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MORPHOLOGICAL CHARACTERISATION AND MOLECULAR SEX DETERMINATION OF HUMAN REMAINS FROM THE 15th–17th CENTURIES IN LATVIA

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Sex determination is one of the most important and initial steps in human profile identification from archaeological material. The aim of the current study was to evaluate the application of molecular approaches alongside morphological methods for sex determination in archaeological human skeletal remains. Human skeletal remains were excavated from three cemeteries: St Gertrude Old Church, Dom Square and St Peter's Church, of 15th–17th century burials in Rīga, Latvia. Morphological and molecular genetic methods, including amplification of genes AMELX/Y *and* SRY *were used to analyse seven skeletal remains. The conducted analyses of morphological features identified sex in all seven cases (two females and five males). By molecular analyses of mediaeval DNA it was possible to determine sex in five of seven (71%) samples. In all positive cases full agreement between morphological estimation and molecular genetic methods was observed. To conclude, DNA analysis can be considered for sex identification in cases with no signs of sexual dimorphism (juvenile skeletons) or partially preserved skeletons.*

Key words: *archaeological material, ancient DNA, anthropological characteristics, amelogenin,* SRY*.*

INTRODUCTION

Determination of the sex from human skeletal remains is of vital importance in the disciplines of bioarchaeology and forensics. Without precise ascertainment of the sex, accurate estimation of age at death and stature are invidious, as the rates of growth, development and degeneration vary by sex and by population. Morphological sex estimation of human skeletal remains is mainly based on morphological traits of pelvis and skull. It has been calculated that the pelvic bone provides the highest accuracy, ranging from 80% to over 90%, which highly depends on skeletal preservation, population-specific sexual dimorphism, as well as experience of the observer (Hoyme and Iscan, 1989; Mays and Cox, 2000; Bruzek, 2002). However, such estimation is most accurate in adult individuals with complete maturation, and thus differences in skeletal morphology. Therefore, morphological methods are less reliable for younger age groups (Saunders, 1992) or partially recovered skeletons, when neither the pelvic bone nor skull is sufficiently preserved.

Recent advances in molecular biology have enabled to identify sex using ancient DNA (aDNA) (Bauer *et al*., 2013). Currently, analysis of aDNA is assumed to be reliable, highly accurate and additional to morphological examinations. It is increasingly used for sex determination, although exceedingly small amounts or highly degraded aDNA, especially from poorly preserved remains of the skeletons, limit this approach. In recent publications, the genes amelogenin (*AMELX/Y*) and sex determining region Y (*SRY*) have been extensively examined (Bauer *et al*., 2013, Zapico *et al*., 2013). The *AMELX/Y* genes are located on X chromosome (Xp22.1–p22.3, *AMELX*) and on Y chromosome (Yp11.2, *AMELY*). X and Y copies of the *AMEL* do not undergo homologous recombination; therefore, it is the mostly used genetic marker for sex determination in modern anthropology, to date. However, point mutations or large deletions in the primer binding sites of *AMELX* and *AMELY* may occur leading to miss-determination of the human gender (Tozzo *et al*., 2013). Thus, simultaneous analyses of several additional genetic markers need to be considered. As an alternative, the gene *SRY*, located on Y chromosome (Yp11.3), is noteworthy. Due to the gene's specific location on the Y chromosome, the genetic marker is male specific and thus could serve as an attractive molecular tool for proper sex typing.

In this study, comparative analysis, including morphological characterization and molecular sex determination, of seven human skeletal remains excavated from the three largest 15^{th}–17^{th} century cemeteries in Latvia is presented. We employed a set of genetic markers, namely *AMELX/Y* and *SRY*, for sex determination by a widely known technique. Consequently, we obtained sex determination results corresponding to descriptive data from morphological analysis.

MATERIALS AND METHODS

Archaeological sites and morphological analysis of human skeletal remains. In this study, seven samples of human skeletal remains from the three largest medieval cemeteries in Latvia, dated 15^{th}–17^{th} centuries, AD, were available for sex estimation using morphological and molecular genetic analyses. Only cases meeting the criteria of good preservation of skeletons and full confidence in sex determination (i.e. adults) were included. Four individuals were from the St Gertrude Old Church cemetery (SGCC), two from the Dom Square cemetery (DSC), and one from the St Peter's Church cemetery (SPCC) (Table 1). For all individuals, a burial period could be obtained using stratigraphy and archaeological finds (Lūsēns, 2008; Actiņš *et al*., 2009; Spirģis, 2012). The age of each individual was established in order to inspect additional internal factors that might have influence on the preservation of skeletal remains, specifically, bone density and, consequently, preservation of aDNA (Waldron, 1987; Mays, 1992; Stojanowski *et al*., 2002). All individuals were assigned to five- or ten-year age categories according to degenerative changes in the pubic symphysis and the auricular surface by standard methods described previously (Lovejoy *et al*., 1985; Meindl *et al*., 1985; Brooks and Suchey, 1990; Buckberry and Chamberlain, 2002).

All individuals had well-preserved pelvic bones, which enabled sex estimation using a number of morphological traits. Skeletal remains Nos. 1, 2, 3, 4 and no. 7 had sufficiently preserved skulls, which were used to obtain additional morphological data.

Table 1

HUMAN SKELETAL REMAINS USED IN THIS STUDY

No. of the individual	Cemetery*, burial No.	Century, AD	Part of human skeleton used for molecular analysis
1	SGCC, 285	16–17	Ulna
2	SGCC, 489	16–17	Thoracic vertebra
3	SGCC, 496	16–17	Lumbar vertebra
4	SGCC, 587	16–17	Humerus
5	DSC, 139	16–17	Sacrum
6	DSC, 202	16–17	Thoracic vertebra
7	SPCC, 152	15–16	Rib

* SGCC, St Gertrude Church cemetery; DSC, Dom Square cemetery; SPCC, St Peter's Church cemetery.

Table 2

BONE FEATURES SCORED IN THE PELVIS AND THE SKULL FOR MORPHOLOGICAL SEX ESTIMATION FROM HUMAN SKELETAL REMAINS

Bone features	Scoring method	Reference
Pelvis:		
Subpubic concavity	Observation (present/absent)	Phenice, 1969
Ventral Arc	Observation (present/absent)	Phenice, 1969
Ischiopubic ramus ridge	Observation (present/absent)	Phenice, 1969
Composite arch	Observation (present/absent)	Genovés, 1959
Preauricular sulcus	Observation (present/absent)	Milner, 1992
Subpubic angle	Measurement of the angle	Phenice, 1969
Sciatic notch	Scoring of morphology (score 1–5)	Buikstra and Ubelaker, 1994
Skull:		
Glabella	Scoring of morphology (score 1–5)	Ascádi and Nemeskéri, 1970
Supraorbital margin	Scoring of morphology (score 1–5)	Ascádi and Nemeskéri, 1970
Mastoid process	Scoring of morphology (score 1–5)	Ascádi and Nemeskéri, 1970
Nuchal Crest	Scoring of morphology (score 1–5)	Ascádi and Nemeskéri, 1970

In total, seven features were scored in the pelvis, and four features in the skull for sex identification (Table 2). The features were chosen according to their accuracy and the preservation in the skeletal material.

Isolation of aDNA and PCR amplification. To eliminate contamination of modern DNA, the surface of the bones was cleaned with 5% sodium hypochlorite (NaOCl, Sigma, Germany) solution for 20 minutes, and rinsed with nuclease-free water (ThermoScientific, Lithuania). The bone was irradiated for two hours with ultra-violet (UV) light with 6 J/cm2 at 254 nm on each side before processing and left to dry overnight at room temperature. A part of the bone was cut out with a cutting disc for analysis and pulverised using a dentistry mill (Kavo, USA). All instruments and surfaces involved in the process were treated with NaOCl solution prior and after each procedure for decontamination. Only one bone at a time was processed and all operations involving bones were carried out in a room dedicated for this purpose only. All standard precautions like dedicated protective clothing and disposables were taken (Donoghue, 2008).

DNA was extracted from between 150 and 190 mg of bone powder using the GENECLEAN® Kit For Ancient DNA (MP Biomedicals, USA) according to the manufacture's protocol. The volumes of the resulting DNA aliquotes were measured, and DNA concentrations were estimated using a NanoDrop® (ND-1000) spectrophotometer. Simultaneously, in order to control contamination, blank samples were included in each aDNA isolation.

PCR primers specific for *AMELX/Y* and *SRY* genes were synthesised by Metabion (Germany) according to the sequences published previously as follows:

5′-CCCTGGGCTCTGTAAAGAATAGTG-3′ and 5′-ATCAGAGCTTAAACTGGGAAGCTG-3′ for *AMELX/Y* and 5`-ATAAGTATCGACTCGTCGGAA-3′ and 5′-GCACTTCGCTGCAGAGTACCGA-3′ for *SRY* (Santos et al., 1998; Mannucci et al., 1994). *Pfu* DNA polymerase (ThermoScientific, Lithuania) was chosen for *SRY* amplification, and *Taq* polymerase (ThermoScientific, Lithuania) — for *AMELX/Y* amplification. The PCR mixture for 12.5 µl of total reaction volume contained 1 x *Taq* buffer with $(NH_4)_2SO_4$ (ThermoScientific, Lithuania), 2.5 mM $MgCl_2$ (ThermoScientific, Lithuania), 10 mM dNTPs (ThermoScientific, Lithuania), 0.4 µM of forward and reverse *AMELX/Y* primers, 1.25 µl of *Taq* DNA polymerase (ThermoScientific, Lithuania) and 2 µl of bone DNA extract. Similarly, the PCR reaction mixture for 12.5 µl of total reaction volume contained 2 × *Pfu* buffer with Mg_2SO_4 (ThermoScientific, Lithuania), 10 mM dNTPs (ThermoScientific, Lithuania), 0.4 µM of forward and reverse *SRY* primers, 1.25 µl *Pfu* DNA polymerase (ThermoScientific, Lithuania) and 2 µl DNA extract. PCR conditions as follows: 94 °C — 5 min, then 45 cycles of 94 °C — 40 sec, 62 °C/58 °C — 40 sec (for *AMELX/Y* and SRY, respectively) and 72 °C — 1 min, followed by a final elongation step of 72 °C — 5 min. All reactions were carried out using a gradient Eppendorf PCR machine (Eppendorf, Germany).

PCR products (10 µl) were separated by 15% native polyacrylamide gel (PAAG) electrophoresis in 1 × TBE buffer for 3 h at 100 volts, 25 mA. The gel was stained with ethidium bromide (Sigma, Germany) for 20 min and visualised using an UV-wavelength transilluminator (UVP, USA).

The band with expected length was cut out from the gel and frozen, later crushed and dissolved in nuclease-free water (ThermoScientific, Lithuania) to extract PCR products. The extracts were then sequenced using an ABI Prism 310 Genetic Analyser (Applied Biosystems, USA) to verify the results. Sequences were analysed with software ContigExpress, a part of the Vector NTI computer program (Invitrogen, USA).

Modern DNA aliquotes (10 ng/µl) isolated from whole blood samples of ten individuals (five males and five females) with clearly expressed sex attributes were used as positive controls for verification and optimisation of PCR.

RESULTS

Morphological determination of sex. All human skeletal elements used for sex estimation in this study were available for individuals from No. 2 to No. 4 and No. 7 (Table 3). Despite this, the observed traits in all seven human skeletal remains were distinctly dimorphic, especially regarding the pelvic area (Fig. 1). The noticeable female traits were clearly present in the pelvic area of individuals No. 1 and No. 2, but absent in No. 3 to No. 7, thus providing evidence for sex determination. Moreover, score 1 (female) or 5 (male) were given for the particular traits in the skull and the pelvic area in most cases. In individual No. 1, score 2 (female, presumptive) was given after studying the mastoid process and the nuchal crest in the skull, which were more pronounced than expected in a female. In individual No. 2, an identical score was given for preauricular sulcus, which was present, but not very pronounced (Fig. 2, arrow 4).

Determination of sex by molecular genetic analyses. DNA extraction was successful in all seven samples involved in the study with detectable DNA yields (> 1.7 ng/µl). DNA was extracted in two batches for bones Nos. 1, 2, 3 and 4, resulting in comparable DNA concentrations for each studied sample (data not shown). However, as the contaminating DNA of bacterial or fungal origin usually represents the vast majority of the aDNA sample, concentration measurements were used for informative purposes only.

In order to optimise amplification reactions and prior to experiments of aDNA, PCR amplification of *AMELX/Y* and *SRY* gene fragments were performed with modern DNA samples. As was expected, in the case of *AMELX/Y* gene one PCR product was obtained for female (*AMELX*, 106 bp), and two PCR products for male (*AMELX* and *AMELY*, 106 and 112 bp, respectively) (Fig. 3). In contrast, a successful amplification of relatively short PCR products (93 bp) of *SRY* gene was observed only for males (data not shown).

Table 3

CHARACTERISATION OF HUMAN REMAINS BY MORPHOLOGICAL METHODS BASED ON TRAITS IN PELVIS AND SKULL

No. of the individual	Sex											Age (years)
	Pelvis*							Skull**				
	SC	SA	IRR	VA	CA	SN	PS	G	MP	NC	SM	
1	-	-	-	-	F	F	F	F	F?	F?	F	40–50
2	F	F	F	F	F	F	F?	F	F?	F	F	30–35
3	M	M	M	M	M	M	M	M	M	M	M	20–25
4	M	M	M	M	M	M	M	M	M	M	M	50–60
5	M	M	M	M	M	M	M	-	-	-	-	25–30
6	M	M	M	M	M	M	M	-	-	-	-	45–50
7	M	M	M	M	M	M	M	M	M	M	M	50–60

* SC, subpubic concavity; SA, subpubic angle; IRR, ischiopubic ramus ridge; VA, ventral arc; CA, composite arch; SN, sciatic notch; PS, preauricular sulcus; **G, glabella; MP, mastoid process; NC, nuchal crest; SM, supraorbital margin; F, female; M, male; -, not determined.

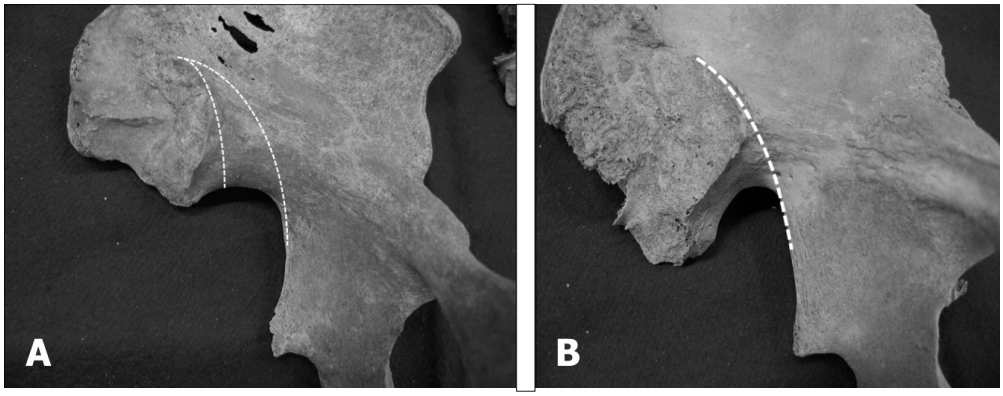

Fig. 1. The representation of the composite arch in pelvis. A, individual No. 2, the presence of the trait, female. B, individual No. 6, the absence of the trait, male.

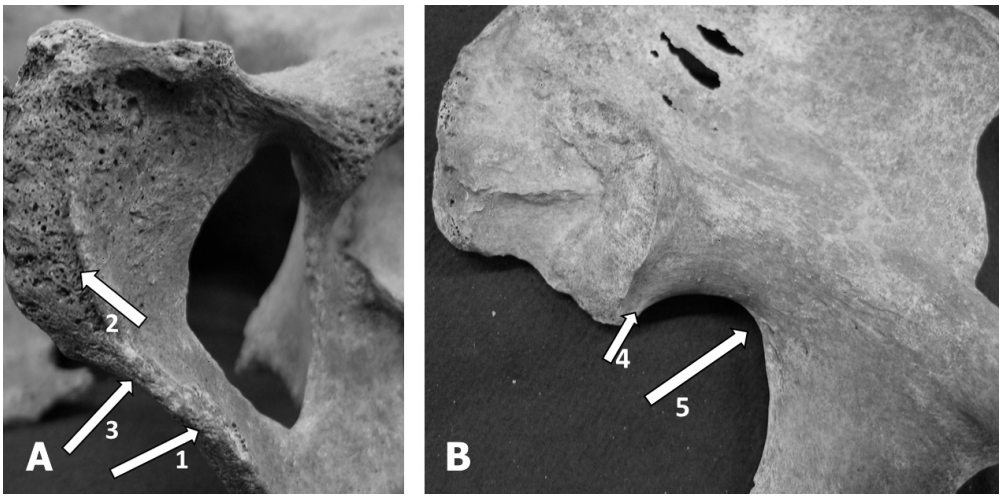

Fig. 2. The representation of the distinct female traits in pelvis. The left pelvis of individual No. 2 showing distinct female traits. A: 1, subpubic concavity, 2, ventral arc, 3, ischiopubic ramus ridge. B: 4, preauricular sulcus, 5, sciatic notch.

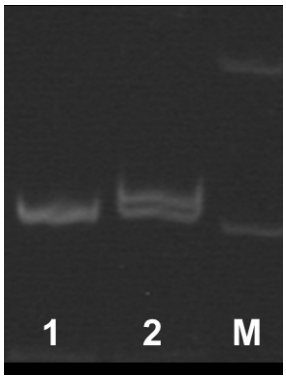

Fig. 3. Polyacrylamide gel (PAAG) electrophoresis pattern showing *AMELX/Y* PCR products. Lane 1, *AMELX* (106 bp) PCR product (female), lane 2, *AMELX* (106 bp) and *AMELY* (112 bp) PCR products (male), lane M, DNA size marker.

Further, amplification of both genetic markers for aDNA samples was performed. Interpretable results of molecular analyses were obtained for five bone samples (Table 4). Two samples appeared female-positive and one sample male-positive by both genetic markers, two samples were positive for *SRY* marker only. Two aDNA samples, one obtained from humerus (skeleton remain No. 4) and one obtained from rib (skeleton remain No. 7), failed to provide a specific PCR result. These samples were repeated with confirmed results, thus eliminating human or technical errors in the laboratory. All results were verified by sequencing analyses of the extracted PCR products from polyacrylamide gels and were concordant to PCR amplification data (data not shown).

Comparison between morphological and molecular genetic sex-typing. Genetic analyses for sex determination were applied to the samples without prior information about sex of the individuals. In five out of seven (71%) cases the results of the anthropological and molecular methods were in agreement (Table 4). In both remaining PCR methods-negative cases (individuals No. 4 and 7) the male gender was determined by anthropological estimation, as all bone elements were available for the assessment. Therefore, two categories of our results were observed: i) molecular genetic data completely confirmed morphological results, ii) molecular genetic data uncertain but is not inconsistent with descriptive and estimative anthropological results.

Table 4

SEX DETERMINATION IN HUMAN SKELETAL REMAINS

No. of the individual	Anthropological analysis*	Molecular genetic analysis**		
		Gene		Interpretation*
		AMELX/AMELY	*SRY*	
1	F	+/-	-	F
2	F	+/-	-	F
3	M	+/+	+	M
4	M	-	-	Uncertain
5	M	-	+	M
6	M	-	+	M
7	M	-	-	Uncertain

* F, female; M, male; ** +/-, one PCR product (*AMELX*); +/+, two PCR products (*AMELX* and *AMELY*, respectively); +, positive PCR; -, negative PCR.

DISCUSSION

This is the first aDNA study in Latvia describing the sex determination of human skeletal remains, excavated from Latvian 15^{th}–17^{th} century burials, by morphological and molecular genetic methods.

Sex data provides important information of the past burial practices, social status within the population, and differences in mortality rates between different age and sex groups. Thus, multiple measures, including aDNA approach, are taken to identify biological profiles. While recent advances in molecular genetics offer new approaches for sex identification, certain limitations arise from the nature of aged specimens. A number of specific measures discussed elsewhere (Grigorenko *et al*., 2009) need to be taken to prevent experimental errors, including, but not limited to contamination by modern DNA or cross-contamination, that might arise during either excavation or manipulation with skeletons. Furthermore, the presence of PCR inhibitors often co-purified with DNA complicates PCR and may lead to the fail. Several strategies in DNA preparation methods (Ye *et al*., 2004) are feasible in order to produce stable, reliable and robust results. In this study, we were able to obtain sufficient molecular data for the sex identification for five out of seven (71%) bone samples, two females and three males. These samples were excavated from St Gertrude Church cemetery and Dom Square cemetery, and all were dated to the 16^{th}–17^{th} century. Importantly, PCR-positive aDNA samples were isolated from different bones: ulna, thoracic vertebra, lumbar vertebra and sacrum, thus indicating that molecular analysis can be performed for different skeletal parts.

In many cases, low amounts of aDNA and the degraded nature of nucleic acid make molecular sex determination challenging. In the current study, PCR amplification failed in two samples. Although the yield of isolated aDNA was comparable in all samples, possible fragmentation of aDNA was not evaluated. Therefore, it could be hypothesised that DNA in PCR-negative samples were fragmented. This assumption is supported by the longest post mortem interval for one case, when skeletal remains were excavated from the oldest cemetery (St Peter's Church) in this study, dated in 15^{th}–16^{th} centuries. Also, both "uncertain" cases were assigned to 50–60 age at death category. The latter observation is corroborated by the fact that internal factors, such as age at death of individual, can have influence on aDNA preservation and are mainly attributed to degenerative diseases, such as osteoporosis, of ageing individuals (Brickley and Ives, 2008). These results could also be at least partially linked to several external factors relating to the nature of the specimens, variation in temperature, humidity or salt concentration of the soil. Furthermore, soil biota additionally influences the preservation of DNA content and its structural integrity (Willerslev *et al*., 2005; Grigorenko *et al*., 2009).

In order to improve studies with aDNA and achieve a strong PCR pattern in gel electrophoresis, primers, amplifying shorter *AMELX/Y* PCR amplicons (55/58 bp or 80/83 bp) (Bauer *et al*., 2013; Haas-Rochholz *et al*., 1997), or the pyrosequencing method that enables analysis of very short (44–45 bp) fragments and as small as 0.5 ng amount of template DNA (Li *et al*., 2012), can be considered for future studies. Such a methodological option is particularly applicable for the analysis of heavily degraded aDNA and is already in use by other researchers (Li *et al*., 2012; Bauer *et al*., 2013). Also, since more than two genetic markers are advisable for correct sex interpretation (Santos *et al*., 1998), more recent X-short tandem repeat (STR) markers DXS7424, DXS8378, DXS6803, GATA172D05, DXYS156 and steroid sulphatase (*STS*) gene can be alternatively considered (Bauer *et al*., 2013; Tozo *et al*., 2013). Evidently, simultaneous application of several target genes minimises the possibility of erroneous sex determination and becomes a powerful tool to unambiguously determine the sex, as a considerable number of regions on both gonosomes can be studied in parallel.

In conclusion, this study demonstrates that molecular genetic data in addition to anthropological results provide a powerful tool for sex determination from archeological osteomaterial and might serve as supplementary mean for archaeological and forensic applications, particularly in cases of incomplete skeletal preservation or in sexing juvenile skeletons. Application of at least two genetic markers should be considered in human profile identification when sex determination is impossible by morphological traits.

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15.–17. GADSIMTA LATVIJAS ANTROPOLOĢISKĀ MATERIĀLA CILVĒKU KAULU MORFOLOĢISKAIS RAKSTUROJUMS UN TO DZIMUMA MOLEKULĀRĀ NOTEIKŠANA

Dzimuma noteikšana ir viens no svarīgākajiem un primārajiem soļiem cilvēka profila identifikācijai arheoloģiskajos izrakumos iegūtā antropoloģiskā materiāla izpētē. Šī pētījuma mērķis bija molekulāro metožu pielietošanas iespēju izvērtēšana dzimuma noteikšanai antropoloģiskajā materiālā līdztekus morfoloģiskām metodēm. Pētījumā izmantots cilvēka kaulu materiāls, kas iegūts arheoloģiskajos izrakumos no 15.–17. gadsimta Sv. Ģertrūdes baznīcas, Doma laukuma un Sv. Pētera baznīcas apbedījumiem Rīgā, Latvijā. Morfoloģiskās un molekulārās ģenētikas metodes, ieskaitot *AMELX/Y* un *SRY* gēnu amplifikāciju, tika pielietotas septiņu indivīdu skeletu kaulu analīzē. Pēc morfoloģiskajām pazīmēm dzimums tika noteikts visos septiņos gadījumos (divas sievietes un pieci vīrieši). Ar DNS molekulāro analīzi dzimumu izdevās noteikt piecos paraugos no septiņiem (71%). Visos pozitīvajos gadījumos tika novērota pilnīga sakritība starp morfoloģisko un molekulārās ģenētikas metožu rezultātiem. Secinājumi: arheoloģiskajos izrakumos iegūtā antropoloģiskajā materiālā DNS analīzes pielietošana dzimuma noteikšanā ir apsverama gadījumos, kad nevar izmantot morfoloģiskās metodes (nepieauguši indivīdi; fragmentārs kaulu materiāls).