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ACCURATE SEX DETERMINATION USING ANCIENT DNA ANALYSIS FOR HUMAN SKELETAL REMAINS FROM DIFFERENT HISTORICAL ARCHEOLOGICAL SITES IN TURKEY

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ABSTRACT

The extraction of DNA from archaeological human skeletal remains provides valuable data about past societies for anthropologists, archaeologists and palaeontologists. Traditionally, anthropological methods are limited if sex determination of infant, juvenile or fragmented human remains are examined. However, studies on ancient DNA from human remains are best way for sex determination. In represent study, two DNA extraction protocols (phenol chloroform/commercial kit) were performed. A total of 37 samples taken from the 27 skeletons were exposed to different environmental conditions obtained from 8 different archaeological sites across Anatolia region of Turkey. Contamination precaution was applied as per preservation of the ancient samples. PCR was done using X-STR kit from DNA samples and the success rates of DNA extraction methods following amplification were completed. After the ancient DNA analysis, morphological results were compared to PCR-based amplification results in terms of sex determination. Due to the good preservation of ancient samples and methods of DNA extraction and amplification performed, 25 of the 37 ancient samples (67.5%) could be amplified successfully. When anthropological sex determination was compared to molecular analysis, it was seen that 85% of the results were consistent. In this study, femoral and petrous bones had better results than the teeth samples. Based on the results of this study, we can say that at least two samples should be taken from each individual for ancient DNA studies to confirm molecular results. Molecular sex determination will be useful fragmentary human remains when it impossible, to determine an individual's sex from morphological features.

KEYWORDS: Sex determination, degraded DNA, Anatolia, X-STR, bones and teeth

1. INTRODUCTION

Sex determination of archaeological human remains is essential, and basic anthropological characteristics play a role in the demographic structure of the past societies. Sex has been traditionally determined by bone morphometric and morphological analysis of skull and pelvis in adults Sex determination from archaeological evidence depends on the inference from grave materials as well (Nikita, 2017).

However, anthropological methods are not always possible to determine the sex. If fragmentary and damaged skeletal, or the pelvis and skull have been lost or sexually dimorphic characters are not presented on the bones, sex determination is often impossible (Faerman, 1998; Malaver and Yunis, 2003; Ubelaker, 2008). In juvenile skeletal remains, morphological sex assessment is unreliable if sexual dimorphism are not fully developed. Fragmentary human skeletal remains of the unknown sex is a serious concern in demographic studies of past societies because of the inaccuracy of the results. In case of mass burials, it is difficult to determine the gender because more than one person is buried side by side in the same grave. Genetic studies have been considered a powerful tool to investigate the human past (Evison, 2014). Thus, molecular analysis can be an alternative to anthropological methods for the accurate determination of sex (Baberova et al., 2012).

In ancient DNA studies, it is necessary to determine the sex of human remains in order to define the variations of migration pathways, particularly in genetic diseases, and in the geographical areas where ancient societies lived (Singh and Garg, 2014). Sex can be determined from human skeletal remains using ancient DNA (aDNA) analysis. Highly degraded DNA can be amplified from skeletal remains by polymerase chain reaction (PCR) (Saiki et al., 1988). One of the most common DNA-based sex determination of human skeletal remains is amelogenin gene amplification (Faerman, 1998). Amelogenin is a protein that plays an important role in the development of tooth enamel in mammals and is located in X and Y-chromosomes. Amelogenin primers used for sex determination are amplified different base pair (bp). The X-Y homologus amelogenin gens (AMEL X and AMEL Y) are amplifiable at 106 and 112 bp (Faerman et al., 1998). Sex determination of ancient human skeletal remains was determined by different lengths of base pairs as male and female. When separated by electrophoresis, the single band at 106 bp. indicates that the sample is female, the two bands at 112 bp. indicate that the sample is male (Sullivan et al. 1993). However, in ancient DNA studies, allele dropout may occur during amplification because of the low amount of degraded aDNA and that leads to in inappropriate results (Kimpton et al., 1994; Schmerer, 2001; Kashyap et al., 2006; Schmidt et al., 2003).

Short Tandem Repeats (STRs) is a genetic marker for the identification of unknown human skeletal remains, and is a valuable source for anthropological studies (Parsons, 2007; Hasan et al., 2014). Genetic markers are commonly being used, due to the small size and their contribution in detection of sample contamination (Bodowle et al., 1996; Zietkiewicz et al., 2012). Considering that the quality and quantity of DNA is associated with allele drop, it is very important for aDNA studies to obtain the best quality DNA for successful genotyping with the STR technique (Kimpton et al., 1994; Ivanov et al., 1999; Ambers et al., 2016). Genetic markers are their small size and because they allow detection of sample contamination, commonly use ancient DNA studies (Keyser-Tracqui et al., 2003).

The aim of this study is to demonstrate the application of ancient DNA analysis for sex determination from human skeletal remains when determination through anthropological methods is limited and not possible. Authentic DNA is obtained from bones and teeth using two different DNA extraction methods from 8 archaeological sites in Anatolia.

2. MATERIAL AND METHOD

Due to the high amount of damage to the ancient DNA, and the poor preservation the extraction process is difficult (Liritzis et al., 2020). DNA was extracted from ancient samples using two different DNA extraction methods. A total of 37 samples were taken from the 27 human skeletons excavated from 8 different archaeological sites of different climatic condition and different historical periods in Anatolia (Fig 1). The archaeological site Giresun Island is situated in the north of the Turkey. The average temperature outside the stone graves in the hottest month is about 23 °C and in the coldest month, 6°C. The average yearly precipitation is 133 mm. The degree of humidity and the amount of humic acids was not determined. However, due to the warm climate and humidity, the amount of humic acid in the soil is high (İncekara, 2017). The archaeological site The Van Castle Mound is located in the east of the Turkey. The average temperature in the hottest month is about 22°C and 3,6 °C in the coldest month. The average yearly precipitation is 59 mm. due to the continental climate the amount of humic acid and humidity is low. The specimens obtained from Nysa Ancient City, Beybag/Mugla and The Teos Ancient City are situated west of the Turkey and has same diagenetic environments.

The average temperature in the hottest month is about 34°C and the 14 °C in coldest month. The av-

erage yearly precipitation is lower than other regions that leads lower amount of humic acid in the soil. Using morphological methods, 8 females and 13 males were identified. Five human skeletal remains and a child skeletal remained undetermined. The type of ancient samples and their historical period with archaeological sites are shown on Table 1.

Table 1. Knowledges about the ancient samples used in the study

Sample Number	Archaeological Site	Year of excavation	Type of samples	Period of samples
1	The Van Castle Mound	2014	Femoral	The Middle Age
2	The Van Castle Mound	2014	Femoral	The Middle Age
3	Teos Ancient City/İzmir	2014	Femoral	HellenisticRome
4	Teos Ancient City/İzmir	2014	Femoral	HellenisticRome
5	Van Kalecik/Ablagens	2014	Teeth (Molar)	Early Iron Age
6	Van Kalecik/Ablagens	2014	Teeth (Molar)	Early Iron Age
7	Van Kalecik/Ablagens	2014	Teeth (Premolar)	Early Iron Age
8	Van Kalecik/ Catak	2007	Teeth (Molar)	Early Iron Age
9	Van Kalecik/Catak	2007	Teeth (Molar)	Early Iron Age
10	Van Kalecik/Catak	2007	Teeth (Molar)	Early Iron Age
11	Van Kalecik/Kalecik	2007	Teeth (Molar)	Early Iron Age
12	Giresun Island	2011	Femoral Teeth (Molar)	The Middle Byzantine
13	Giresun Island	2011	Femoral Teeth (Molar)	The Middle Byzantine
14	Giresun Island	2011	Teeth (Premolar)	The Middle Byzantine
15	Giresun Island	2011	Teeth (Molar)	The Middle Byzantine
16	Giresun Island	2011	Teeth (Premolar)	The Middle Byzantine
17	Nysa Ancient City/Aydın	2015	Petrous bone Teeth (Incisor)	Byzantine
18	Nysa Ancient City/Aydın	2015	Petrous bone Petrous bone Teeth (Molar)	Byzantine
19	Nysa Ancient City/Aydın	2015	Teeth (Molar) Petrous bone	Byzantine
20	Nysa Ancient City/Aydın	2015	Fibula Femoral	Byzantine
21	Giresun Island	2012	Teeth (Premolar)	The Middle Byzantine
22	Beybag /Mugla	2008	Teeth (Molar)	The Late Byzantine
23	Giresun Island	2012	Teeth (Canine)	The Late Byzantine
24	Giresun Island	2012	Teeth (Incisor)	The Late Byzantine
25	Beybag/ Mugla	2008	Petrous bone Teeth (Molar) Femoral	The Late Byzantine
26	Giresun Island	2012	Femoral Teeth (Molar)	The Middle Byzantine
27	Giresun Island	2012	Femoral	The Middle Byzantine

Fig.1. A map of Turkey showing archaeological excavation sites

2.1. ARCHAEOLOGICAL SITES OF THE SAMPLES

10 femoral bone and 9 teeth samples were used from 11 human skeletons for ancient DNA extractions excavated from Giresun Island, which is located in the north of Turkey. The excavated ancient samples were dated from 2011-12 to the Middle Byzantine period (Doksanaltı et al., 2011). During the Roman Empire, the ancient name of Giresun Island was Aretias-Khalkeritis. Giresun-Aretias/Khalkeritis island was the only settlement in Eastern Pontus Region. As it was located at a strategic point along the East Black Sea and Caucasus route and it had a small harbor and anchorage area, it was inhabited from the Archaic Period to Late Antiquity and Late Middle Ages (Doksanaltı and Ekici, 2017).

Sampling was made on 5 petrous, 1 femur and 1 fibula bones along with 3 teeth from 4 skeletons excavated from Nysa Ancient City, which is located in the west of Turkey. It was established in B.C. 3rd century in the Hellenistic Period and was located in the direction of important transportation and trade roads that were densely used in ancient times. The information about Nysa was learned firstly from Strabon's narrations from ancient times. It was determined with the excavation which was conducted in the city that the city life continued through the periods of Rome, Late Rome and Byzantine up to A.D. 13-14th centuries although in the latter periods, it lost its function and was used as a reservoir (Strabon, 2005).

2 femoral bones of different individuals from Teos Ancient City were analyzed. This archeological site is one of the 12 ancient cities of Ionia, which was founded on a small peninsula in İzmir, and it was a port city in the Roman-Hellenistic period. There are ports in the north and south of this ancient city. Today, not much remains from the northern port. However, the southern harbor is well preserved and the remains of the artifacts date back to the Roman period (Kadıoğlu, 2018).

Beybağ is located between the ancient cities of Lagina and Stratonikeia in Muğla. Beybag was used as a settlement from mid-10th century to the beginning of the 13th century (Arıhan et al., 2017). 4 ancient bones of 2 skeletons excavated in 2008 from this site were sampled.

The Ancient City of Van and the Castle Mound is located in Van province in eastern Turkey. The capital of the Urartian State, which was ruled by a kingdom between the 9th and 6th centuries BC, is known as the Van Fortress (Tuspa) (Konyar et al., 2014). From there, femoral bones of the two skeletons dated to the Middle Age (early period) were sampled.

The necropolis of Van Kalecik is located in an area of stela to the north of the Van Castle, which was the capital of the Urartian State (Yılmaz et al., 2008). The sampled dental tissue found in the archeological excavations carried out in 2007, was dated to the Early Iron Age.

The Necropolis of Van Ablagens, dated to the Early Iron Age is located in the north of Van (Yılmaz, 2016). Three dental tissues of different individuals obtained from this archeological site in 2014 were sampled.

Catak is a settlement in Van, and an Early Iron Age settlement (Yılmaz et al., 2014). Three sampled dental tissues from different individuals used for DNA extractions were excavated from this archeological site in 2007.

2.2. Samples

Following the archeological excavations, the skeletal remains were stored at room temperature in carton boxes. However, skeletal remains which were obtained from Nysa Ancient City were kept at cool temperatures and dried until DNA analysis since a genetic analysis would be done. Totally 37 samples including femoral (n=10), fibula (n=1), and petrous bones (n=5) as well as teeth (n=21) were chosen for molecular genetic investigations, as those were the most promising ones amongst the available tissues according to the literature (Prinz et al., 2007; Damgaard et al., 2015; Higgins et al., 2015; Lazaridis et al., 2016). Prior to conducting our chosen DNA sexing methods on the human skeletal remains, most of the materials from Anatolia collection were known as morphological sex.

2.2.1. Choice of samples

It is very difficult to know and estimate the preservation of DNA by macroscopic examinations from ancient bones. Considering that the quality of the DNA in the ancient specimens depends on the site conditions of the archeological excavation, the age of the studied sample also partially affects the quality of the DNA. A number of studies that support the reliability of femoral, petrous bone and teeth are mostly the only sources of DNA for molecular analysis (Alakoç, 2009; Adler, 2011; Higgins, 2013; Pinhasi et al., 2015; Geigl and Grange, 2018). Contamination is the potential risk for genetic analysis while working with ancient human remains of DNA. As selecting ancient samples from 8 different excavation areas, some criteria were taken into consideration. Morphologically well-preserved long cortical bones, especially femoral and petrous bone without many cracks, were preferred. Intact and disease-free ones from most diseased teeth (preferentially molars) were selected for ancient DNA analysis. Burnt or heated teeth were not included in DNA analysis.

2.3. DNA EXTRACTION

2.3.1. Precautions for contamination risk

Contamination is always a potential risk while working with human DNA taken from skeletal remains. It is essential to minimize contamination of the bones by modern DNA. Several precautionary measures were applied to prevent contamination in this study.

1. As it was priorly known that DNA analysis would be performed on the samples taken from Nysa Ancient City and The Van Castle Mound, gloves and face masks were used during the excavation. Selected bones were put into a sealed, clean plastic bag to prevent cross-contamination.
2. After each sampling, and before all the steps, the working area was cleaned by concentrated bleach, and then, cleaned with water, then exposed to UV light.
3. All instruments were bleached, and plastic ware and solutions were exposed to UV light.
4. Sample Pre-treatment, DNA extraction, quantitation, amplification, and data analysis were performed in separated laboratory areas.
5. The gloves were changed while handling each sample. In every step, disposable gloves, face masks, and bonnets were used.
6. The porcelain mortar to be used in the pulverization step was sterilized.
7. Filtered tips, sterile tubes, and falcon tubes were sterilized along with their pocket.
8. Non-disposable dental tools and instruments which were used for DNA extraction and PCR were also sterilized.

2.3.2. Physical and Chemical Sample Pretreatment

The mechanical and chemical processing of the samples was performed using the combination of Watt (2005) procedures. For the petrous bone and the long cortical bones; the soil was firstly removed by a scalpel blade, and then, cleaned with a soft brush and a mill to remove the outer layers and surface contamination. After the surface of bones was cleaned with Mikrozid® AF, sterilization agent, and the samples were dried in a closed laminar flow cabinet and exposed to UV light (245 nm) for 5 minutes on both sides. When the physical pre-treatment was completed, chemical processing was performed. At this stage, the SDS solution with distilled water was prepared by using Rohland and Hofreiter (2007). Petrous bone and long bone diaphyses were cleaned with % 10 SDS solution using disposable toothbrush; afterward, UV was irradiated for 5 min on both sides. Physical Pre-treatment was not performed for teeth samples. The external surfaces of the teeth samples were cleaned with a soft toothbrush which had been washed with %10 SDS, and purified water

(DNA/RNA free). Then, each sample was exposed to UV light (245 nm) for 5 min on both sides.

2.3.3. Bone Powdering (Pulverization)

The fibula and femoral bones were cut into small pieces from the diaphyses small pieces (0,5 cm-1 cm in diameter) by hacksaw. The petrous bone was completely powdered; it was not cut into pieces. The tip of the hacksaw was changed for each sample and before this process; tips were exposed to UV light for 10 minutes. Bone pieces were sterilized with Mikrozid® AF, after the samples which were dried by exposure to UV light (245 nm) 5 min. Before powdering, porcelain mortar was kept in the refrigerator at -85 °C for 3 hours. About 2-3 grams of bone pieces were powdered using porcelain mortar with liquid nitrogen and the bone powder (35-50 mg) was stored in a sterile sample tube at -20 ° C.

2.3.4. Decalcification

About 35-50 mg bone powder was incubated in 20 ml ethylene diaminetetra-acetic acid (EDTA) solution (0,5 M, pH 7,5) in a 50 ml falcon tube and was placed on a rotary shaker at 4°C. EDTA solution was replaced every 24 hours following by 3000 rpm centrifugation. On the completed third day, the EDTA solution was removed and the bone powder was washed with ddH_2O. Decalcification was completed following the protocol described by Cemper-Kiesslich (2014).

2.4. DNA EXTRACTION FROM BONES

The phenol/chloroform method was performed as a modification of the method described by Barnett and Larson (2012). In the method which was used for this study phenol/chloroform was combined with guadine thiocyanate (GnSCN).

Two different DNA extraction methods (phenol/chloroform and Qiagen forensic kit) were applied in this study for the bone samples. The organic DNA extraction method (phenol/chloroform) was preferred to use only for teeth samples, because of the limited yield of the dental pulp.

2.4.1. DNA Extraction Method 1

- The decalcification of bone powder (0.4g) samples were mixed with 175 µl Tris EDTA Buffer (TE), 100 µl 10 mM NaCl, 100 µl Triton X-100, 100 µl proteinase K (20 mg/ml) and 200 µl Denaturation Solution (Sol D) containing N-lauryl sarcosin, Na-citrate thiocyanate, Na-Guadine thiocyanate and then incubated at 56^{o}C for overnight.
- The next day, 50 µl proteinase K and 100 µl Sol D was added to the mixture and incubated at 56 ^{o}C for overnight.
- Vortexed for 10 seconds after the addition to one volume phenol (pH:7,5) and one volume to chloroform isoamyl alcohol (24:1); then, centrifuged at 12,5000 rpm for 10 minutes.
- The supernatant was transferred into a 1,5 ml micro centrifuge tube, added one volume cold isopropyl alcohol and Na-Acetate 1/10 total of the volume and samples were precipitated at −20 °C for 4 hours.
- Following the centrifuge at 14000 rpm for 20 min, removed all traces phases.
- 300 µl 70% cold ethanol was added and centrifuged at 14000 rpm for 15 min
- Ethanol was carefully discarded and after the pellet was air-dried, DNA was eluted 20 µl ddH_20 and stored at -20 ^{o}C.

2.4.2. DNA Extraction Method 2

Bone samples that were physically and chemically cleaned were taken into the microcentrifuge tube with approximately 0.4 g of bone powder. The DNA extraction was applied using the Qiagen Forensic Tissue Kit ® (Qiagen, USA) and its specifications. During the DNA extraction, the analysis of different negative controls were applied on them to observe the possible DNA. According to Qiagen Forensic Kit, the decalcification procedure was not performed on bone samples.

2.4.3. DNA Extraction from Teeth

The inlet cavity was prepared using a round diamond bur (W&H aerator) on the cleaned surface of the teeth. Using long cylindrical diamond bur (W&H aerator) entrance hole to pulp chamber was opened. Pulp powder was collected into sterile tubes.

After the pulp was obtained from the teeth, it was transferred into sterile 1.5 ml tubes and weighed. Then, 0,5 M 250 µl EDTA (pH 7,5) was added into tube and was incubated in rotary shaker at 4°C for 20 minutes. After centrifuging at 3000 rpm for 15 minutes to remove all traces of EDTA, it was washed with ddH_20. After decalcification, nearly 0.05 g pulp tissue was used for DNA extraction. The decalcified tooth samples were incubated in 90 µl TE buffer, 50 µl Sol D, 20 µl proteinase K, 25 µl NaCl and 25 µl Triton X-100 at 56 ^{o}C for overnight. The next day, 50 µl Sol D and 10 µl proteinase K was added to the mixture, and then incubated at 56 ^{o}C for overnight. When the incubation process was completed, one volume of phenol chloroform isoamyl alcohol (25:24:1) was added into the tube and vortexed. After centrifuging at 12500 rpm for 10 minutes, the supernatant was transferred into a new sterile 1,5 ml centrifuge tube. One volume of cold isopropyl alcohol and Na-Acetate 1/10 total of the volume was added and stored at -20 ^{o}C for 5 hours, then it was centri-

fuged at 14000 rpm for 15 minutes. The aqueous phase was discarded and it was washed with absolute ethanol. Ethanol was removed and the pellet was air-dried. DNA was eluted with 20 µl ddH_20 and stored at -20 °C.

2.5. DNA Quantitation

All DNA extracts were quantitated by Nanodrop ND-1000 spectrophotometer. The average DNA yield was between 8,5 and 17,6 ng/ µl from approximately 0,4 g of bone powder for DNA extraction method 1. DNA yield was between 2,1-6,3 ng/µl from the same amount of bone powder for DNA extraction method 2. 3,04-5,6 ng/µl DNA was obtained from 0,05-0,09 pulp samples using phenol chloroform DNA extraction method.

2.6. PCR Amplification

For STR typing to determine sex, all the samples were PCR amplified by using Investigator® Argus X-12, which contains (Amelogenin ve DXS7132, DXS7423, DXS8378, DXS10074, DXS10079, DXS10101, DXS10103, DXS10134, DXS10135, DXS10146, DXS10148, HPRTB) Qiagen PCR amplification kit. PCR mix was prepared according to Qiagen X-STR PCR Amplification kit procedures. A total of 25 µl of PCR reaction was amplified on GeneAmp® PCR System 9700 thermal cycler.

For all, PCR amplification was carried out according to the Qiagen PCR amplification protocol. Amplification conditions were: initial denaturation step at 94 °C for 4 minutes, the first step was followed by 5 cycles of denaturation at 96 °C for 30 seconds, at 63 °C for 2 minutes, and at 72 °C for 75 seconds. The second step was followed by 27 cycles at 94 °C for 30 seconds, at 60 °C for 2 minutes, at 72 °C for 30 seconds, and extension at 68 °C for 65 seconds. PCR amplified products were separated by capillary electrophoresis on ABI Prism 3130 Genetic Analyzer, using POP-4 polymer. Peak sizing and typing were analyzed by Gene-Scan software.

3. RESULTS AND DISCUSSION

Researchers interested in ancient DNA analysis can learn about ancient cultures and how archeologists interpreted them. Contemporary societies' demographic information and the relationships with other archeological communities have been revealed in recent years through various studies in this field.

The demographic structure of archeological societies is tried to be explained with the joint studies of archeologists and anthropologists as the number of females, males, and children in the graves is determined and the cultural applications such as the items in graves are considered. Moreover, the determination of the ancient population's structure has many difficulties. For example, the determination of sex with the paleo-demographic methods includes a set of restrictions (it can not be identified for individuals under 15 ages since they hadn't developed completely yet) so it creates the fields that the molecular anthropological approaches can contribute to the paleo-demography. Furthermore, the bone samples from the various archeological contexts are fragile and they are mostly obtained in parts or deficiently, so these situations reveal the restrictions in the building of the paleo-demographic structure and create the fields working with the molecular anthropological techniques.

The sex determination of archeological human remains is essential for the reconstruction of the past population in paleoanthropological studies. Especially, the determination of sex plays an important role in understanding the mortality rates between sexes of infant and juvenile as well as the burial and patterns by disease for adult human remains in anthropological studies. Sex can be determined using traditional morphological and morphometric analyses. However, for infant and juvenile or fragmentary adult skeletons, these methods are unreliable.

The aim of this research was to demonstrate the application of ancient DNA analysis from human skeletal remains for the determination of sex in cases where anthropological methods are limited. In this study, DNA was extracted from totally 37 bone and teeth specimens that were taken from the 27 human skeletons and were investigated for molecular sex determination. Human skeletal remains were obtained from 8 different excavation sites which had different climatic characteristics and different historical periods in Anatolia. In general, bone and teeth samples were well preserved.

Two different DNA extraction methods (phenol/chloroform and Qiagen Forensic Kit) were used for 16 bone samples (petrous bone femoral bone and fibula) in this study. Approximately 0,4 g of bone powder was used for both of the two DNA extraction methods. The amplification results of the DNA extraction methods are shown in Table 2. As a result of DNA extraction method 1, 13 of the 16 (%81) and for the DNA extraction method 2, 7 of the 16 (%43) was successful. The phenol/chloroform DNA extraction method was used for totally 21 teeth samples. Because of the limited yield of the dental pulp, Qiagen Forensic Kit could not be used. The amount of pulp and amplification results are shown in Table 3. Only 6 tooth samples of human skeletal remains were able to be amplified.

X-allele drop was seen in one of the tooth samples (tooth sample number:14) and only the Y allele was present in the amelogenin. In ancient DNA studies, due to the fact that the amount of DNA is limited,

the allele drop can be seen (Alghafri et al. 2018). Therefore, variations that may occur during amplification since it can cause allel drop. In the case of the Y allel drop, interpretation of the results is difficult, whereas the drop of the X allel did not lead to a wrong result.

Table 2. ***DNA extraction methods and amplification results of ancient samples. Ancient bones and dental pulp amplified in PCR are symbolized by (+). Non-amplified samples in PCR are symbolized by (-). DNA extraction method 2 was not preferred for teeth samples and was symbolized by (x).***

Sample for DNA Analysis	Method 1 (Phenol Chloroform) PCR Result	Method 2 (Qiagen Forensic Tissue Kit) PCR Result
1(Femoral)	+	+
2(Femoral)	+	+
3(Femoral)	+	-
4(Femoral)	+	-
5(Teeth/ Molar)	+	X
6 (Teeth/ Molar)	+	X
7(Teeth/Premolar)		X
8(Teeth/ Molar)	+	X
9(Teeth/ Molar)	+	X
10(Teeth/ Molar)	-	X
11(Teeth/ Molar)	-	X
12(Femoral)	+	-
12(Teeth/Molar)	+	X
13(Teeth/Molar)	+	X
13(Femoral)	+	+
14(Teeth/Premolar)	-	X
15(Teeth/ Molar)	-	X
16(Teeth/Premolar)	+	+
17(Teeth/ Incisor)	-	-
17(Petrous bone)	+	-
18(Teeth/ Molar)	-	X
18(Petrous bone)	+	-
18(Petrous bone)	-	-
19(Teeth/Molar)	-	-
19(Petrous bone)	+	+
20(Fibula)	+	+
20(Femoral)	+	+
21(Teeth/Premolar)	-	X
22(Teeth/ Molar)	-	X
23(Teeth/Canine)	-	X
24 (Teeth/Incisor)	-	X
25(Petrous bone)	+	-
25(Femoral)	-	-
25(Teeth/ Molar)	-	X
26(Teeth/ Molar)	-	X
26(Femoral)	+	-
27(Femoral)	+	-

Subsequent to the DNA extraction, the amount and purity of DNA can be measured by spectrophotometric method or Real Time PCR. In addition, the use of agarose gel electrophoresis after DNA extraction may not be very accurate in ancient DNA studies. Because of the limited yield, DNA that was recovered from poorly preserved ancient samples, may not be seen in the agarose gel visualized by ethidium bromide. This does not mean that there is no DNA, and the band appereance does not mean that pure DNA has been obtained or that the seen band has been caused by contamination.

In this study, DNA amount and purity were determined by using nanodrop N-100 spectrophotometer. This method, which is more advantageous than agarose gel electrophoresis, has the sensitivity to detect DNA that is too limited and can not be imaged on the agarose gel. The DNA quantities of the 16 bone samples using DNA extraction method 1 (phenol/chloroform with GuSCN) according to ND-1000 spectrophotometer changed between 4,3 ng/µl of minimum and 14,2 ng/µl of maximum and as for the tooth samples, it was measured between approximately 2,2 ng/µl of minimum and 4,3 ng/µl of maximum. DNA was measured using DNA extraction method 2 (Qiagen Forensic Kit) between 2,1 ng/µl and 5,1 ng/µl for bone samples.

The X-STR kit used in the amplification of the DNA obtained by ancient samples was valuable. The Qiagen Investigator Argus X-STR kit performed well even with 0,5 ng of DNA. When the study is evaluated in this respect, we think that the quality of DNA is more important than the amount of DNA obtained, particularly in ancient DNA studies. Positive and negative controls were used for each amplification and they confirmed the validity of the amplification procedure.

Although ancient DNA studies have similar protocols to modern DNA studies, they are more difficult to study due to the contamination and DNA degradation encountered in aDNA studies. Especially, the contamination problem may change the results. Moreover, contamination with contemporary DNA is a serious problem, and many precautions against contamination must be adopted. STR (short tandem repeat) are commonly used for identification in the forensic sciences and genetic studies that have an important role in the use of degraded and very limited amounts of DNA (Budowle et al. 1996; Parsons, 2007; Xing et al. 2018; Coble and Butler, 2005). We performed a study of Investigator® Argus X-12 Kit (Qiagen, Germany) using totally 37 bone and

tooth samples taken that from the 27 human skeletons investigated for genetic sex determination. 6 of them were unidentified skeletal remains (1 child and, 5 unidentified, because of the fragmentary and poor preservation); so, sex could not be determined using anthropological studies.

DNA was extracted from the dental pulp of 21 teeth for sex determination using phenol chloroform with GuSCN. The DNA extraction method and the amelogenin loci were detected in 10 of them. Adler et al. (2011) and Damgaard et al. (2015) successfully obtained high amounts of endogenous DNA from the dentin part of the tooth. The dental pulp is a good DNA resource and it is preferred in ancient DNA studies (Pfeiffer, 1999; Alakoç, 2007; Tilotta, 2010; Higgins and Austin, 2013, Drosou et al. 2017). However, in recent years, petrous bone has been commonly preferred since it provides significantly higher endogenous DNA (Pinhasi et al. 2015; Lazaridis et al. 2016; Hansen et al. 2017). In this study, femoral and petrous bones had better results than the teeth samples. Amelogenin loci could not be amplified in 12 dental pulps. It is thought that the amount of pulp obtained from the teeth was not sufficient and a loss of DNA during decalcification of the teeth occurred. It has to be taken into account when choosing the samples in ancient DNA study. According to Fischer (1993), DNA can be successfully extracted without decalcification from ancient samples. Some researchers successfully extracted DNA from historical tooth samples without decalcification and amplified using the commercial multiplex kit (Silva et al. 2018)

As the amount of dental pulp was low, only one of the DNA extraction methods was applied. In addition, because of the low amount of pulp obtained from the dental teeth, the repetition of DNA extraction from non-amplification tooth samples was limited. When these results are evaluated, it is considered that working with cortical bones and petrous bone is more suitable for aDNA studies if the amount of dental pulp is low or other part of teeth will not be used. Studying with dental pulp has to be taken into account.

Based on the results of the molecular sex determination, 8 of the individuals could not be determined. Due to the good preservation of ancient samples and methods of DNA extraction (particularly method 1) and amplification performed, 25 of the 37 ancient samples could be amplified successfully. In 3 individuals, the results of the X-STR and anthropological analysis were not concordant. Sex determination as female for 4 individuals was confirmed by PCR amplification using the AmpFLSTR Identifier PCR Amplification kit and also repetition studied with Investigator® Argus X-12 Kit. The dental pulp and femoral bone of the individuals 12, 13 and 26 excavated from Giresun Island showed the same result in molecular analysis confirming that the sex was female. These historical samples were excavated from the mass burial in Giresun Island. When the results are evaluated in this respect, there will be an error in morphological sex determination of human skeletal remains because of the minimum number of individuals who are not fully determined. The results were summarized in Table 3. It is important that skeletons obtained from archeological excavations are carefully removed from the field and stored after removal, taking into account the molecular studies that can be performed. At this point, it is very important for archeologists working in the excavation to have knowledge about molecular studies.

Table 3. Morphological sex of skeletons compared to molecular sex identified using DNA based methods

Sample Number	Sex Molecular Analysis Data	Sex Anthropology Data	Bone Type	Period of samples and Archaeological Site
1	Female	Female	Femoral	The Middle Age The Van Castle Mound
2	Male	Male	Femoral	The Middle Age The Van Castle and Mound
3	Male	Unkown (incomplete)	Femoral	Hellenistic-Rome Teos Ancient City /Izmir
4	Male	Unkown (incomplete)	Femoral	Hellenistic-Rome Teos Ancient City /Izmir
5	Male	Male	Teeth (Molar)	Early Iron Age Van Kalecik/Ablagens
6	Male	Male	Teeth (Molar)	Early Iron Age Van Kalecik/Ablagens

7	Undetermined	Male	Teeth (Premo-lar)	Early Iron Age Van Kalecik/Ablagens
8	Female	Male	Teeth (Molar)	Early Iron Age Van Kalecik/Catak
9	Male	Male	Teeth (Molar)	Early Iron Age Van Kalecik/ Catak
10	Undetermined	Male	Teeth (Molar)	Early Iron Age Van Kalecik/Catak
11	Undetermined	Male	Teeth (Molar)	Early Iron Age Van Kalecik/Kalecik
12	Female	Male	Femoral Teeth (Molar)	The Middle Byzantine Giresun Island
13	Female	Male	Femoral Teeth (Molar)	The Middle Byzantine Giresun Island
14	Undetermined	Male	Teeth (Premo-lar)	The Middle Byzantine Giresun Island
15	Undetermined	Female	Teeth (Molar)	The Middle Byzantine Giresun Island
16	Female	Female	Teeth (Molar)	The Middle Byzantine Giresun Island
17	Male	Unkown (child)	Petrous bone	Byzantine Nysa Ancient City/Aydın
	Male	Unkown (child)	Teeth (Incisor)	
18	Undetermined	Unkown (incomplete)	Petrous bone	Byzantine Nysa Ancient City/Aydın
	Female		Petrous bone	
	Female		Teeth (Molar)	
19	Male	Unkown (incomplete)	Teeth (Molar)	Byzantine Nysa Ancient City/Aydın
	Male		Petrous bone	
20	Male	Unkown (incom-plete)	Fibula	Byzantine Nysa Ancient City/Aydın
	Male		Femoral	
21	Male	Male	Teeth (Premo-lar)	The Middle Byzantine Giresun Island
22	Undetermined	Female	Teeth (Molar)	The Late Byzantine Beybag/Mugla
23	Undetermined	Female	Teeth (Canine)	The Middle Byzantine Giresun Island
24	Undetermined	Female	Teeth (Incisor)	The Middle Byzantine Giresun Island
25	Female	Female	Petrous bone	The Late Byzantine Beybag/Mugla
			Teeth (Molar)	
	Undetermined			
	Undetermined		Femoral	
26	Female	Male	Femoral	The Middle Byzantine Giresun Island
	Undetermined	Male	Teeth (Molar)	
27	Female	Female	Femoral	The Middle Byzantine Giresun Island

4. CONCLUSION

The studies on ancient DNA from archeological remains make it possible to get genetic data from the past. Many archeological and anthropological excavations have been done in Anatolia including many civilizations for thousands of years. By means of the new methods of DNA isolation, amplification and sequencing improved in recent years in Turkey, and results can be increased and a higher amount of data can be obtained. The field of aDNA has, however, largely focused on mitocondrial DNA (mtDNA) analyses in Turkey. Mitochondrial DNA Hypervariable region I and Hypervariable region II of ancient human samples were amplified haplogroups of these ancient samples. They were identified and evaluated together with the data of other published communities by researchers. Particularly Eastern and Southeastern Anatolia was the center of the domestication of animals. The studies in Turkey aim to understand how and when domestic animals (sheep and goat) were transported across Anatolia to the West from the domestication center by using ancient DNA. In order to achieve that mitochondrial DNA, Hypervariable regions were amplified and sequenced.

In this study, we improved and optimized DNA-based method using GuSCN with phenol chloroform, and compared it to Qiagen Forensic Tissue Kit for the sex determination from human skeletal remains excavated from different archeological locations in Anatolia. Following the DNA extraction and using X-STR with amelogenin markers, sex was successfully determined in 19 out of the 27 (%70) individuals. The sex identifications from 9 of the 10 femoral bones (%90), from 4 of the 5 petrous bones (%75) and from dental pulp 11 of 21 dental pulps (%52) were successful. In ancient DNA studies, the preservation of samples, storage condition and historical period might change the DNA extraction methods. When we compared and evaluated DNA extraction methods with X-STR in this scope, it was seen that the DNA extraction method 1 (phenol/chloroform) was 87,5 more successful in bone samples. When anthropological sex determination was compared to molecular analysis, it was seen that 85% of the results were consistent. Based on the results of this study, we can say that at least two samples should be taken from each individual for ancient DNA studies to confirm molecular results.

If the bones are fragmented and poorly preserved, traditional anthropological methods based on morphological and morphometric analysis are insufficient to determine sex. In recent years, ancient DNA research has provided reliable and alternative methods, particularly for juvenile skeletal remains for sex identification. When precautions are taken carefully to prevent the contamination problem, which is frequently encountered in ancient DNA studies, it will be facilitated to obtain pure DNA, and for the amplification of the targeted gene, the reliability of the results will be ensured.

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