Comparison and Development of A Rapid Extraction Method of DNA from Ancient Human Skeletal Remains of Turkey

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Abstract

The use of genetic technology in forensic science is applied primarily to distinguish between individuals who may be the source of biological material associated with archeological remains. DNA sequences from ancient fossils have great potential for studies of phylogeny, biogeography and molecular evolution. DNA from fossils also facilitates the rigorous testing and calibration of mutation rates among related taxa, sex test and molecular divergence time 2, 3. In order to isolate pure and high amount of DNA from fossil bones, these fossils are believed to be chemically well preserved because of the low oxygen content and cold temperatures of the water in which they were deposited 9. In this study, a rapid and quantitative aDNA extaction methods from human skeletal remains was developed for application of forensic science and archeometry. For that reason, DNA was extracted from ancient human bones from Mugla in Turkey. Extraction of DNA was carried out using the laboratuary handling and cleaning protocol. After cleaning of bone, small piece of ancient bones were ground to powder with a mixer mill. Aliquots of the powder were subjected to a calfication method and extracted with 0.5 M EDTA (pH 8.3) for 48 hours at 56 °C. After addition of proteinase K, solution of bone was incubated at 37 °C. Genomic DNA from supernatant was extracted automatically by using EZ1 Automatic Nucleic Acid Isolation System (Qiagen, Germany) with investigator kit (Qiagen, Ilden, Germany) and different DNA extraction methods which are modified by researcher from ancient bones. EZ1 Nucleic acid isolation method; This tehnique is quite useful for high yield and quality of aDNA isolation from human skeletal remains. In this method, no further purification was needed for molecular analysis. Amount and purity of extracted DNA from ancient bones were measured by Spectrophotometer. In addition to spectrophotometric measurement, extracted DNA was applied to 1 % agarose gel, stained and imaged under ultraviolet (UV) irradiation. As a result, 50 ng pure DNA was extracted from ancient bones.

INTRODUCTION

An important goal of any forensic investigation involving unidentified human fossil remains is positive identification. Forensic specialists including anthropologists and odontologists may evaluate the remains, estimating the individual's sex, stature, age at death, and ancestry. Any identifiable characteristics such as fingerprints, if flesh is still intact, unique skeletal features, and dental arrangement are also noted. However, when antemortem reference records are not available for comparison or remains are fragmented or otherwise in a state in which definitive conclusions cannot be made as to the person's identity or sex determination. DNA analysis may be required. Indeed, identification through analysis of DNA from human skeletal remains has been used in numerous cases, beginning in 1989 [10]. Advancements in techniques and applications occurred in the 1990's [14511] reviewed in [12], and in 1991 the Armed Forces DNA Identification Laboratory was established for the identification of the remains of U.S. military personnel [7]. Bones encountered by the forensic biologist often vary in their degree of degradation, and if more than one bone is available, the scientist may sensibly choose which bone to analyze based on its appearance; a bone in good condition would logically contain 'better' DNA than a more weathered sample. However, the lack of any proven association between the degradation level of a bone sample and the quality or quantity of its DNA means that it is currently difficult or impossible to reliably predict DNA typing success for aged bones. Further, because of the high levels of variability among aged skeletal samples that have been studied to date (including bone type, age, the environment where it was found, etc.), DNA preparation and typing techniques that have been shown to work well on one sample may not be useful for another. If criteria were available for relating the appearance of aged bone to the DNA within, the

efficiency, affordability, and reliability of analysis could be greatly enhanced. Given the type of skeletal material (long bone, flat bone, tooth, etc.) and its level of weathering, valuable predictions could be made about the probable amount of obtainable DNA and its level of degradation, thus leading the scientist to the best genetic loci and polymerase chain reaction (PCR) primers for analysis. DNA fragment length isolated from ancient DNA is typically small [12]. To more exactly pinpoint useful size ranges for analysis, PCR can be used to determine the largest size class of DNA existing in each sample by targeting a series of amplification products. Progressively larger segments of DNA can be amplified until a negative result is obtained, this cutoff size can indicate how degraded a sample is. Amplicon sizes can then be compared within a weathering stage to see what generalities can be observed, within a stage and among stages, to discern any statistically meaningful differences in degradation levels.

MATERIAL AND METHOD

COLLECTION OF SAMPLES

A subset of 100 bones from the total set obtained from Mugla in Turkey was analyzed in this study. Upon recovery of the skeletal remains, the bones were described in terms of sex, estimated age, and some of the skeletal weathering stages. In order to allow ratings on individual bones, a new staging system was developed at Archeometry Laboratory in Selcuk University, Arts and Science Faculty, and assigned as period or era each bone based on visual inspection for the DNA study (Table 1). The bone samples of more than 100 individuals were chosen to study the genetics of this skeletal population.

Figure 1

Table 1: The ancient ruins of Koranza and Necropal area are situated in the region of modern city Mugla in Turkey. More than hundred ancient tombs has been excavated and a lots of grave gifts and skeletal remains were found in this graves.

NUMBER OF BONE SAMPLES	CODES	PERIOD	LOCATION SITE	EXCAVATION REGION
2	05BM13	ROMA	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM22	GEÇ HELLENİSTİK-ERKEN ROMA	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM09	GEÇ KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM29	HELLENİSTİK-4.YY	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM40	HELLENİSTİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM21	GEÇ HELLENİSTİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM05	GEÇ GEOMETRİK-M.Ö.730-680	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM23	HELLENİSTİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM05	GEÇ KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM40	HELLENİSTİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM13	ROMA	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM37	ROMA	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM85	HELLENİSTİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM118	GEOMETRİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM13	GEÇ HELLENİSTİK-ERKEN ROMA	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM39	GEÇ KLASİK-M.Ö.377	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM27	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM01	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM14	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM41	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM22	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM02	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM17	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM64	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM29	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM37	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM31	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM01	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM42	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM26	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM30	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM58	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM95	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM106	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM18	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM100	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM10	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM02	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM11	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM25	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM05	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM55	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM29	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM13	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM42	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM39	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM25	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM23	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM45	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA

CONTAMINATION CONTROLS

All DNA extractions and PCR setups were carried out in a dedicated ancient DNA laboratory following the suggested protocols for contamination controls and detections.[6] All bone samples and extraction reagents were exposed to UV irradiation. Furthermore, All post-extraction manipulations were conducted by H.C.Vural. Disposable laboratory coats, gloves, fitler tips, dedicated pipetmen, and disposable laboratory ware were used throughout the analyses. Benches and equipment were frequently treated with a 20% bleach solution. Sterile water was aliquoted and irradiated by placing the tubes directly on a light source of 254 nm for 30 min.[14] Two extractions were prepared for each bone sample by two researchers to test reproducibility and aDNA quality. The amount of contaminant DNA in this study was probably not significant.

SAMPLE PREPARATION AND DNA ISOLATION

Approximately 1 cm_3 of bone was cut from the source section using a Dremel MultiPro tool and was collected in a tube. Samples were then immersed in filter-sterilized wash buffer (1% SDS, 25 mM EDTA) and 0.1 mg/ml proteinase K, and incubated for one hour at room temperature.

Following the incubation, the wash buffer was poured off and each sample was washed with 1ml of sterile dH_2O six consecutive times. Samples were allowed to air dry. Bone powder from the dried bone samples was collected in one of two ways. Bone was either ground to powder drilled using a the Dremel tool both fitted with 1/16 microfuge tube and weighed. Four hundred microliters of digestion buffer (20 mM Tris, 100 mM EDTA, 0.1% SDS) and 0.4 mg/ml proteinase K was added to each ground bone sample and incubated overnight at 56 ºC. A standard phenol/chloroform organic extraction was performed on each of the samples. DNAs were precipitated using 3M sodium acetate and 95% ethanol, vacuum dried, and resuspended in TE buffer (10 mM Tris, 1 mM EDTA) based on the original mass of the bone powder. Furthermore, After addition of proteinase K, solution of bone was incubated at 37[[[°]]]C. Genomic DNA from supernatant was extracted automatically by using EZ1 Automatic Nucleic Acid Isolation System (Qiagen, Germany) with investigator kit (Qiagen, Ilden, Germany) from ancient bones. Amount and purity of extracted DNA from ancient bones were measured by Spectrophotometer. In addition to spectrophotometric measurement, extracted DNA was applied to

1% agarose gel, stained and imaged under ultraviolet (UV) irradiation. As a result, 50 ng pure DNA was extracted from ancient bones. Several precautions were taken to prevent contamination during the experiments. Grinders and drills used to generate bone powder were washed with 70% EtOH and 10% bleach, and were UV irradiated between each sample prep. Pre-amplification and post-amplification steps were carried out in separate rooms. Finally, negative controls and reagent blanks were included in all experiments (Figure 1 and 2).

Figure 2

Figure 1: Genomic DNA was isolaled from fossil bone tissue remains, respectively, Lane 1, 2, 3-13 with Bio Robot EZ1. aDNA samples submitted to electrophoresis in 1% agarose gel. Sample codes, respectively, 05BM13, 05BM22, 06BM09, 05BM29, 06BM40, 05BM21, 07BM05, 05BM23, 06BM39, 07BM13, 05BM64, 05BM30, 05BM106 illustrated in the table 1

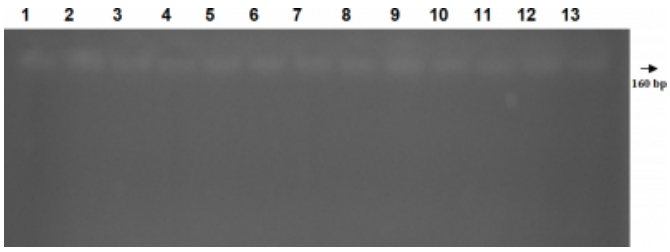

Figure 3

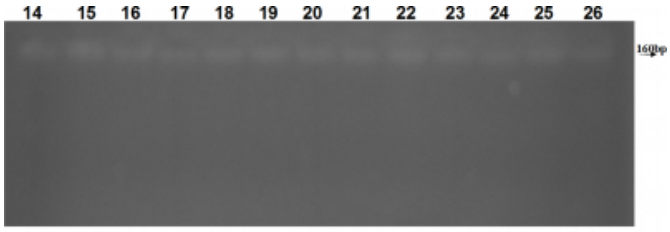

Figure 2. Genomic DNA was isolaled from fossil bone tissue remains, respectively, Lane 14, 15, 16 - 26 with Bio Robot EZ1. aDNA samples submitted to electrophoresis in 1% agarose gel. Sample codes, respectively, 06BM45, 06BM23, 06BM42, 06BM29, 06BM55, 06BM11, 07M02, 06BM10, 05BM100, 06BM18, 05BM95, 05BM58, 05BM30 illustrated in the table 1.

As for the other methods which is used in this study; powdered bone for DNA extraction, was generated by grinding bone fragments under liquid nitrogen in the classic 8000M Mixer/Mill existent in our laboratory. The bone powder was then placed in 6,000-8,000 molecular weight cut off dialysis tubing and immersed in 0.5 M EDTA, pH 7.3, at 4 °C until complete decalcification had occurred. Due to the high collagen content of the skeletal material and the low temperature used for decalcification, complete decalcification required approximately 2-3 weeks. The EDTA extract was washed extensively with HPLC water and concentrated by passage adding the 200 ml to a Amicon Microcon YM30 Filter (Milipore CAT # 42410) placed in an eppendorf tube. Each fitler was washed five times with HPLC water, and the washes were processed as blanks in order to detect cross-contamination between YM30 filtrations. To decontaminate the filtration unit, it was boiled between samples. Experiments were performed to test the efficacy of bleach or EDTA pretreatment in reducing DNA contamination of the skeletal material. Bleach treatment involved immersing the powdered bone in 20% bleach for 2 min followed by extensive HPLC water washing. The EDTA

protocol consisted of a 2 day treatment with 0.5M EDTA at 55 °C. Following each pretreatment, DNA was extracted by immersing the powdered bone in 0.5 M EDTA at 4°C until complete decalcification occurred. As the other method, physically powdered bone (750 mg) was suspended in 1.6 ml extraction buffer (0.1 M EDTA, 0.5% N-laurylsarcosine-Na salt, 100 mg/ml proteinase K), vortexed and incubated overnight at 37 °C with continuous vertical rotation. After phase separation by centrifugation at room temperature at 12.000 r.p.m. for 10 min, 250 µl supernatant was transferred to a 1.5 ml eppendorf tube and 3.5 µl 1 µg/µl Dextran Blue (Fermentase), 250 µl 4 M NH_4-acetate and 500 µl 96% EtOH were added and mixed by vortexing. Dextran Blue has large size (greater than 2 million molecular mass), effectively coprecipitates low concentrations of DNA and colours the pellet. PCR is inhibited in a dose-dependent manner at concentrations of Dextran Blue only>125 µg/ml. It remains in the well during the gel run and thus does not interfere with sequence recordings [8]. The DNA was precipitated at -70°C for 7 min and centrifuged at 14.000 r.p.m. at 4°C for 15 min. The pellet was redissolved in 20-30 µl deionised water. The remaining extract was stored at -20°C.

AUTHENTICITY OF ANCIENT DNA

Precautions commonly taken for aDNA work (i.e. the use of protective clothing and the processing of various types of control samples such as mock extractions and no-template samples) were followed during all steps of sample preparation, DNA extraction and amplification in order to minimise the risk of false positive results due to modern contamination[15]. All archaeological sample material was extracted twice in independent processes.

ANCIENT DNA QUANTITY

Genomic DNAs isolated from fossil bone remains were showed by spectrophotometric analysis. DNA quality and concentrations were evaluated nearly 1.8. Genomic DNA from supernatant was extracted automatically by using EZ1 Automatic Nucleic Acid Isolation System (Qiagen, Germany) with investigator kit (Qiagen, Ilden, Germany) from ancient bones. Amount and purity of extracted DNA from ancient bones were measured by Spectrophotometer and then extracted DNA was applied to 1% agarose gel, stained and imaged under ultraviolet (UV) irradiation. As a result, 50 ng pure DNA was extracted from ancient bones. Several precautions were taken to prevent contamination during the experiments. EZ1 Nucleic acid isolation method; This tehnique is quite useful for high yield and quality of aDNA isolation from human skeletal remains. In this methods, no further purification was needed for molecular analysis.

RESULT AND DISCUSSION

Studies on aDNA are primarily useful for the advancement of knowledge in a new field of research. The results acquired so far open a new frontier on problems related to aDNA characteristics as data are collected from different archeological sites. At present, one of the most concrete conclusions to be drawn is that multidisciplinary studies play an essential role in studying an archeological site using biomolecular research techniques. On the other hand, it seems clear that each archeological site exhibits peculiar characteristics. Consequently, bone diagenesis features and aDNA damages may be different or may be present to different extents. Thus aDNA extraction methods and PCR strategies must be adjusted to the particular characteristics of each site. As far as mtDNA is concerned, experience has shown that these molecules are amplifiable most of the time. Nuclear genes, in the past only occasionally amplifiable, today can be successfully rescued even taking advantage of the physical features of the aDNA, since some damages are substrates of DNA enzymes. Moreover, it is well established that animal remains are extremely useful in authenticating aDNA data from a given archeological site. Exploring the genetic structure of ancient populations through the application of molecular biology techniques can answer a number of these questions. It is noteworthy that the knowledge of DNA nucleotide sequences of ancient animals, plants, and bacteria might also provide a spin-off to many other fields, including phylogenetic relationships of extinct animals, plant breeding, and the spread of infectious diseases. These studies provide invaluable contributions toward understanding the characteristics of burial conditions and the physical history of archeological sites. This kind of information helps by acquiring preliminary data on bone preservation conditions, such as histological evaluation, before performing aDNA analysis. The analysis of bone preservation can highlight archeological sites at risk for aDNA investigations, prone to rapid bone destruction and DNA degradation. This subject is indispensable for establishing sex and age of individuals and for skeleton reconstruction useful in selecting bones for DNA extraction. They are also irreplaceable in the recognition of skeletal traces of genetic (i.e., thalassemia), infectious (i.e., tuberculosis), or acquired diseases, population genetics, phylogeny, taxonomy, and relationships, and nourishment

custom.

From the experiments and results presented here it seems clear that skeletal appearance can be used as a reliable metric of aDNA quantity or quality, and indeed statistically significant result obtained was that more weathered burials resulted in higher DNA yields (other factors being equal). Likewise, with the preservation of contamination risk as to the weathering stage of an individual bone is a useful measurement of DNA quantity or quality. On the other hand, bone type plays a substantial role in DNA results, particularly the ability to successfully produce an amplicon. This should be taken into account when selecting skeletal material for processing. Owing to its extreme sensitivity precautions need to be made, particularly running reagent blanks and negative controls to test for contamination, however with these in place DNA results can be obtained from material that would otherwise generate a negative result. This article presented leads to a number of conclusions. DNA quantity, while related to PCR results, can act as a hard and fast assay for downstream PCR success; bones with the lowest level of DNA were capable of generating results. DNA quality does not relate to bone/skeletal appearance. This study does not invalidate the notion that contamination must be strictly controlled and false results must be removed from ancient DNA studies. Instead, this study clearly demonstrates how tiny amounts of modern human DNA may become a major problem in ancient DNA studies. The greatest efforts should still be made to minimize contamination levels. We employed a simple, effective EZ1 isolation method to extract sufficiently pure aDNA required for successful PCR. The results confirm that aDNA can be extracted and amplified in ancient skeletal remains as old as era. The quantity and purity of extracted aDNA was evaluated by means of agarose gel electrophoresis and UV spectrophotometry. As expected, there was clear band of DNA corresponding to genomic DNA. Our findings indicate the high quantity and quality for retrieved aDNA. Furthermore, the main aim of the current research was improved the best effect uncontaminated aDNA isolation protocol for molecular identification of excavated skeletal remains, and at the same time we aimed set up our aDNA extraction and amplification facility and testing the authenticity of extracted aDNA, and to observe and eliminate any potential contamination of aDNA with modern DNA.

CONCLUSION

The protocol was efficient in extracting genomic DNA from all fossil bone remains. Analysis of whole genomic DNA in agarose gel (Figure 1 and 2) and amplified fragments (106 and 112 bp) by PCR (the other article) demonstrated that the extracted DNA had high molecular weight, one of the most important aspects for successful amplifications of larger fragment. Spectrophotometric measurements indicated differences in aDNA concentration and purity, according to the bone tissue origin (Table 1). The OD260/OD280 ratio values satisfied those suggested by Sambrook et al. (1989), approximately 1.8. This protocol proved to be advantageous because of its simplicity, quickness and affordable reagents, besides the high molecular weight DNA and purity achieved in a variety of fosil bone tissues from the total set obtained from Mugla in Turkey. Furthermore, there is no phenol in aDNA purification, known as a strong PCR inhibitor. A collaborative arrangement such as this, in which all research using modern DNA is conducted in one laboratory while the ancient specimens are analyzed in another laboratory, is critical for successful aDNA studies.

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