined archeological-genetic (C) analysis of child skeletal remains together with archeological analysis of adults, are diagrammatized in Figure 1. Categorization of the analyzed child skeletal remains into age cohorts indicates a clear trend of increased mortality of children in the Infans I, II and Juvenis cohorts. Moreover, in these categories, boys are present more often than girls of the same age (Figure 2).

These findings are consistent with demographical analyses of historical and recent populations, where boys predominate over girls in young age cohorts. In Bell-Beaker culture, exact population data are not available; however, according to analyses of Bell-Beaker graveyards performed thus far, men predomi-

1A



1B



1C

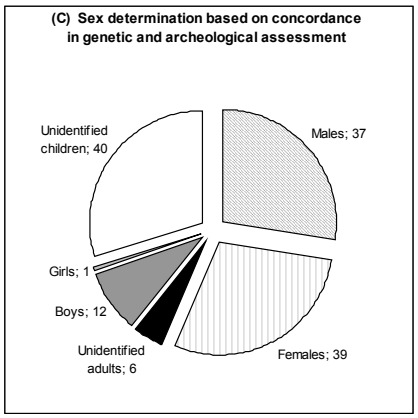


Fig. 1. Archeological (children) and anthropological (adults) (A), genetic (B) and combined (C) sex determination of Hoštice 1 za Hanou burial site. Genetic analysis performed only on children.

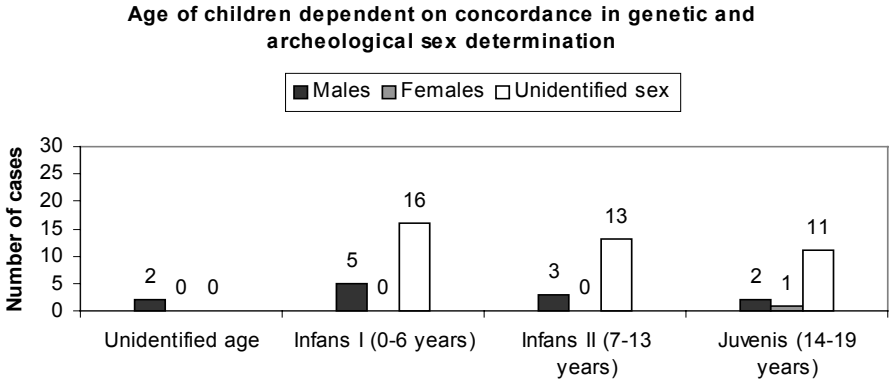


Fig. 2. Children with determined sex divided by age. Number of boys in Infans I, II and Juvenis cohorts predominates over the number of girls in same age cohorts

nate over women in the ratio 53% : 47% (DVOŘÁK and DROZDOVÁ 2007).

The study has shown that in seven cases the results were divergent between archeological and genetic analyses, when body imposition or grave content did not correspond with genetic sex of the imposed body. The archeological status of these cases is depicted in Figure 3.

Case H814: Age 15-19 years, well preserved skeletal remains; body imposition is on the right side and corresponds with the women rite. However, the body lacked any gifts or female attributes. By genetic analyses, the gender was determined as male. It is possible that this burial represents an exception in body imposition.

Case H823: Age 15 years, well preserved skeletal remains; body imposition is on the right side with ceramics behind; thus female according archeological analyses. Genetic analyses revealed the presence of a Y chromosome. It is possible that this burial represents an exception in body imposition.

Case H829: Age 7 years, skeletal remains very fragmentary; position of the body according to deposited gifts probably indicates female type. However, both markers were positive for male signals.

Case H834: Age cannot be determined. Skeletal remains fragmentary; sex determined as male according to body imposition, but with some female attributes. Genetic analysis was negative for SRY as well as for the Y allele of amelogenin.

Case H855: Age 15-22 years, very well preserved skeletal remains; according to archeological analysis sex determined as female, with a number of female attributes, including knobs. However, PCR analysis revealed amplification of SRY as well as the Y allele of amelogenin. Results may indicate a potential specific exception in burial rite.

Case H924: Age 3-4 years, very fragmentary skeletal remains; unclear body imposition. According to several findings of ceramics, sex determined as female. However, both SRY and Y allele were positive.

Case H942: Age 8 years, well preserved remains; sex determined as female according to body imposition and burial gifts. Surprisingly both genetic markers were positive for male. This case may probably represent a specific exception in burial rite.

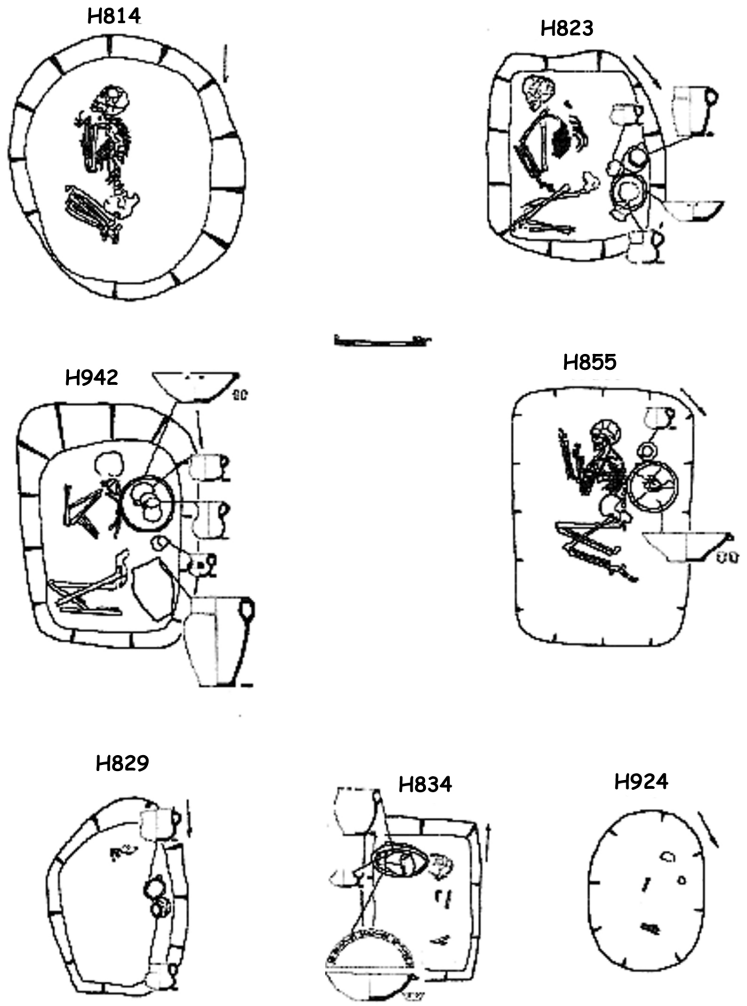


Fig. 3. Schematic layouts of discussed cases having different archeological and genetic sex determination. Where possible, grave inventory is indicated

Generally, the burial rite may represent a particular way of affiliation of an individual to a community; usually, there exists a gender specific grave content or gifts reflecting social hierarchy in a community (TUREK 2002). In the case of the Bell Beaker people, the burial rite included typical, strictly followed, body imposition in a gender dependent manner (man on left side, head northwards, woman on right side, head southwards) accompanied by gender-specific attributes such as leg plates, bone pendants or earrings, daggers, needles or bodkins. The several known exceptions in the burial rite of the Czech branch of the Bell Beaker people were described as a divergence between grave content and the estimated gender. In a few cases, the body was deposited on right side with the head

facing southwards (female type), but accompanied with leg-plates used by archers and numerous ceramics, usually found in male graves (DVOŘÁK and HÁJEK 1990, TUREK and FOSTER 2000, TUREK 2006) or by other male attributes, such as copper daggers. Additional details, not mentioned in this work, can be referred to in TUREK 2002. At present, little is known about to what extent the child burial rite and grave contents are gender-specific; however, it is highly possible that the adult burial rite was followed for children as well (PODBORSKÝ *et al*. 1993). Our study further supports this hypothesis by the high concordance (~62%) between genetic and archeological sex determination in children samples suitable for analysis (Tab. 1).

Our findings demonstrate possibilities of genetic analysis applied in old and fragmentary bone material where standard anthropological assessments are limited. The data obtained in most cases validate the sex determined by archeological characterization; however in seven cases we revealed specific exceptions to the burial rite of Bell Beaker culture. Interestingly, compared with previous work (TUREK 2002), these child or young burials were identified as genetic males with female impositions and/or female attributes in six cases. In one case we found a genetically identified woman buried with several female attributes, but with a male imposition. We therefore conclude that genetic analysis of skeletal remains allows further insights into the burial rite of the Bell-Beaker people, especially in respect of child burials, but in a broader context also opens new and interesting questions about their demography, sex-ratio, pathophysiology and kinship.

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Streszczenie

Metodami nowoczesnej genetyki molekularnej oznaczono płeć dzieci i osobników młodocianych z liczącego 4000 lat eneolitycznego cmentarzyska ludności kultury pucharów lejkowatych na stanowisku Hoštice 1 za Hanou (Środkowe Morawy, Republika Czeska). Ponieważ, zarówno ze względu na stan zachowania materiału, jak i brak cech płciowych na kościach dziecięcych, oznaczeń metodami antropologicznymi nie można było wykonać, przeprowadzono analizę starożytnego DNA (aDNA), a jej wyniki skonfrontowano z danymi archeologicznymi i anatomicznymi. Rytuał pogrzebowy badanej kultury pozwalał na taką analizę, ponieważ wyposażenie grobów oraz sposób chowania zmarłych zróżnicowane były płciowo (mężczyźni na lewym boku, z głową zwróconą na północ, kobiety na prawym boku z głową na południe). Zasady tej nie zawsze przestrzegano w odniesieniu do pochówków dziecięcych.

Pobieranie materiałów do badań aDNA przeprowadzono z zachowaniem warunków ochrony przed zanieczyszczeniem współczesnym DNA. Obecność specyficznych dla płci sekwencji amelogeniny i *SRY* badano metodą PCR (reakcji łańcuchowej polimerazy). Spośród 55 próbek pobranych z kości dzieci pochowanych na cmentarzysku 2 wyłączono ze względu na zły stan zachowania kości. Oznaczenia markerów amelogeniny i *SRY* z 53 próbek uznano za prawidłowe. Pozytywne wyniki dla obu markerów uzyskano w 21 próbkach; w 5 próbkach nie udało się stwierdzić żadnego z dwóch markerów, a w 27 próbkach ujawniono jeden marker – amelogeniny lub *SRY*, niemniej próbek tych nie włączono do końcowej oceny.

Zweryfikowane wyniki analizy genetycznej skonfrontowano z ocenami archeologicznymi, a następnie oszacowano, na połączonych danych, proporcję płci wśród zmarłych z cmentarzyska. Zaobserwowano wysoką zgodność (ok. 62%) pomiędzy oznaczeniami genetycznymi i archeologicznymi. W 7 przypadkach stwierdzono jednak rozbieżność oznaczeń archeologicznych i genetycznych. Przypadki te mogą dotyczyć odstępstw od rytuału pogrzebowego.