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The optimization of aDNA extraction protocol and sex determination of Bronze Age individuals from Oylum Höyük (Kilis, Turkey)

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Abstract: Oylum Höyük falls within the borders of the province of Kilis, located in the southern part of Turkey. It is an important Bronze Age settlement dating back to 5500 BC. In this study, the sex of 36 individuals found in Oylum Höyük was examined using morphological and genetic methods. In the morphological method, sex was identified in 14 female and 13 male individuals. The remaining 8 individuals could not be identified due to the poor preservation of their skeletal remains. Genetic sex was determined with the amplification of sex-specific X, Y centromeric alphoid repeats and ZFX-ZYF gene regions of DNA, mostly extracted from teeth. Based on ZFX-ZYF gene regions, sex was successfully determined using the PCR-RFLP method. The amplification results of ZFX-ZYF gene regions were also tested by applying the PCR-RFLP method. The amplification of X, Y centromeric alphoid repeats was successful in 29 individuals, while sex determination based on ZFX-ZFY fragments was carried out in 22 individuals. The vast majority of the results determined by analysis of ancient DNA were consistent with the morphological method. However, these 2 methods produced slightly different results for 5 individuals. Finally, the results indicated that ancient DNA analysis is a reliable method for sex determination, especially in poorly preserved and fragmented skeletal remains.

Key words: Ancient DNA, sex determination, Bronze Age, Oylum Höyük

Oylum Höyük (Kilis, Türkiye) bronz çağı bireylerinde aDNA izolasyon protokolünün optimizasyonu ve genetik cinsiyet tayini

Özet: Oylum Höyük Türkiye'nin güneydoğu bölgesinde Kilis ili sınırları içinde bulunur. Oylum Höyük MÖ 5500 tarihli önemli bir Bronz çağı yerleşim yeridir. Bu çalışmada Oylum Höyük'te 36 bireyin cinsiyeti morfolojik ve genetik yöntemler kullanılarak araştırıldı. Morfolojik cinsiyet tayinine göre 14 birey dişi, 13 birey erkek olarak belirlendi. Kalan 8 bireyin cinsiyeti iskelet kalıntılarının kötü korunmuş olması nedeniyle tespit edilemedi. Genetik cinsiyet, çoğunlukla dişten izole edilen DNA'nın cinsiyet-özel X,Y sentromerik alfoid tekrarlarının ve ZFX, ZFY gen bölgelerinin amplifikasyonu ile belirlendi. ZFX, ZFY gen bölgelerine bağlı cinsiyetler PCR-RFLP metodu ile başarılı bir şekilde belirlendi. X,Y sentromerik alfoid tekrarların amplifikasyonu 29 bireyde sonuç verirken, ZFX, ZFY gen bölgesi kullanılarak yapılan cinsiyet belirlemede ise 22 bireyde başarı elde edildi. Eski DNA analizi kullanılarak elde edilen sonuçların büyük çoğunluğu morfolojik yöntemle uyumlu sonuçlar verdi. Diğer taraftan bu 2 yöntemin 5 birey açısından farklı sonuçlar verdiği tespit edildi. Sonuç olarak eski DNA analizinin özellikle iyi korunmamış ve parçalanmış iskelet kalıntılarında cinsiyet tayini için güvenilir bir yöntem olduğu düşünülmektedir.

Anahtar sözcükler: Eski DNA, cinsiyet tayini, Bronz Çağı, Oylum Höyük

Introduction

Oylum Höyük is a Bronze Age site, located on the Gaziantep-Kilis highway, next to the village of Oylum. The site is approximately 460 m in length and 370 m in width. It consists of 2 ridges, a northern ridge 22 m in height and a southern ridge 37 m in height, with a neck connecting them. This settlement area is situated along the ancient trade road extending from Aleppo to Anatolia. It is also one of the largest Bronze Age settlement areas in the Gaziantep district (1). The first scientific research in Oylum Höyük was initiated in 1985 (1). The earliest layers of the site date back to the Late Chalcolithic period, approximately 4000 BC. The layers from the Late Chalcolithic to the Early-Middle Bronze Age (4000-2000 BC) on the eastern side of the site have been studied in detail. The earliest levels on the western terrace (Late Chalcolithic I - Ubeid), Late Bronze Age remains on the south slope, Late Bronze-Hellenistic layers on the north hill, a church belonging to the Late Roman period, and Medieval remains on the western slope have been examined. The excavations have uncovered many burials, especially from the eastern slopes of those layers from the Bronze period (2-6). In the Bronze Age grave tradition of Oylum Höyük, infants aged 0-3 years were buried in jar graves, which were usually placed horizontally or perpendicular to the ground. Other individuals were buried in extended or hocker position in simple graves, pithoi, and room graves (2-6).

The identification of sex is one of the most important applications in the examination of skeletal samples in anthropological studies. It is also an essential resource in the reconstruction of the demographic and historical structure of past societies. Traditionally, sex has been easily determined from an adult skeleton depending on the conditions of preservation. Morphological methods may be unreliable, however, for fragmentary adult skeletons, child, and infant remains. Particularly, the characters of sex identification are not developed for children and juvenile individuals and morphology-based identification alone is often impossible. Recent advances in molecular anthropology have enabled researchers to identify sex using methods involving ancient DNA (aDNA) (7-9). The use of aDNA fragments specific to the X and/or Y chromosomes can provide an ideal solution for sex determination problems. Although aDNA research has been widely used throughout the world, there have only been a few molecular anthropology studies in Turkey (7). In the present study, we aimed first to optimize the process of DNA extraction from ancient skeletal samples for future research in our laboratory. Secondly, we aimed to determine the sex of some adult and adolescent individuals from Bronze Age layers of the Oylum Höyük excavation site using both morphologic and molecular methods. In this way, we were able to compare morphological and molecular data sets and test the reliability of sex identification analysis based on aDNA.

Materials and methods

Materials

The skeletal samples used for this analysis consist of 36 individuals from the Oylum Höyük (Kilis) excavation site; these individual were found between 1989 and 2007 and belong to the Early to Middle Bronze Age (Table). The material used for genetic testing was selected from teeth and bone fragments without any paleopathological lesions. Bone samples consisting of dense, spongious tissue such as vertebra, rib, and femur were used in most of the examinations. Parts of well-preserved teeth were also chosen for 8 individuals. For 1 sample, however, a first upper right incisive tooth was also included in the sex identification process because the individual had only an incisive tooth (Table).

Morphologic sex identification was performed utilizing morphological differences in the cranium, mandible, pelvis, and long bones. Age of death was estimated using dental wear, ectocranial suture closure, and auricular surface, pubic symphyseal, and a complex method of WEA for adults and epiphyseal fusion and tooth eruption and calcification for adolescents (10-12).

Genetic sex determination

Contamination prevention

All of the procedures followed to prevent contamination from modern sources and to monitor contamination were as previously reported (13,14). The bone samples were always handled while wearing

Table. Information on the archeological number, age, and element origin of samples and the results of morphological and genetic sex identification.

No.	Skeleton Number	Age	Element	Anthropologic Evaluation	Centrometric Alphoid Repeat	ZFX / ZFY	No.	Skeleton Number	Age	Element	Anthropologic Evaluation	Centrometric Alphoid Repeat	ZFX / ZFY
1	OY'89 N165 E75 Lot 8/6	43-56	Rib	Female	-	-	19	OY'00 OBY 18	17-20	Throcal Vertebra	Female	Female	Female
2	OY'89 N165 E75 Lot 11/8	Adult	Phalanx	Unknown gender	Male	Male	20	OY'00 X9 OBY 48	adult	Naviculare	Unknown gender	Male	-
3	OY'89 N10 W30 Lot 7/1	Adult	Rib	Female	-	-	21	OY'00 X9 OBY 32	adult	Patella fragment	Male	Male	Male
4	OY'89 N165 E75 Lot 16/5	15-20	Rib	Unknown gender	Male	Male	22	OY'00 Y11 OBY 310	17-25	Cervical Vertebra	Male	Male	-
5	OY'90 N165 E70 2	17-25	L. Mast. Proc.	Male	-	-	23	OY'00 X9 OBY 23	32	R M_1	Male	Male	Male
6	OY'90 N165 E70 Lot 27/14	19	L. Dist. Fibula	Female	Female	Female	24	OY'00 Y42a-c OBY 25	17-25	R I^1	Female	Female	Female
7	OY'90 N170 E75 1	Adult	Rib	Male	Male	Male	25	OY'00 OBY 107	adult	R M^1	Female	Female	Female
8	OY'90 N165 E70 Lot 48/28	25-35	Rib	Unknown gender	-	-	26	OY'00 X42a-c OBY14	adult	R C	Female	Male	Male
9	OY'90 N170 E75 16	25-35	Parietal fragment	Female	Female	-	27	OY'00 Y11 OBY 358	adult	R C	Unknown gender	Male	Male
10	OY'91 AA11d Tomb 7	25-35	Rib	Unknown gender	Male	Male	28	OY'00 X41a-c OBY 13	25-35	R P_1	Male	Male	Male
11	OY'97 I31b OBY 24 M7	17-25	L P^1	Male	Male	-	29	OY'00 Y11 OBY 342/2	adult	L MT-V	Male	Male	Male
12	OY'98 OBY 48	25-35	Cervical Vertebra	Female	Male	Male	30	OY'00 X41a-c OBY 12	28-37	Rib	Male	-	-
13	OY'99 Y10 OBY 102	33-45	L. Cond. femoris	Male	Male	Male	31	OY'00 X42b-d OBY 2	adult	R Mast. Proc.	Male	Male	Male
14	OY'99 Y11 OBY 259	17-19	Scapula fragment	Male	-	-	32	OY'00 X41a-c OBY 9	35-39	L. Cavitas glenoid.	Male	Female	Female
15	OY'99 Y9 OBY 19	18-25	L C	Female	Female	Female	33	OY'00 X42b-d OBY 3	adult	L C	Female	-	-
16	OY'99 Y11 OBY 266 Sk.4	15-17	L Patella	Female	-	-	34	OY'00 Y22a-c Lot 1 OBY 11	25-35	Rib	Unknown gender	Male	-
17	OY'99 Z11a OBY 295 Sk.12	Adult	R Talus	Unknown gender	Male	-	35	OY'07 J22a OBY 47/2	34-43	Throcal Vertebra	Male	Male	Male
18	OY'00 X9 OBY 44	17-25	Throcal Vertebra	Female	Female	Female	36	OY'07 J22a OBY 39	25-35	Cond. femoris frag.	Male	Male	Male

sterile latex gloves, face masks, and laboratory coats. All reagents were purchased as DNase and RNase-free molecular biology grade chemical. All reagents and equipment were bleached and UV irradiated (1.0 J/cm^2, 254 nm for 45 min) before use. PCR tubes were prepared in laminar flow cabinets equipped with UV decontamination facilities. The negative PCR (no DNA) and positive PCR (including DNA of modern female and male samples) controls were used to check whether amplified products were obtained from the aDNA samples. The correct electrophoretic migrations of the X- and Y-specific centromeric alphoid repeat fragments were assessed by running authenticated controls in parallel. Finally, the accuracy of identifications based the centromeric alphoid repeat fragments was tested to benefit from amplification and analysis of zinc finger (ZFX/ZFY) gene fragments.

aDNA extraction and PCR amplification

Prior to extraction, potential agents contaminating the surface of the samples were removed using physical and chemical methods. To eliminate possible surface contamination, 2-3 mm of the external layer of bones was removed with a sterile disposable scalpel blade and the samples were then soaked in a 6% sodium hypochlorite solution for 10 min and washed with ddH2O (15). Samples were exposed to UV light for 5 min. The bones were crushed in a mortar and pestle and then transferred to sterile falcon tubes and washed with ddH2O.

The genomic DNA was extracted from samples using the method described by Sambrook et al. (16), with some alterations. The efficiency of the extraction protocol was enhanced by decalcifying samples (17). Decalcified bone (200 mg) was incubated in 1000 µL of extraction buffer (50 mM Tris–HCl, pH 8.0/100 mM NaCl/100 mM EDTA, pH 8.1/1% SDS/39 mM DTT/500 µg/mL proteinase K) overnight at 56 °C. Next, the samples received 0.7 M NaCl and 1% CTAB to selectively remove polysaccharides before being incubated at 65 °C for 10 min. After phenol extraction, the DNA was precipitated by adding 0.1 vol of 5M NaCl, 2 vol of 99% ice cold ethanol, and 0.01M $MgCl_2$, which is done to increase the yield in precipitations of low concentration or small nucleic acid pieces. The DNA was washed with 70% ethanol to remove the sodium salt and re-dissolved in 100 µL of TE buffer (10 mM Tris, pH 8.0/1 mM EDTA, pH 8.0). The DNA samples were purified with a silica-based DNA extraction kit (Fermentas K0513).

A 130 bp fragment of X chromosome-specific alphoid repeat and 170 bp fragment of Y chromosome-specific alphoid repeat, defined by Lin et al. (1995), were amplified by PCR (18). Another gene region selected for sex identification was a 209 bp of ZFX-ZFY fragments (19). Primers were used as follows: X1, 5'-AATCATCAAATG GAGATTTG-3' and X2, 5'-GTTCAGCTCTGTGAGTGAAA-3'; Y11, 5'-ATGATAGAAACGGAAATATG-3' and Y22, 5'-AGTAGAATGCAAAGGGCTC-3'; ZFX, 5'-CTGGAGAGCCACAAGCTGAC-3' and ZFY, 5'-TTGCTGTGGACTGCCAAGAG-3'. The 25 µL reaction volume included 0.5 U of Taq polymerase (MBI Fermentas), 2.5 µL of 10× reaction buffer (100 mM Tris-HCl, pH 8.8, 500 mM KCl, and 0.8% Nonidet P-40), 1 mg/mL BSA (AppliChem), 5 pmol of each of the primers (İontek, İstanbul, Turkey), 0.2 mM of each of 4 dNTPs (MBI Fermentas), 2.5 mM $MgCl_2$, and 2 µL of DNA template (≈ 25-50 ng of DNA). PCR amplifications were performed in a DNA Engine PTC-200 thermal cycler (MJ Research, Watertown, MA, USA) under the following amplification conditions: 3 min at 94 °C, followed by 34 cycles of 30 s at 94 °C, 30 s at 47 °C for X-Y primers and 58 °C for ZFX-ZFY primers, and 30 s at 72 °C. Reactions were terminated with a 5 min final extension step at 72 °C. Amplification products were analyzed by 2% (wt:vol) agarose gel electrophoresis (Figure 1).

The amplified ZFX-ZFY fragments were subjected to restriction fragment length polymorphism (RFLP) analysis to confirm sex determination by X-Y alphoid repeat fragment (19). The amplification products of this fragment included an additional polymorphic position in the Y chromosome; this was more than was expected in the X chromosome. Hence, the PCR product (10 µL) was digested overnight with 10 units of HaeIII restriction enzyme (New England Biolabs, Beverly, MA, USA). The digestion fragments of PCR products (209 bp) running on 3% agarose gel exhibited 2 distinct bands (172 and 37 bp) for females (XX) and 4 distinct bands (172, 84, 88, and 37 bp) for males (XY) (Figure 2).

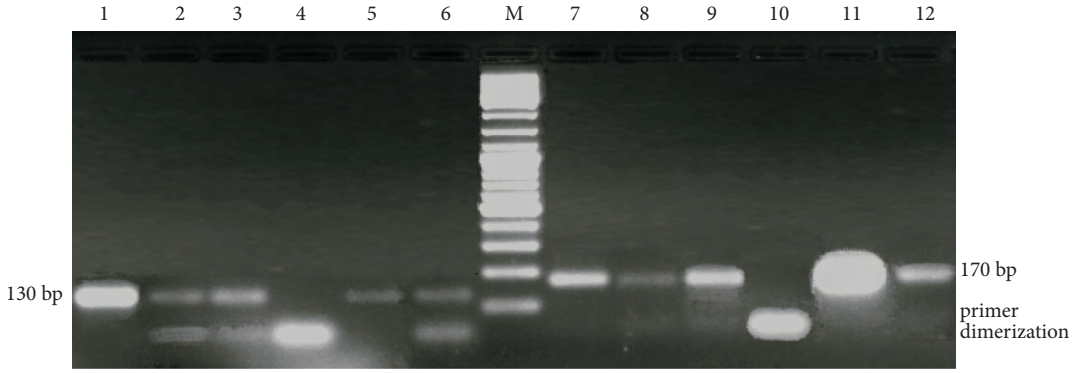

Figure 1. The PCR products of the X and Y chromosome-specific alphoid repeat fragments. X chromosome-specific alphoid repeat fragment (130 bp, Lane 2, 3, 5, 6), lane 1: positive control (modern DNA), lane 4: negative control (no DNA). Y chromosome-specific alphoid repeat fragment (170 bp, Lane 7, 8, 9, 12), lane 10: negative control (no DNA), lane 11: positive control (modern DNA). (M: molecular weight marker, 100 bp, Fermentas).

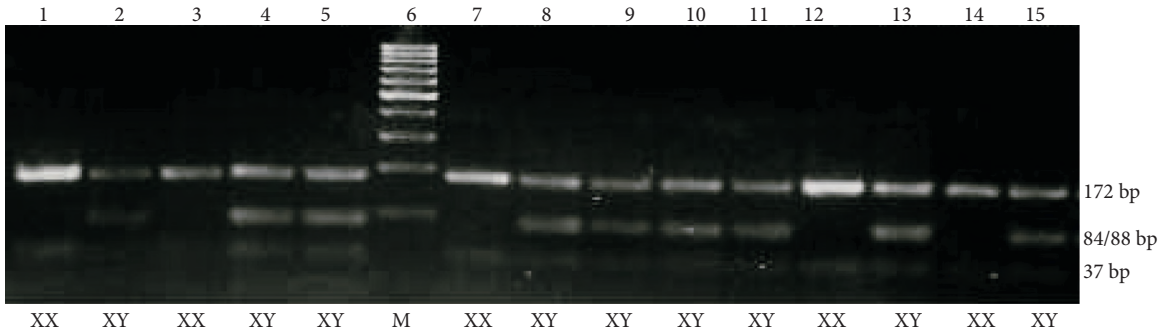

Figure 2. PCR-RFLP patterns of the ZFX-ZFY gene fragments. Female (XX) is separated on the 172 bp and 37 bp fragments and male (XY) is separated on the 172 bp, 84/88 bp, and 37 bp fragments after Hae III digestion of PCR products (209 bp).

Results and discussion

The preservation conditions of the skeletal elements in the Oylum Höyük series were poor and considerably fragmented because of tomb raiding and the acidic nature of the sediments. These conditions can affect the accuracy of morphological sex identification and the resulting studies in anthropological science (17). Genetic sex determination using aDNA is an important alternative approach. The biggest problems encountered in genetic studies using archeological material include the difficulty of DNA extraction and PCR amplification and the prevention of contamination (17,18,20). These problems occur in samples that are not well preserved because of the mostly fragmented nature of genomic DNA. In the present research, teeth and well-preserved bone fragments that did not include paleopathological lesions were selected from the skeletal elements for DNA extraction. A higher amount of DNA was extracted from teeth in the present study since many previous aDNA studies indicate that ancient DNA is better preserved in teeth (8,21-23). PCR amplifications were successfully performed in 8 out of 9 tooth samples included in this study (Table). Further successful extractions were obtained from dense, spongious bone tissues. On the other hand, aDNA extractions of remaining samples were carried out utilizing tissue taken from ribs and only half of the samples were used successfully (Table). These DNA extractions may have been unsuccessful as a result of deamination and dehydration processes that occurred while the samples were buried in Oylum Höyük. After the aDNA extraction process, the concentration and purity of the aDNA sample were accepted as a success. Gel electrophoresis of the extracted DNA was performed to guarantee the size, shape, and color of short DNA fragments. The success rate of aDNA extraction increased with the use of a silica-based DNA extraction technique. This technique can be applied to short DNA fragments

(<500 bp) and is useful for removing PCR inhibitors from extraction homogenates rather than increasing the amount of aDNA. In our study, in spite of agents such as BSA and Triton X-100, which are used to increase the activity of DNA polymerase, most of the PCR reactions resulted in failure. The failure of aDNA amplification can be considered a result of PCR inhibitors such as humic acids, tannins, porphyry products, phenolic compounds, hematin, and type I collagen (22,23). The extracted DNA was observed under gel electrophoresis to be a white-green color. This color confirms the existence of PCR inhibitors in the samples. The PCR inhibitors could be removed from the DNA samples with the use of the silica-based DNA extraction technique. In addition to silica, the use of $MgCl_2$ in the precipitation step of DNA extraction protocol allowed our study to retain more short and fragmented DNA. During the amplification period, negative control reactions were performed to determine possible contamination by modern resources. Positive control reactions were used to show differences in the quality and quantity of ancient and modern DNA (Figure 1).

Sex determination is one of the most important studies in anthropology because it assists researchers in understanding demographic structure, marriage and burial patterns, differential mortality rates between sexes, and differential patterns of disease by sex (17). The sex of 36 individuals was investigated by applying morphologic and genetic methods. Morphological analysis revealed the presence of 13 females and 15 males, while the sex of 8 individuals could not be determined (Table). Overall, the sex of approximately 78% of the skeletons was identified using the genetic method (Table). In previous studies, this figure has been reported to be between 44% and 85% for amelogenin-based genetic sex identification in anthropologic studies (13). The amplification of X, Y centromeric alphoid resulted in the determination of 8 females and 20 males (Table). The evaluation of PCR-RFLP results of ZFX-ZFY gene fragments revealed the sex of 22 out of 36 individuals. The sex of morphologically unidentified samples was also investigated using the genetic method. While 7 of 8 samples were identified by the genetic method (sample nos. 2, 4, 10, 17, 20, 27, and 34), the sex of 1 individual was never determined (sample no. 8). On the other hand, the morphologically determined sex of 7 individuals was not identified using either of the gene regions. This may be due to the degraded and fragmented nature of the aDNA. The sex of 3 morphologically known individuals was not confirmed by aDNA analysis (sample nos. 12, 26, and 32). Concordance between the morphologic and genetic methods occurred in 21 out of 24 individuals (87.5%). We also observed that the results of different gene regions were broadly consistent with each other. On the other hand, genetic sex identification of 2 females and 1 male produced results that differed from those determined by the morphologic method. Morphological sexing error rates (determined by genetic sexing) were set at approximately 12% in some studies (17). In this study, the rate of incorrect sex identification by the morphologic method was found to be 10.71%. This is probably caused by the relatively poor preservation conditions of the skeletal remains.

In conclusion, the sexes of skeletal remains excavated from the Oylum Höyük archeological sites were identified using both morphologic and genetic methods. An improved aDNA extraction protocol was optimized for future research, for use in sex determination, the detection of infection or genetic diseases at an individual level, or for the clarification of relationships between populations. The present study was also the first of its kind carried out in Turkey.

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References

1. Özgen E. Gaziantep-Kilis Regional research survey of the Höyük: Oylum Höyük Excavation Results Meeting. 4: 239-248, 1987.
2. Özgen E. Oylum Höyük, 1987. Excavation Results Meeting. 10: 95-102, 1989.
3. Özgen E. Oylum Höyük, 1988. Excavation Results Meeting. 11: 203-210, 1990.
4. Özgen E, Tekin H, Ensert HK. Oylum Höyük excavation studies in 1991 and 1993. Excavation Results Meeting. 16: 95-105, 1995.
5. Özgen E, Helwing B, Engin E et al. Oylum Höyük, 2002. Excavation Results Meeting. 25: 339-344, 2004.
6. Özgen E, Engin A, Acara Eser M et al. Oylum 2006: Mozaikli Bazilika Excavation. Excavation Results Meeting. 29: 411-426, 2008.
7. Matheson CD, Loy TH. Genetic sex identification of 9400-year-old human skull samples from Çayönü Tepesi, Turkey. J of Archaeo Science 28: 569-575, 2001.
8. Sivagami S, Rao AR, Varshney UA. Simple and cost-effective method for preparing DNA from hard tooth tissue, and its use in polymerase chain reaction amplification of amelogenin gene segment for sex determination in an Indian population. Forensic Sci lnt 110: 107-115, 2000.
9. Eliášová I, Mazura I, Smejtek L. DNA analysis of ancient skeletal remains. Folia Biol (Praha) 56: 47–50, 2010.
10. Workshop of European Anthropologist. Recommendations for age and sex diagnoses of skeletons. J Hum Evol 9: 518-549, 1980.
11. Lovejoy CO, Meindl RS, Pryzbeck TR. Chronological metamorphosis of the auricular surface of the Ilium: A new method for the determination of adult skeletal age at death. Am J Phys Anthropol 68: 15-28, 1985.
12. Kaur H, Jit I. Age estimation from cortical index of the human clavicle in Northwest Indians. Am J Phys Anthropol 83: 297-305, 1990.
13. Brown K. Ancient DNA applications in human osteoarchaeology: achievements, problems and potential. In: Cox M. and Mays S. (eds.) Human Osteology in Archaeology and Forensic Science. Greenwich Medical Media, London; 2000: pp. 455-473.
14. Bouwman AS, Chilvers ER, Brown KA et al. Brief communication: identification of the authentic ancient DNA sequence in a human bone contaminated with modern DNA. Am J Phys Anthropol 131: 428-431, 2006.
15. Kemp BM, Smith DG. Use of bleach to eliminate contaminating DNA from the surface of bones and teeth. Forensic Sci Int 15: 453-61, 2005.
16. Sambrook J, Fritsch E, Maniatis T. Molecular Cloning - A Laboratory Manual. 2nd Ed. Cold Spring Harbor Laboratory Press. New York; 1989.
17. Kaestle FA, Horsburgh KA. Ancient DNA in anthropology: Methods, applications, and ethics. Am J Phys Anthropol 35: 92-130, 2002.
18. Lin Z, Kondo T, Minamino T et al. Sex determination by polymerase chain reaction on mummies discovered at Taklamakan desert in 1912. Forensic Sci Int 75: 197-205, 1995.
19. Stacks B, Witte M. Sex determination of dried blood stains using the polymerase chain reaction (PCR) with homologous X-Y primers of the zinc finger protein gene. J Forensic Sci 41: 287-290, 1996.
20. DeGusta D, White TD. Technical report on the use of skeletal collections for DNA analysis. Ancient Biomolecules 1: 89-92, 1996.
21. Alvarez GA, Munoz I, Pestoni C et al. Effect of environmental factors on PCR-DNA analyses from dental pulp. Int J Leg Med 109: 125-129, 1996.
22. Kemp BM, Monroe C, Smith DG. Repeat silica extraction: A simple technique for the removal of PCR inhibitors from DNA extracts. J Archaeol Sci 33: 1680-1689, 2006.
23. Scholz M, Giddings I, Pusch CM. A polymerase chain reaction inhibitor of ancient hard and soft tissue DNA extracts is determined as human collagen type I. Anal Biochem 259: 283-6, 1998.