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Identification Gender of Ancient Human DNAs from Koranza Skeletal Remains in Turkey Using Molecular Techniques

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Abstract The ancient ruins of Koranza and Necropal area are situated in the region of modern city Mugla in Turkey. More than hundred ancient tombs have been excavated and a lot of grave gifts and skeletal remains were found in this graves. According to this finds, the date of this graves goes back to the 7th century B.C. Sex identification of ancient human is essential for the exploration of gender differences in past population. Gender has been traditionally determined through the identification of grave goods and by bone morphometric analysis. In order to identify gender of ancient human, molecular techniques especially typing for a length variation in the X-Y homologous amelogenin gene (AMEL X and AMEL Y) for that reason, DNA sequences specific to the X and Y chromosomes may provide an ideal solution. In this study, we describe here ultra-sensitive sex analysis of forensic and fossil samples by amplifying X and Y homolog of the amelogenin gene (AMEL X and AMEL Y) for sex determination on molecular levels. As a result, sex gender of ancient human bones was determined related with DNA fragments with different length of base pair as male and female.

Keywords Ancient; Fossil bone; DNA isolation; PCR; aDNA; Forensic samples; Gender identification
Abbreviations: aDNA (ancient DNA)

Introduction

The use of molecular technology in forensic science is applied primarily to distinguish between individuals who may be source of biological materials associated with archaeological remains. In addition to, the study of ancient DNA plays an important role in archeological and palaeontological research but also in pathology and forensics. Ancient DNA analyses are widely used for evolutionary and phylogenetic study of mankind in anthropology and archeology. Especially, sex identification of ancient human is essential for the exploration of gender differences in past population. Investigation of gender differences plays an important role in archaeologic reconstriction of the structure of past societies and particularly demography of historical socities based on skeletal remains from cemeteries (Stone A.C., et.al., 1996). Gender has been traditionally determined through the identification of grave goods and by bone morphometric analysis. However, traditional morphometric analysis fails to identify the gender of incomplete skeletal remains. A method for determining the sex of human skeletons was developed using molecular genetic techniques. The amelogenin gene, found on the X and Y chromosomes, was examined using the polymerase chain reaction (PCR) and a nonradioactive dot blot procedure. DNA was analyzed from 50 individuals of known sex and 100 bones from the total set obtained from the ancient ruins of Koranza and Necropal area are situated in the region of modern city Mugla in Turkey. More than hundred ancient tombs have been excavated and a lot of grave gifts and skeletal remains were found in this graves. According to this finds, the date of this graves goes back to the 7th century B.C. With burials there are two types of information which can be compared - the biological sex of the burials, and the nature of the grave goods interred with them; these data can be

analysed to categorise the objects that typically accompany male and female burials, and anomalies from the norm can be identified. To do this the archaeologist needs a reliable method of identifying the sex of human remains. Current physical anthropological methods work well with complete remains, but with incomplete skeletal material, cremations or juvenile/infant remains the accuracy of these methods decreases and the element of subjectivity increases. Much research effort has been put into the development of a simple, PCR-based test to identify the sex of human remains. The extraction of ancient DNA from human remains and the amplification of sequences from the X and Y chromosomes in theory should provide a robust and objective method of identifying the genetic sex of an individual. In order to identify the gender of ancient human, typing for a length variation in the X-Y homologous amelogenin gene (AMEL X and AMEL Y) (Manucci A., et. al., 1994 and Mitchell R.J., et.al., 2006). For that reason, DNA sequences spesific to the X and Y chromosomes may provide an ideal solution. This sex-typing test is easily performed using the AMEL 106 and 112 bp primers. In mammals, AMEL is reported to possess seven exons and two extra exons (8 and 9) are found in human (Figure 1). Amelogenin plays a crucial role in enamel structure and mineralization, but the function of its various domains is far to be understood. Evolutionary analysis seems to be a promising way to approach structure and function relationships. In this paper, we focus on amelogenin

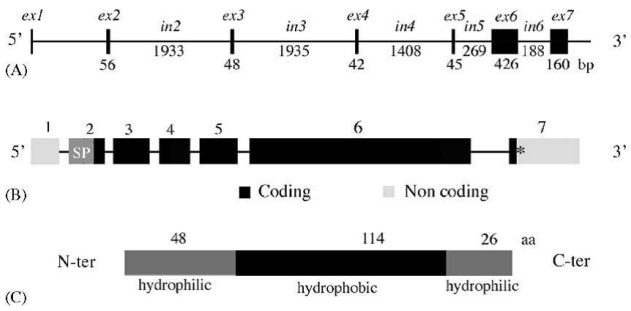

Figure 1 Amelogenin gene. (A) Gene structure. The five first exons (ex1-ex5) are small (42-56 bp) and exons 6 (426 bp) and 7 (160 bp) are large. (B) Coding and uncoding exons. Exon 1 is uncoding, most exon 2 sequence codes the signal peptide, and the only first three nucleotides of exon 7 are coding for the protein. (C) Linear representation of the native protein

genes (AMEL) where the amelogenin gene is encoded on the short arm of both the X (AMELX) and Y (AMELY) chromosomes in humans. Although AMELX and AMELY are homologous, they are different in size and sequence and utilized for the PCR-based sex test (Manucci A., et. al., 1994). Genetic difference between male and female is the presence or absence of chromosome Y. Therefore, many of existing methods for sex identification are based exclusively on the detection of sequences specific for chromosome Y (Bondioli et al. 1989, Hagelberg E, 1991, Schröder et al. 1990, Wayda et al. 1995). However, the absence of a signal does not necessarily mean that the sample is of female origin, as negative results may also be generated by experimental errors. Thus, the detection of Y- and X-chromosome specific sequences is advantageous. Amelogenin gene (AMEL), the origin of which 112 has been traced back to the Precambrian period (Delgado et al. 2001) has been used as a model in the field of molecular phylogenetics (Delgado et al. 2005, Toyosava et al. 1998) as well as in the analyses of biological traces aiming at determining the sex in humans and animals (Sullivan et al. 1993, Ennis and Gallagher 1994, Reklewski et al. 1996, Lechniak and Cumming 1997, Pfeiffer and Brenig 2005). In forensic science, designing a single primer pair for the amelogenin provides the investigation of X and Y sequences simultaneously for sex identification in ancient bones. With only a single PCR reaction and agarose gel electrophoresis, molecular sexing was reliable and fast. Furthermore, the contamination with human DNA is sometimes a problem during the laboratory analysis. However, the amplification of the human AMEL gene with the same primers resulted in a different and clearly distinguishable banding pattern. Our primer design procedure and PCR amplification provided an excellent tool to DNA from ancient bone. In conclusion, our study shows how the use of genetic markers of different mutability might provide an insight into the history of past necropolises. It also provides genetic data on ancient Koranza and Necropal area specimens that could help to confirm or disprove models developed from modern genetic data to explain population history. Finally, it provides an excellent tool to select samples of interest for

interpopulation analyses. In conclusion, we have established that DNA can be recovered from ancient bone, often less degraded than the DNA recovered from soft tissue remains, and that aDNA sequences can be amplified consistently from many archaeological samples. In addition, archaeologists and conservators will need to learn of the potential for genetic information in excavated skeletal remains, and to develop appropriate methods for the removal and storage of samples for future study. We are now beginning to work on a wider range of bone samples, and although the oldest bones we have studied so far date to only about 5000 years BP (Hagelberg et al. 1989), the analysis of chloroplast DNA from a 17-20 million-year-old magnolia leaf (Golenberg et al. 1990) suggests that in exceptional circumstances DNA may survive indefinitely, which augurs well for the study of hominid evolution. Although archaeology, anthropology and forensic science will probably profit most from PCR bone analysis, these methods will also be applicable to studies of evolutionary and population biology, for example in resolving questions of the phylogenetic relations between extinct and living taxonomic groups. In the present work, we have established a sensitive and reliable aDNA extraction and a PCR reaction based on amplifying part of intron 1 of the AMG- amelogenin encoding gene- is more suitable. The method facilitates analysis of samples with degraded DNA, such as frequently encountered in archaeological specimens, with high confidence. Furthermore, this study demonstrates the applicability of the methods for gender determination in skeletal remains from different periods in Koranza and Necropal area are situated in the region of modern city Mugla in Turkey.

1 Material and Method

1.1 Collection of Samples

A subset of 100 bones from the total set obtained from 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. According to this finds, the date of this graves goes back to the 7th century B.C. Upon recovery of the skeletal remains, the bones were described in terms of sex, uncontaminated or clear and pure DNA, estimated age, and skeletal weathering stages. In order to allow ratings on individual bones, a new staging system was developed in our laboratory and assigned to each bone based on visual inspection for the DNA study (Table 1).

1.2 Contamination Prevention of Fossil Human Bones

Ancient DNA research has proved a powerful tool for studying archaeological remains, but technical problems, as the degradation into small bits (100 to 200 bp on average), the oxidation, the effects of background radiation and the exogenous contamination, continue to make these degraded molecules extracted from fossil and subfossil difficult to study. The only solution is to adopt strict laboratory precautions. To prevent any possible risk of contamination, several precautions were adhered to: These; with DNA extraction and PCR studies, sample preparation workings were done dedication of equipment to the different laboratory rooms. Furthermore, no-template controls were used in every PCR run to monitor contamination of reagents.

1.3 Decalcifications of Bone and Genomic DNA Extraction

Extraction of DNA was carried out using the laboratory handling and cleaning protocol (Rӧmpler H., et. al., 2006). After cleaning of bone with chromatographic water, small piece of ancient bones were ground to powder with a mixer mill. Aliquots of the powder were subjected to a calcification method. 150 mg of bone powder was extracted with 0.7 ml of 0.5 M EDTA (pH 8.3) for 48 hours at 56^{o}C. After addition of 1U of proteinase K, solution of bone was incubated at 37^{o}C. Genomic DNA from supernatant was extracted automatically by using EZ1 Automatic DNA Isolation System (Qiagen, Germany) with investgator kit (Qiagen, Ilden, Germany) from ancient bones. Amount and purity of extracted DNA from ancient bones were measured by Shimadzu UV 1700 Spectrophotometer. Extracted DNA was then stored at -20 ℃ until assay for the amelogenin was performed (Figure 2).

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 Hellenistik-Erken Roma	Börükçü	Yatağan/Muğla
2	06BM09	Geç klasik	Börükçü	Yatağan/Muğla
2	05BM29	Hellenistik-4.yy	Börükçü	Yatağan/Muğla
2	06BM40	Hellenistik	Börükçü	Yatağan/Muğla
2	05BM21	Geç hellenistik	Börükçü	Yatağan/Muğla
2	07BM05	Geç geometrik-m.ö.730-680	Börükçü	Yatağan/Muğla
2	05BM23	Hellenistik	Börükçü	Yatağan/Muğla
2	06BM05	Geç klasik	Börükçü	Yatağan/Muğla
2	05BM40	Hellenistik	Börükçü	Yatağan/Muğla
2	06BM13	Roma	Börükçü	Yatağan/Muğla
2	05BM37	Roma	Börükçü	Yatağan/Muğla
2	05BM85	Hellenistik	Börükçü	Yatağan/Muğla
2	05BM118	Geometrik	Börükçü	Yatağan/Muğla
2	07BM13	Geç hellenistik-Erken Roma	Börükçü	Yatağan/Muğla
2	06BM39	Geç klasik-M.ö.377	Börükçü	Yatağan/Muğla
2	06BM27	Klasik	Börükçü	Yatağan/Muğla
2	05BM01	Klasik	Börükçü	Yatağan/Muğla
2	07BM14	Klasik	Börükçü	Yatağan/Muğla
2	05BM41	Klasik	Börükçü	Yatağan/Muğla
2	06BM22	Klasik	Börükçü	Yatağan/Muğla
2	06BM02	Klasik	Börükçü	Yatağan/Muğla
2	07BM17	Klasik	Börükçü	Yatağan/Muğla
2	05BM64	Klasik	Börükçü	Yatağan/Muğla
2	06BM29	Klasik	Börükçü	Yatağan/Muğla
2	06BM37	Klasik	Börükçü	Yatağan/Muğla
2	05BM31	Klasik	Börükçü	Yatağan/Muğla
2	06BM01	Klasik	Börükçü	Yatağan/Muğla
2	05BM42	Klasik	Börükçü	Yatağan/Muğla
2	05BM26	Klasik	Börükçü	Yatağan/Muğla
2	05BM30	Klasik	Börükçü	Yatağan/Muğla
2	05BM58	Klasik	Börükçü	Yatağan/Muğla
2	05BM95	Klasik	Börükçü	Yatağan/Muğla
2	05BM106	Klasik	Börükçü	Yatağan/Muğla
2	06BM18	Klasik	Börükçü	Yatağan/Muğla
2	05BM100	Klasik	Börükçü	Yatağan/Muğla
2	06BM10	Klasik	Börükçü	Yatağan/Muğla
2	07BM02	Klasik	Börükçü	Yatağan/Muğla
2	06BM11	Klasik	Börükçü	Yatağan/Muğla
2	06BM25	Klasik	Börükçü	Yatağan/Muğla
2	07BM05	Klasik	Börükçü	Yatağan/Muğla
2	06BM55	Klasik	Börükçü	Yatağan/Muğla
2	06BM29	Klasik	Börükçü	Yatağan/Muğla
2	07BM13	Klasik	Börükçü	Yatağan/Muğla
2	06BM42	Klasik	Börükçü	Yatağan/Muğla
2	06BM39	Klasik	Börükçü	Yatağan/Muğla
2	06BM25	Klasik	Börükçü	Yatağan/Muğla
2	06BM23	Klasik	Börükçü	Yatağan/Muğla
2	06BM45	Klasik	Börükçü	Yatağan/Muğla

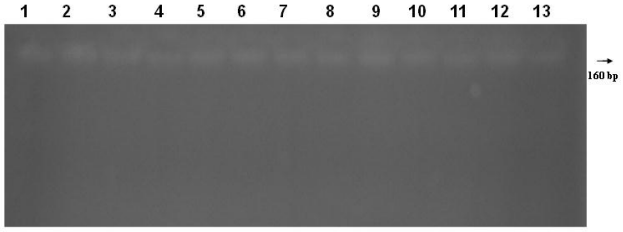

Figure 2 Genomic DNA was isolaled from fossil bone tissue remains, respectively, Lane 1, 2, 3-13 with Bio Robot EZ1. aDNA samples submitted to electrophoresis in 1% agarose gel. Sample codes, respectively, 05BM13, 05BM22, 06BM09, 05BM29, 06BM40, 05BM21, 07BM05, 05BM23, 06BM39, 07BM13, 05BM64, 05BM30,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

1.4 Digestion of Fossil DNA by Restriction Endonucleases

In order to have a better distinguish between product sizes of AMG gene, the PCR products were digested by EcoRI/HindIII (Fermantase Life Science) restriction enzyme.

1.5 Restriction digestion of DNA protocols

Protocol 1 containing; 2 µl 1Xbuffer, 0.5 µl Lambda EcoRI/HindIII, 0.2 µl 10XBSA, 13 µl water and 2 µl genomic DNA. 55℃ and 2 hours incubation respectively in lane 2 and 3 identified with protocol 1.

Protocol 2 containing; 2.5 µl 10XBuffer, 0.6µl Lambda EcoRI/HindIII, 1 µl RNase, 16.9 µl water and 4 µl genomic DNA. 37 ℃ and 3 hours incubation respectively in lane 4 and 5 identified with protocol 2. After digestion, the reaction mixture was electrophorosed through 0.8% agarose in 50xTAE buffer. The sample was also tested for nuclease activity (Figure 3).

1.6 Polymerase Chain Reaction (PCR) Amplification of Sex Determination

Ancient DNA (aDNA) sex identification was used to aid in the verification of individual identification through comparisons to historical documentation of burials and small sizes human fossil skeletal bones estimations of sex. The PCR reaction is manipulated through primer design to favour the amplification of the Y fragment over the X fragment thus minimizing the occurrence of 'false female' results for male samples. In this study, the primers for PCR amplifications used are as follows:

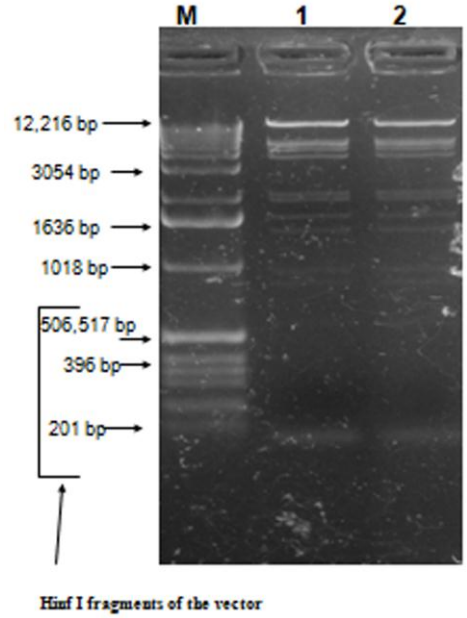

Figure 3 Screenning of agarose gel electrophoresis of fossil bone DNA digested with restriction endonuclease. M: 1 kb ladder size standard marker or Hinf I fragments of the vector. Lane 1-2, genomic DNA isolated from fossil bone by Bio Robot EZ1 digested with restriction endonuclease

Sequence Amel-A (5'– CCCTGGGCTCTGTAAAGA ATAGTG –3')

Sequence Amel-B (5'– ATCAGAGCTTAAACTGGG AAGCTG –3')

These primers amplify a small region in intron 1 of the amelogenin gene that encompasses a deletion polymorphism giving a product of 106 bp for the X allele and a product of 112 bp for the Y allele, so both products should be present in males, but only one in females. 0,5 mg genomic DNA was amplified in a mixture composed of 5 µL 10XPCR Taq buffer (pH 8.8), 2 mM $MgCl_2$, and 10 mM dNTPs (dGTP, dATP, dTTP, dCTP) at each, 0.5 mM of each primer, and 0.3 U DreamTaq polymerase (Advanced Biotechnologies Ltd., Fermantase Life Science). Amplification was submitted to denaturation at 94 ^oC for 10 min, 50 amplification cycles with denaturation at 94 ^oC for 30 s, annealing at 60 ^oC for 10 min and extension at 72 ^oC for 1 min in a thermocycler (Biorad, Germany). PCR blank reactions did not show spot contamination during the collection of the data (Figure 4, 5). Studies of ancient DNA from museum and fossil samples can

provide valuable information toward a better understanding of degraded DNA preserved in postmortem specimens. This information helps to improve molecular techniques designed to recover and analyze old DNA to be used for evolutionary studies and as well as for forensic analysis. Our comparison of commonly used ancient DNA extraction techniques based on glass bead-based methods usually cause noticeable loss of genomic DNA during purification. We also found that the choice of extraction buffer may be critical to the success of recovering endogenous DNA from different types of tissue (for example, soft tissue, and bone material) preserved under different physical and chemical conditions. We have obtained results only either at the lowest or at the highest amounts of aDNA extracts analyzed. Multiple steps were taken during DNA amplification procedures to decrease the effects of PCR inhibitors found in the amplification reaction. For fossil material, PCR mixes were set up in dedicated hood in the ancient DNA laboratory using appropriate contamination control procedures and then brought to the main molecular genetics or archaeometry laboratory for thermocyling. For all ancient and modern reactions, amplification products were not detected in the negative extraction (Figure 4 and 5).

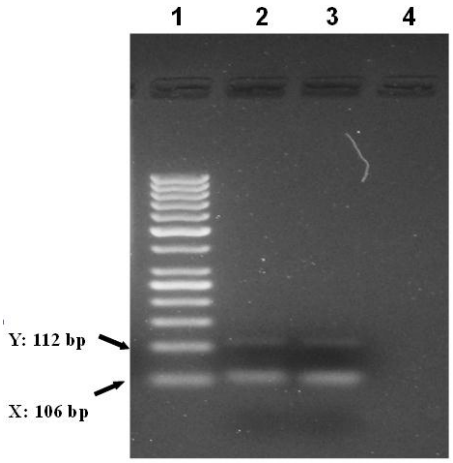

Figure 4 Sex determination based on amelogenin amplification (Amel A/B) in male and female fossil bones. Amel A/B indicate PCR fragments spesific to female 106 bp and male 106/112 bp. Agarose gel electrophoresis of PCR products male and female DNA templates. Lanes 1-4, Lane 1, 50 bp ladder size standard marker, Lane 2 male and lane 3 female, Lane 4 negative control water blank

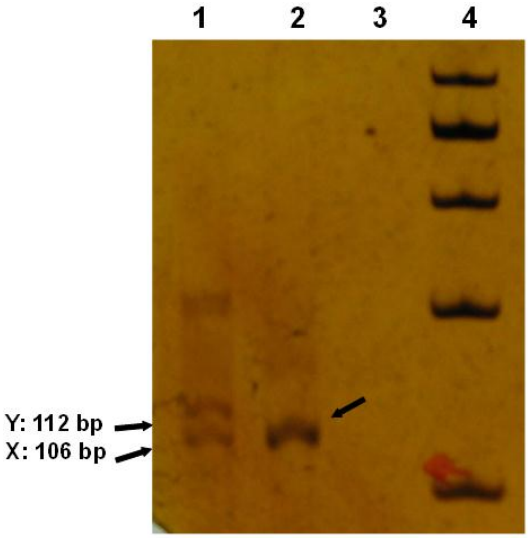

Figure 5 Polyacrilamide gel electrophoresis of PCR products in male and female fossil DNA templates or 106/112 bp amelogenin gene PCR products. Respectively, Lanes 1-4, Lane 1: amelogenin male sample 106/112 bp, and lane 2: amelogenin female PCR fossil sample 106 bp, Lane 3 negative control water blank or none DNA, Lane 4, 100 bp ladder size standard marker

1.7 Gel Electrophoresis of PCR Amplicons

PCR product was separated by electrophoresis on 2 % agarose gel in 1XTAE buffer (45Mm Tris, 1mM EDTA, pH 8), stained with ethidium bromide. In addition to electrophoresis of agarose gel, PCR products were completely loaded in 1.5 % acrylamide:bisacrylamide gels, stained with $Ag(NO_3)$ and agarose gel systems were visualized under UV, and Poly Acrylamide Gel Electrophoresis systems were illuminated from above using an white fluorescent light source (Figure 4, 5). We isolated the samples from a histological section of the burial place material and repeated the procedure three times. In each of the three repeated approaches, amelogenin could be amplified in all samples showing a successful DNA extraction (Figure 2). Amplification products generally showed weak signals in agarose gel analysis, presumably due to low amount of extracted material. Nevertheless, high-resolution polyacrylamide gel electrophoresis demonstrated that the ancient DNA is derived from a female individual, as in all amelogenin PCR products only the X-Chromosome specific 106 bp fragment was visualized (Figure 5).

1.8 Evaluation of Y Chromosome Lineage PCR-RFLP Analysis

Here we present Y chromosome lineage assays based on a more widely accessible technique Polymerase Chain Reaction- Restriction Fragment Lenght Polymorphism (PCR-RFLP). PCR products (male human bones) are always observed with following restriction endonuclease digestion protocol. Digested DNA fragments vary from 201 bp to 12.216 bp, but no single assay requires this range of DNA fragment sizes to be discriminated. All Y-chromosome lineages can be assigned correctly by examining restriction fragments between 201 bp to 12.216 bp, and these fragment sizes are easily distinguished following standart 2% agarose gel electrophoresis. RFLP protocol: Protocol containing 2 µl 10 x buffer, 0,5 µl Lambda EcoRI/Hind III , 0.2 µl 100 x BSA, 13 µl water and 2 µl PCR products belonging to female aDNA. 55 ℃ and 2 hours incubation. These protocols that require only a minimal molecular biology setup fulfil two niche roles. PCR-RFLP protocols require little experimental equipment and limited technical expertise.

1.9 Microsatellite DNA Profiling and Authentication

DNA was extracted from each sample at least twice. From each extract, at least four PCR amplifications were made. Only if two of four amplification reactions resulted in consistent allele determinations, and if this could be reproduced in another extract, were the alleles rated reproducible and thus authentic.

1.10 Statistical Analyses

Statistical analyses were performed in Microsoft Excel. Single factor Analysis of Variance (ANOVA) was used to examine the effect of weathering stage, sex, age, and bone type on the DNA quantity and quality results from the Koranza bone samples. ANOVA was also used to examine the effect of skeletal weathering on amplification success of multiple bones from a single individual, as well as the effect of skeletal weathering, bone weathering, and bone type on amplicon size. DNA quantities for sex and age statistics were averaged when multiple bones originated from a single individual. Amplification success DNA quantity was examined using a t-test with equal variance assumed. In all cases results were considered significant at $p<0.05$.

2 Results and Discussion

There has been growing interest in PCR amplification of DNA from ancient remains and preserved bone tissues, which is expected to provide important information in population and evolutionary studies. The extraction method from fossil bones employed in this study. In the present article, partial genealogical reconstruction was obtained using biparental, paternal, and maternal genetic systems in a sample of 100 human skeletal remains of which is provided by A. Ahmet TIRPAN and fellowships. The ancient ruins of Koranza and Necropal area are situated in the region of modern city Mugla. More than hundred ancient tombs have been excavated and a lot of grave gifts and skeletal remains were found in this graves. According to this finds, the date of this graves goes back to the 7th century B.C. Sex identification of ancient human is essential for the exploration of gender differences in past population. Investigation of gender differences plays an important role in archeology reconstruction of the structure of past societies and particularly demography of historical socities based on skeletal remains from cemeteries (Faerman M., et. al., 1995). To the best of our knowledge, no equivalent molecular analysis has been undertaken so far. Such a study was possible because the Koranza necropolis was mainly composed of relatively well-preserved skeletons. In this study, genomic DNA from 100 individual remains from Koranza Skeletal Remains in Turkey was extracted within one month after removal of bone from cave. Before extraction, cleaned specimens were stored at room temperature. Under optimal analysis conditions, they immediately were stored in lab frezeer. Before DNA extraction from bone, decalcification procedure was applied to all bone. In this stage, addition of an aliquot of proteinase K to bone powder was carried out at 56 ^{o}C in order to remove proteins such as collagen, osteocalcin and noncallogen structure from DNA. After removal of protein, genomic DNA extracted from each sample was extracted automatically by using EZ1 DNA isolation system (Qiagen- Germany) with investiagator kit (Qiagen). They monitored amount and purity of genomic DNA by UV spectrophotometer (Shimadzu UV 1700) at 260 nm/280 nm as 1,8 value. Image of DNA on 1 % agarose gel (W/V) in TAE buffer and

Polyacrylamide Gel Electrophoresis systems were illuminated from above using and white fluorescent light source was given in Figure 5. In this study, DNA based sex determination test was used to aid in the verification of individual identification in order to compare to historical documentation of burials. Applying sex determination test based on primers specific to human amelogenin on the short arm of both the X (AMELX) and Y (AMELY) chromosomes on genomic DNA from males and females. Therefore, single copy X-Y homolog related amelogenin was amplified under optimal PCR condition. The PCR amplification from DNA of different ancient bones in male and female using AMEL primers was displayed a sex-spesific band pattern showing a length variation characteristic for AMELX and AMELY. Female related to AMELY showed a single 106 bp band whereas male an additional band of 112 bp was observed from 50 bones (Figure 4). However, DNA from 10 bones didn't amplify the AMEL fragments due to contamination of DNA during preservation of bone on buriel. The typing results for the amelogenin locus are given in Figure 4 and 5. With the exception of fifty samples positive results could be obtained for all bones leading to a success rate of 90%. Ancient DNA (aDNA) and proteins provide valuable clues to questions about nutrition, domestication, population genetics, kinship reconstruction and human evolution. By investigating ancient biomolecules with the use of newer molecular biology techniques and robust procedures of inference genetic data from both archaeological remains and living populations, molecular anthropology has begun to draw more informed conclusions about human evolutionary history. Ancient DNA can shed light on the relationships between populations and how they dispersed through the ancient world and validate evolutionary hypotheses inferred from archaeological, linguistic and historical records. Also, aDNA can help solve archaeological puzzles and build up a picture of the demography of past societies by identifying the sex of skeletons that cannot be determined by osteology and to assess the degree of maternal relatedness in multiple burials. The remarkable thing about sexual differentiation is its diversity. That males are the heterogametic sex, larger than females, more aggressive than females, and the 'non-default' mode of sexual differentiation are concepts not valid throughout most of the animal kingdom. Sex chromosomes are characteristic only of land animals. In birds, the heterogametic sex is female and the sex chromosomes are not related to those of mammals. External factors such as temperature determine sex in lower vertebrates, and there is no similarity among sex-determining genes of different species.

3 Concluding Remarks

Ancient DNA has been proven to be useful genetic markers for studies of kinship, parentage, gene mapping and sex determination. They are highly polymorphic, easily scored, single-locus markers that are readily applied to small or degraded DNA samples, including museum specimens (Ellegren, H., 1991). Genetic typing of ancient bones means trying to obtain multilocus STR profiles from highly degraded, minimal, or even no detectable amounts of human DNA. Additionally, there are several other factors hampering a successful amplification, e.g. burial conditions such as temperature, humidity, and huminic acids. Our results were according to those findings since there was absolutely no correlation between the age of the bone and the amplification success. Therefore, our idea was to create an experimental approach to systematically investigate the age and environmentally caused degradation of DNA. Each 1 ml of DNA extract was employed to amplify 15 STR loci plus amelogenin sex determination simultaneously. The artificial aging process led to an amplification pattern which is supposed to be typical for aDNA with weaker signals or total allelic drop outs for the longer PCR products as described in Ref. Burger J., et.al., (1999), thus providing a hint for simulating the original degradation process as it occurs in bone tissues and for amplification of authentic aDNA from historical bones since the typing patterns were similar. The present method to determine sex employs PCR and a nonradioactive dot blot procedure to examine sequences from the amelogenin gene on the X and Y chromosomes (Herr C. M., et. al., 1990). One ancient individual was classified as a male based on morphology, but the PCR products hybridized only with the X specific

oligonucleotide. This may be the result of insufficient DNA, or mutations in the priming or oligonucleotide binding sites on the Y chromosome copy of the amelogenin gene. Another molecular technique for sex determination that has been applied to skeletal remains was developed by Sullivan et al. (1993). For this method, which also uses the amelogenin gene, a short DNA fragment from intron 1 is amplified that contains a ***6*** bp deletion in the X chromosome sequence that is not present in the corresponding Y chromosome sequence. Gill et al. (1994) employed this technique to determine the sex of skeletal remains found near Ekaterinburg, Russia, that were reputed to be those of the Romanovs, their doctor, and three servants. The method described in this paper, like the technique designed by Sullivan et al. (1993), solves the problem encountered when using the Y repeat sequences. Our method, however, appears to be more sensitive to very low quantities of DNA, such as those likely to be found in ancient bones. A dilution series testing the sensitivity of the primers indicates that they can begin amplification from as few as one or two copies of the gene**.** Sex determination using DNA can be valuable for both forensic and archaeological research. Standard osteological methods, however, are less expensive and more rapid when the skeletons of adults are complete and the bones are in good shape. For archaeological research, the use of DNA to determine the sex of juveniles provides an opportunity to extend traditional mortuary analyses through the inclusion of children of known sex. Molecular analyses can also address questions regarding the sex of adult skeletons that fall in the overlapping range of male and female morphological variation. By using this method in combination with routine genotyping more information about a material under investigation can be obtained. In addition, the amplification of the AMEL gene can also be used as an internal control.

In conclusion our findings show that the PCR assay based on the AMEL gene is reliable for sex identification of fossil bone remains in Koranza and Necropal area are situated in the region of modern city Mugla in Turkey. The advantage of this assay is that neither additional control amplicons with a second locus specific autosomal primer pair nor restriction endonuclease steps are necessary for sex determination and control of the PCR reaction. However, despite these objections and characteristic features of aDNA mentioned above, it can be shown that the molecular approach is the most powerful tool for the identification and reconstruction of kinship of skeletal human remains of archaeological excavations. These validated protocols allow the assignment of unknown men to every major branch of the global human population. Hopefully these protocols will encourage new research groups to implement a broader range of anthropological surveys, archaeological excavations and archaeometry studies etc. Furthermore, there is not the only parameter that determines the overall specificity and sensitivity of the PCR reaction; primer design and optimization of PCR parameters also have a profound effect. Results of the present our work demonstrate that the primers utilised in this test (Amel A and Amel B) provide robust and highly efficient amplification. It is envisaged that this test will prove to be an advantageous addition to other methods of forensic DNA analysis. The size difference between the amplified segments of X and Y copy of AMG was not big enough to be detected clearly on agarose or polyacrylamide gel electrophoresis (PAGE). For that reason, we searched the list of commercial restriction enzymes and find a new enzyme capable of recognizing and cleaving the PCR product for Y copy of AMG, but not the X copy. The molecular determination of gender based on AMG PCR/Restriction enzyme digestion was compared with anthropometric reports. At the beginning stages of the project the molecular sex determination was both different from anthropometric reports and also not reproducible. However, after optimizing the procedure and setting guidelines to eliminate the risk of contamination we were able to have reliable and reproducible molecular sex determination. According to study, 36 female from 100 samples, 10 of them do not proliferate in the rest of the samples are composed of male subjects.

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