

Sex determination of medieval skeletal remains: evaluation of anthropological, odontological and genetic methods

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Abstract

During 2019, 28 grave constructions were excavated at cemetery Metaljica (Hadzici municipality, Bosnia and Herzegovina). Archaeological excavation has revealed 17 well preserved skeletons that were subjected to anthropological, odontological and genetic analyses. To determine sex by genetic analysis, amelogenin and DYS391 loci were taken into consideration. Concordance between results of anthropological, odontological and genetic analyses applied in this research occurred in one sample out of 17. In four samples, sex was determined only by genetic analysis, since sexual dimorphism indicators were not preserved. Concordance between odontological and genetic determinations was observed in two samples, while affirmative results of anthropological and genetic analysis were obtained in only one sample. Discrepancy in the majority of samples can be attributed to the state of preservation of skeletal remains, interpopulation differences and allele drop-out. Moreover, anthropological and odontological analyses were not applicable to juvenile/subadult skeletons, since sexually dimorphic characteristics relevant for metric and morphological analysis are not developed well at that developmental period. This study emphasizes the importance of combining anthropological, odontological and genetic methods in order to determine sex of archaeological skeletal remains accurately.

Introduction

Alongside age determination, sex determination is an important element within the forensic and bioarchaeological analysis of human archaeological skeletal remains (Tekeli *et al.*, 2020; Navitainuck *et al.*, 2021). According to The Cambridge Dictionary of Human Biology and Evolution by Mai *et al.* (2005), sex represents “biological category based upon

reproductive attributes and roles in sexually reproducing species”. Traditional anthropological and odontological approach of sex determination is based on sexual dimorphism, which refers to differences in metric and morphological properties of skeletal remains and teeth between males and females (Banerjee *et al.*, 2016; Horbaly *et al.*, 2019). In general, differences between males and females reflect in size and shape of bones, where male bones are larger and

more robust in comparison with smaller and gracile female bones. The most commonly used bones with traits reliable for sex estimation are postcranial and pelvic bones (Spradley, 2016). A study conducted by Navitainuck *et al.* (2021) showed that morphological traits of the cranium and pelvis are at an advantage compared with metric traits of pelvis and scapula, due to their higher utility in the purpose of sex estimation. The other research conducted by Inskip *et al.* (2018), focused on *os coxae* and skull traits, revealed their high sex estimate accuracy, with an accent on *os coxae* or pelvic traits. In comparison with non-metric, odontometric approach is more accurate and based on the differences of tooth size and proportions, where the linear measurements like buccolingual (BL) and mesiodistal (MD) dimensions of teeth crowns are the most commonly used for sex determination (Joseph *et al.*, 2013). If the skeletons are damaged or incomplete, which is common in archaeological skeletal remains, sex assessment by morphological analysis is limited or impossible. In that case, metric methods are considered more appropriate for assessing sex. However, sex assessment equations should be used only when the sample is known to come from the same population from which the functions were derived since the expression of sexual dimorphism is population specific (Chovalopoulou *et al.*, 2018). Therefore, formulae derived from one population group will not be applicable for another group due to differences in sexual size dimorphism (Spradley, 2016). Thus, analysis of genetic markers is a powerful solution for sex assessment when anthropological or odontological analysis is not efficient, especially in cases of non-adult or juvenile skeletal remains, due to the fact that sexual dimorphism development depends on the age of individual (Vaňharová & Drozdová, 2008). Two sex specific genetic markers commonly used for sex determination are amelogenin and SRY (sex determining region Y). Amelogenin exists on both the X and Y chromosome and provides information for the synthesis of amelogenin, which is an essential protein for normal tooth development, while SRY gene occurs on the Y chromosome and codes for transcription factors crucial for development of male specific characteristics (Primorac *et al.*, 2014; Stewart *et al.*, 2017). An aggravating factor for successful extraction

and analysis of aDNA (ancient DNA) from archaeological or recent bones and dental material can be degradation of DNA conditioned by low amount of starting DNA and the presence of polymerase chain reaction (PCR) inhibitors in the soil and environmental factors like soil type, soil pH, temperature, humidity and the presence of microorganisms (Jakubowska *et al.*, 2012; Higgins & Austin, 2013). Laboratory procedures, equipment and experience can contribute to the successfulness of DNA analysis (Siriboonpiputtana *et al.*, 2018). Furthermore, soil pH and type, bone type and size as well as age and sex of individuals can affect survival of bone (Manifold, 2012). Also, biological, chemical and physical agents can lead to morphological changes of bone material (White, 2005). The aim of this research was to determine the sex of 17 skeletons excavated from medieval cemetery Metaljica (Hadzici municipality, Bosnia and Herzegovina) applying anthropological, odontological and genetic analyses

Materials and methods

Samples

In 2019, archaeological excavations within multidisciplinary project “Genetic characteristics of inhabitants of Medieval Bosnia” (No. of project 11/05-14-27684/19) revealed large medieval cemetery at the Metaljica locality near Tarcin (Hadzici municipality, Bosnia and Herzegovina). In 28 excavated graves, 21 human skeletal remains were found, among which 17 skeletons were well preserved for anthropological, odontological and genetic analysis.

Anthropological analysis

Before primary anthropological analysis was carried out, human skeletal remains had been carefully washed in order to preserve skeletal remains (Fig. 1, Fig. 2, Fig. 3). After the process of washing, osteological material was left on paper towels to dry. Drying lasted about seven days. During the entire process of examination (washing, drying, primary anthropological analysis and storage) regulations on temperature and moisture were strictly followed. Temperature ranged from 18 to 21°C, while the humidity was about 50% and not more than 70% (Gob & Drouguet, 2007). Dried osteological material was laid out on the examination table in



Figure 1: Nuchal crest of individual from grave 5



Figure 2: Mandible of individual from grave 18



Figure 3: Fragmented sciatic notch of right innominate

anatomical position in order to build biological profile for each individual. It is necessary to point out that full biological profile was built for none of individuals, due to high fragmentation of human skeletal remains. During anthropological analysis, anthroposcopy method was applied for sex determination. On the other hand, metric methods for sex estimation could not be applied since such formulae have not been even

derived for medieval Bosnian population. These formulae are going to be produced when there will be a large enough (and otherwise representative) sample of medieval Bosnian population available for study. Since sexual dimorphism is mostly manifested on pelvis and cranium, these two regions of skeleton were particularly the subject of analysis. Analysis was conducted following already established standards for sex estimation (Buikstra & Ubelaker, 1994). Degree of preservation for the most of the samples was in the range of 10 to 20%, with the exceptions of the grave 10 (1%) and the grave 17 (9%). The highest degree of preservation of skeletal remains was determined in the grave 26 (40%) and the grave 2 (55%). In the case of the samples from graves 4, 8 and 16, fragmented skeletal material or insufficient amount of skeletal material for anthropological analysis was collected.

Odontological analysis

All samples were previously washed, cleaned from remnants of dirt and photographed. For some samples parts of the jaws were present with several teeth (Fig. 4), while in other samples only teeth were recovered (Fig. 5). Condition of material was also different, from well preserved to poorly preserved. Out of 17 individuals recovered, in 4 samples no teeth and/or no jaws were recovered, so there were classified as "Unidentified" by odontological analysis. Out of remaining 13 individuals, two were children with mixed dentition. Even though deciduous teeth were measured as well, for this analysis only measurements of



Figure 4: Well preserved sample with two parts of maxilla and teeth



Figure 5: Sample where only teeth were recovered

permanent teeth were included. Number of teeth and type of teeth was different in different samples. Total number of permanent teeth analyzed was 50. Teeth measurements were done with veneer caliper, performed by single investigator. Crown measurements included mesiodistal (MD) and buccolingual (BL) diameter as recommended by other researchers (Vodanovic, 2007). Additionally, mesiodistal crown width at cervical level was measured as well. Cervicoocclusal diameter was also noted, but excluded in all cases with severe abrasion to avoid false results. Length of the root and length of the whole tooth were also measured. In samples where parts of jaws were present, additional measurements were taken, such as height of mandible at level of foramen mentale, intercanine distance, etc. During odontological analysis, the results of anthropological analysis were unknown to the researcher. Sex estimation was done by comparison to the average tooth dimensions of contemporary population (Konjhodzic Rasic, 1978).

Genetic analysis

Teeth samples were used for DNA analysis. Prior to analysis samples were soaked in 5% w/v Na-hypochlorite for 10 minutes, then rinsed three times with distilled water and soaked in absolute ethanol (Sigma Aldrich, USA) for five minutes. Washed teeth samples were transferred to a clean paper towel and dried for five days. After drying, all samples were irradiated by UV light for three minutes and grounded to a powder using sterilized IKA Tube mill (IKA®-Werke

GmbH&Co.KG, Germany). Approximately one gram of powder of each sample was placed in sterile 50 mL polypropylene tube. DNA extraction was performed in a laboratory hood dedicated to ancient DNA work, according to an optimized phenol-chloroform-isoamyl alcohol DNA extraction protocol, preceding decalcification with 0.5 M EDTA (Sigma Aldrich, USA) solution during seven days. In order to detect possible contamination during extraction process, negative extraction control was included. Laboratory hood, work surfaces and laboratory equipment were cleaned with Na-hypochlorite and 70% ethanol, and irradiated by UV light. DNA extracts were purified with DNA-free water using Amicon Ultra 0.5 mL centrifugal filter units (Merck, Millipore, Carrigtwohill, Co.Cork, IRL), transferred into 1.5 mL Eppendorf tubes and stored at -80°C. DNA amplification was performed using Investigator® 24plex QS Kit (Qiagen, Hilden, Germany) which includes 23 autosomal STR loci (amelogenin, TH01, D3S1358, vWA, D21S11, TPOX, DYS391, D1S1656, D12S391, SE33, D10S1248, D22S1045, D19S433, D8S1179, D2S1338, D2S441, D18S51, FGA, D16S539, CSF1PO, D13S317, D5S818, D7S820) in GeneAmp™ PCR System 9700 (Applied Biosystems, USA). To check for possible contamination, for all PCR analyses was used PCR negative control. Products of amplification were detected and separated by capillary electrophoresis using 3500 Genetic Analyzer (Applied Biosystems, USA). Data were collected using 3500 Series Data Collection Software and analysed by GeneMarker® HID Software (Soft Genetics, USA). To determine sex, amelogenin and DYS391 loci were taken into consideration.

Results and Discussion

Seventeen out of 28 skeletal remains excavated from medieval cemetery Metaljica near Tarcin (Hadzici municipality, Bosnia and Herzegovina) were included in this research and investigated by three different methods of determining sex (Table 1). None of individuals had all anthropological indicators of sexual dimorphism preserved. For two individuals from graves 6 and 27 sex was not estimated by using anthropological analysis, since the individuals were subadults. There are no standards for sex diagnosing in juvenile/subadult materials considered acceptable by most osteologists (Buikstra & Ubelaker, 1994). For two

Table 1. Results of sex assessment by anthropological, odontological and genetic analysis conducted on skeletal remains from medieval necropolis Metaljica

Sample	Anthropological sex determination	Level of confidence (1-4) ^a	Odonatological sex determination	Level of confidence (1-4) ^b	Genetic sex determination		Level of confidence (1-4) ^c
					Amelogenin	DYS391	
Grave 1	Male	2	Male	2	Unidentified		1
Grave 2	Female	2	Female	2	X Y	Y	2
Grave 3	Unidentified	1	Male	3	X Y	Y	4
Grave 4	-*	-	Unidentified	1	X Y	Y	3
Grave 5	Male	2	Female	2	X Y	Y	4
Grave 6	Unidentified	1	Male	2	X Y	-	3
Grave 7	Male	2	Female	3	X	-	3
Grave 8	-*	-	Unidentified	1	X Y	Y	3
Grave 11	Female	2	Female	2	X Y	Y	3
Grave 15	Male	2	Male	3	Unidentified		1
Grave 16	-*	-	Unidentified	1	X Y	Y	4
Grave 17	Female	2	Female	3	X	Y	3
Grave 18	Male	2	Male	4	X Y	Y	4
Grave 19	Female	3	Male	4	X Y	Y	4
Grave 23	Unidentified	1	Unidentified	1	X	-	3
Grave 26	Unidentified	1	Male	3	Unidentified		1
Grave 27	Unidentified	1	Male	2	X	-	3

*Fragmented skeletal material or insufficient amount of skeletal material with expressed sexual dimorphism for anthropological analysis

^aScale of confidence on the anthropological sex determination: 1- it was not possible to determine sex, 2- sex determination is based on insufficient number of indicators of sexual dimorphism, 3- sex determination is based on more than half indicators of sexual dimorphism, 4- there were present all anthropological indicators of sexual dimorphism

^bScale of confidence on the odontological sex determination: 1- it was not possible to determine the sex, 2- sex determination is based on 1-3 teeth, 3- sex determination is based on 4-8 teeth present, 4- there were more than 8 teeth present and/or additional anthropometric analysis of jaws was possible

^cScale of confidence on the genetic sex determination: 1- it was not possible to generate DNA profile, 2- partial DNA profile with less than half STR loci amplified, 3- partial DNA profile with more than half STR loci amplified, 4- full DNA profile

individuals (graves 3 and 26) sex could not be determined at all, since indicators for sexual dimorphism have not been preserved. Six individuals had only one indicator of sexual dimorphism while individual from grave 19 had the highest number of preserved indicators of sexual dimorphism: nuchal crest, supra-orbital margin, glabella, mental eminence and sciatic notch. The pelvis is not preserved, but only incomplete iliac bones with partially preserved sciatic notches. It is necessary to emphasize that skeletal remains were highly fragmented and that none of skeletal sets had complete pelvic bones, therefore, features such as ventral arc, the subpubic concavity, the ischiopubic ramus ridge and preauricular sulcus (in

female skeletons) were absent. Regarding the sex estimation based on odontological analysis, in our research, the odontometrics was used but the estimation itself was done by comparison to teeth dimensions of contemporary population, therefore certain inaccuracy was expected. Most of the recent researches on odontometric sex estimation worldwide are based on teeth of contemporary populations, therefore certain inaccuracy was expected (Yapes, 2019; Viciano, 2020; Kanchan 2021). For samples from graves 4, 8, 16 and 23 odontological analysis could not be performed because no dental material was available. For samples from graves 18 and 19 sex was estimated with high level of confidence by using odontological

analysis while for samples 3, 7, 5 and 17 determined sex was confirmed with DNA analysis. In general, archaeological samples are too small to represent the base for population odontometric standards. Other researches recommend usage of other dental characteristics (Kazazi, 2018), non-metric dental traits, and/or dental arch dimensions, jaw dimensions, dental indices of specific teeth (Żądzińska, 1999; Gupta, 2016; Kanchan, 2021), which might be impossible to perform in fragmented and poorly preserved archaeological samples. Severe abrasion, usually seen in teeth of archaeological origin, limits the possibility to use all teeth dimensions. Other, non-metric dental traits can be used to establish differences between populations, therefore if noted can help in answering the question of geographic origin of the samples (Vankatesh, 2019; Zukic, 2020).

For samples from graves 1, 15 and 26, genetic analysis could not be done since no markers at amelogenin or DYS391 locus were amplified probably due to the small quantities and degraded DNA molecules present in archeological samples (Dzehverovic et al., 2020). For one sample (grave 6) X and Y alleles were amplified, without amplification at DYS391 locus, while for sample from grave 17 X allele was amplified with observed amplification at DYS391 locus. For those two samples, male sex was established, despite "partial" amplification. Failure to amplify DNA at both loci as well as absence of "complete" amplification (XY at amelogenin and amplified DYS391) in both cases can be explained by the fact that genetic analysis of skeletal remains faces with significant issues such as low amount and quality of aDNA (ancient DNA), presence of contamination and PCR inhibitors as well as small amount of starting material (Quincey *et al.*, 2013). Discrepancy in sex determination between three methods applied in this research can be attributed to highly fragmented skeletal remains, interpopulation differences and allele drop-out (Eliášová and Kubálek, 2009; Bauer, 2013). Also, the fact that juvenile/subadult skeletons do not have developed secondary characteristics relevant for metric and morphological analysis explains non-concordance between results of these analyses and genetic analysis. It is important to have in mind that each of used methods have

limitations which are mainly related to sample preservation which is very small in case of archeological samples. It is also necessary to have in mind that incorrect sex identifications can be made because of variation among populations. Some populations are composed of larger and more robust individuals, both sexes, while some populations are much smaller and gracile. Because of such interpopulation differences, sex assessment could be mistaken. Furthermore, all of the morphological techniques used in sexing skeletal remains depend on the preservation of sexually dimorphic elements. All of them share a nontrivial error rate, even for adult remains. However, if aDNA can be recovered from osseous remains, the sex of any individual (regardless of individual age) can be determined with high precision (White & Folkens, 2005). On the other hand, results of DNA analysis should be interpreted very carefully when it comes to aDNA since small amounts of DNA can give inconclusive results due to the presence of different amplification artifacts (allele drop out, null allele, increased stutter peak, etc.) (Harder *et al.*, 2012; Butler, 2015). Therefore, for sex determination for ancient samples it should be imperative to conduct as many available analyses as possible.

Conclusion

Through an evaluation of anthropological, odontological and genetic data, our research demonstrates that combining these three methods contributes to the accurate sex determination of archaeological skeletal remains. Also, in situations involving poorly preserved or highly fragmented skeletal remains as well as juvenile or subadult skeletal remains without expressed sexually dimorphic characteristics relevant for anthropological and odontological analyses, genetic analysis is efficient solution for sex determination of archaeological skeletal samples.

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