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Molecular Diagnosis Through Genetic Typing of Skeletal Remains in Historical Populations of Situated Turkey

Hasibe Cingilli Vural[1,*], Ahmet Adil Tırpan[2], Evrim Tekeli[1], Seda Akarsu[2] and Babur Akarsu[2]
[1]*Selcuk Üniversity, Science Faculty, Department of Biology, Molecular Biology, Selçuklu, Konya*
[2]*Selcuk University, Art Faculty, Department of Archaeology Turkey*

1. Introduction

1.1 History of archeological excavation site

The Börükçü Location and The Lagina Hekate's Temenos are in the Muğla Province's Yatağan Town, in Turkey. In ancient world, The Börükçü Location and The Lagina Hekate's Temenos had been a part of Caria Region. At the ancient times, The Lagina Hekate's Temenos had a 9 km long holy way to the city Stratonicea, which is at the south of it (Akurgal E., 2000). An uninterrupted settlement has been seeing in this district since early bronze age (3000 B.C.) to present day (Güngördü E., 2003). Caria Region (Figure 1a-6b) had been having a mountainous geography and its name has been coming from "Car" people. They had a peculiar language and they were thinking themselves the natives of Anatolia (Güleç E., et.al., 2006). Anatolia has geographical regions because of its natural elements (Güngördü E., 2003). Since ancient period, these geographical regions has been shaping Anatolian people's life styles and connections betweeen each other. In Aegean region which contains Börükçü Location, The Lagina Hekate's Temenos and ancient Caria Region, mountains lines straight to the shore. These chains of mountains lines to Aegean Sea by becoming peninsulas, islands, promontories. Aegean Sea owns many islands and because of this, it's called the sea of islands. Approximately at the fourth geological period in Aegean Region, lands collapsed underwater, so Aegean islands existed (Baykara T., 1988 and Sevin V., 2001). At the same geological period, all Anatolia had the shape of present time and by the effects like; tectonic movements and outer factors, it still continues to change. Aegean shoreline is quite intricate. The Aegean pit plains are between mountain chains that usually progress in the east-west direction. And these pit plains have been progressing towards to sea by accumulation of silt that carried by rivers. So the ancient coastal cities and ports are left inside the land today.

Because of its physical elements, Aegean Region is divided into two parts, named; "Actual Aegea District" and "Inner Western Anatolia District". There's The Inner Western Anatolia

* Corresponding Author

Fig. 1a-1b. 05BM29 number, were found in tombs dated to the Hellenistic period, length 1.36 m as measured skeletal north-south direction and lies parallel to the floor. Hand side of the skeleton was taken from the hip and right leg below the left foot is on the case thrown. Bone on the spinal cord, a standing bronze coins were found. Dated to the fourth century BC coins on the front of the commander's head in profile Persian, the rear side has a relief of the monument.

District between horst and graben structered Actual Aegea District and The Central Anatolia Region. This district has a high altitude and prevents costal climate entering further inland but doesn't prevent tranportation. Aegean Region is generally under the influence of Mediterranean Climate, i.e. mild, wet winters and hot, dry summers. There are differences in climate between Actual Aegea District and Inner Western Anatolia District. Actual Aegea District is closer to The Mediterranean Climate. On the other hand, Inner Western Anatolia District is colder than Actual Aegea District, because of being far from coasts and its physical elements.

Ancient Caria Region's frontier contains today's Muğla, Aydın provinces' major areas and Denizli province's western end segment. At the ancient period, Caria Region is surrounded by, Ionia and Lydia Regions in the north, Phrygia and Lycia Regions in the east and southeast (Prag J., Neave R., 1999). The west and the south boundary of Caria Region is The Aegean Sea. Carian frontiers are generally known but it's not known exactly or in the course of time, it had undergone many changes like many other ancient regions. Some of the the local kindoms before VII. Century B.C. are known at present, but much earlier period of Ancient Caria Region isn't known much. This region had experienced the control of Lydia state at VII. century B.C. and in the first half of VI. century B.C. After the victory of Ionia cities, The Persians under the command of Harpagos marched to Caria Region and at 545 B.C. Persians took this region under control. Heredot notifies that; except the Pedasians brief resistance, there hadn't been any resistance against Persians in Caria (Mansel A. F., 1988) and (Olmstead A. T., 1960). With this victory, Persian Satrapy period began in Caria and many satraps reigned in this Region. At the begining of V. century B.C., although Persians decisiveness to control the west Anatolia, after some discontentions and movements, a rebellion began in Ionia Region. At the first times, Caria didn't join this rebellion but later Caria joined too. Then rebellion had spread to a large area and continued many years. At 494 B.C. despite some great victories obtained and Ionia cities' heroic battles, rebellion failed.

At 478/477 B.C. Athens established "The Attika-Delos maritime alliance" with the main purpose to fight against Persians. Greek sacred island Delos was the center of this alliance and this alliance had established by taking an oath;"to contineu forever". After a while Caria joined this alliance too but this union couldn't contineu much. Many of Carian and Lycian cities left the alliance and other cities had begun thinking negative abaut this alliance. At the begining of The Peleponnes War, the alliance ended completely (Akurgal E., 2000). At 395 B.C. Caria, itself had turned into a satrapy and its administration had given an indigenous family. After satrap Hecatomnus (395-377 B.C.) Mausolus became the satrap of Caria. He married with his sister Artemisia (Whitley J., 2001). Mausolus expanded the domain of Caria and embraced Hellenic culture. He moved capital from Mylasa to Halicarnassus and expanded the city within Greek style. He brought sculptors and architects to make Hellen culture dominant in the other cities of Caria too. After his death a monumental shrine, called Mausoleum, built by his wife Artemisia. This monumental shrine is known, the seven wonders of the ancient world (Tekin, 2007). In Roman Empire, emperors Augustus's and Hadrianus's monumental shrines were also called Mausoleum so, this shows the influence of Mausoleum of Mausolus in ancient world (Whitley J., 2001). At 353 B.C. Artemisia took over the sovereignty of Caria after the death of her husband Mausolos. After Artemisia, Hidrieos and then Hecatomnos's daughter Ada ruled Caria. Ada overthrown and exiled by Pixodaros, then Pixodaros began to reign Caria. After Pixodaros, Orontobates began to rule Caria. At 334 B.C.

Alexander the Great seized Caria Region and gave the sovereignty to Ada again. The Dominance of Seleucid Empire began in Caria Region after Alexander the Great's death. At 180 B.C. Caria incorporated into the mainland of Pergamon Kingdom. At 133 B.C. this region became a part of the Roman Empire's Asia Province.

In a big probability, in the second half of II. thousand B.C., "Karkiša-Karakiša" in the Hittites' texts and at I. thousand B.C., "Karka" in persians' texts, belons to Carians. Discustions abaut the origin of "Car" people who named "Caria" hasn't ended. They were bleiving that they were the natives of Anatolia and Lydians, Mysians were their relatives (Prag J., Neave R., 1999). Heredotus and some Greek writers had reported that, Carians were Lelegian origined people and they had migrated to west Anatolia from Greek islands. Heredotus had written such: "Carians had come to mainlands from islands. Formerly, they were living in the islands with the origin of Lelegian and their nationality was Minoan. Much later Dorians and Ionians sent out the Carians from islands, thus the Carians went to mainlands. This is what the Gretans tell about Carians, but Carians themselves doesn't accept this, they say that they are the natives of mainland and they have been having the current name ever. They show a very old temple in Mylasa belongs to Zeus Carios that they accept only Mysians and Lydians to this temple because of being brother nations (Whitley J., 2001)

Athenios had written that: "In Philipus of Theangela's book which was about Carians and Lelegs, it was reported that Lelegs were used as slaves by Carians. Geographer Strabon had written the "Car" people as the most former nation of Greece, in Epidaurus, Hermione[1]. Pavsanias, who was a geographer and a traveller, had indicated that one of the Megaran castle's name was "Caria".

Greek had known some nations had lived in Anatolia before them, but they didn't have a clear view on this issue. Today, some academics argue the hypothesis which's main idea is; The "Car" people can be the continuation of Luwians. But the Luwian language which is a member of Indo-European languages family, never match with Carian place names and Carian religion. Carians' consideration about the "double axe" symbol and in the second half of II. thousand B.C. Gretans' usage of this symbol, attracts the attentions on this matching. Carians had a peculiar language and the studies are still going on about the origins of this language. In Ancient Greek epic poet Homer's Lliad, Carians had written thus: People with a coarse language (different from Greek language) . Carian alphabet was similiar to Phoenician alphabet and had between letters 30-37 (Whitley J., 2001).

Carians were qualified as warrior people. They were using crested helmet and carrying handled shield which can be hung on shoulder. They were the first people who were decorating external surfaces of their shields with pictures. And sometimes they were mercenaries in the armies of other states. In his lliad, Homer had written thus: Listen, let me tell you the most accurate, there are the Carians who are close the shore, there are the Paions who have curved springs, and the Lelegians, the Cauconians, divine Pelasgians, Lycians and Mysians are at around Thymbre, Phrygians, there are the Maionians too who fights with horse carts. And Strabon had written about Carians this: "Some authors attracts the attention to Carians' handled shields, shields decorated with symbols and crests which all called Carian, to indicate their enthusiasm for military. İn this topic at least Anacreon says so:"Come, pass your arm into the handle of the shield which is an invention of Carians." And Alcaios writes:"Caria by shaking crests (Güngördü E., 2003). At the ancient period, livestock production was constituting a large portion of the total production in Caria. Cereal

was being produced in the valleys and high plateaus. Alabanda District's sulphur was being used during the cereal production for disinfecting.

Olive and fig production was widespread in the region. Olive production produced much in the valley Maindros and its coastal section where the mediterranean climate is seen. At IV. century B.C., the quality of Carian olive oil was being talked even in Athens. Strabon mentioned about Carian Antiocheia's dried figs called "trifoliate". Furthermore, it's known that dried fig was an important export of ancient Caria Region and it was being exported to Egypt and Italy by ships in large quantities. In addition, viticulture and as a result wine and vinegar production, cabbage and herbs production, reed production used in manufacturing pen, various oils production and amphora manufacturing that used for transporting and storing, had been produced in ancient Caria. And also beekeeping, fishing, marble production and maritime trade had an important place in the economy of Caria.

The city of Stratonicea was one of the ancient cities in Caria Region as; Tralleis, Coscinia, Euhippe, Orthosia, Alinda, Alabanda, Antiocheia, Mylasa, Halicarnassus and Caunos. History of Stratonicea city and the sacred places connected to Stratonicea city is quite old. Ancient sources transfers that, at 270 B.C. Stratonicea city was being founded by Seleucid king I. Antiochus in the name of his wife, queen Stratonice. Queen Streatonice was the stepmother of I. Antiochus before he married with her. Strabon had written abaut Stratonicea city thus: Miletus Poseidon is arrived after Iasus. There are three cities in the interior to sign: Mylasa, Stratonicea and Alabanda. The others are depend on these or depend on the other coastal citie (Prag J., Neave R., 1999). Stratonicea City was being founded in an old Carian town called Chrysauris or Idrias or around it. In the early of II. century B.C. Rhodes dominance began in this city but at 167 B.C. it became independent again. Stratonicea City had an automnous and rich position in the Roman Empire era. After this, in the christianity period, Stratonicea City became a bishop center, depending on Aphrodisias Metropolitan. Some important structures of the Stratonicea city were bouleuterion, gymsasion that was being built in the middle of II. century B.C., theatre, the Roman gate which was the beginning of the holy way to Lagina.

1.2 The archaeology of human skeletal remains

After the archaeological works was led by Osman Hamdi at 1891-1892, excavation and restoration works have been continue by an archaeological research team led by Prof. Dr. Ahmet Tırpan since 1993, in The Lagina Hekate's Temenos The propylon is at the southwest corner in The Lagina Hekate's Temenos. The stoa lays along the pribolos, at the northwest of the propylon (Prag J., Neave R., 1999). There's a water reservoir, almost 150 metres away from the southwest of the propylon. İn the Temenos, there's an oval pool which has an approximately 10 metres diameter. Lagina's ancient inscriptions are talking about a sacred pool. In a big probability, revealed out and restored pool must be this sacred pool. The altar, which is in The Lagina Hekate's Temenos is at the south Hekate's temple and also at the east of propylon. The Börükçü Location is at 100 metres east of the holy way which is between Stratonicea and The Lagina Hekate's Temenos Excavations of Börükçü Location are being made by an excavation team led by Prof. Dr. Ahmet Tırpan from Selçuk University, Archaeological Department.

Between 1967-1970, Prof. Dr. Yusuf Boysal found materials that proves an uninterrupted settlement has been seeing in Lagina and araund since early bronze age. Late geometric

Fig. 2. 06BM02 numbered, dated to the Classical period in the grave, above ground level at a depth of 0.75 m is revealed burial inhumation style. Skeleton of the east-west, head north of the way and placed in the style of Hocker was buried were found.

period was saw in Aldağ and Bozukbağ, classical settlement and tombs was found in Emirler. An archaic period settlement was revealed and many terra cotta materials was found in Hacıbayramlar mound. A sacred area that belongs to Apollon and Artemis and tombs used in classical period was revealed by excavations in Koranza. The Börükçü's finds indicates that the Börükçü Location belongs to the same period as the settlements above. It was determined that an intensive habitation existed from VII. century B.C. to II. century B.C.

At the excavations of Börükçü, abundance of weaving workshops and olive oil workshops attracted the attention. Researches' and excavations' results, distance to other places, owned roads, water sources and wells araund, have shown that, Börükçü Location was contemporary with other settlements but different from them (Mansel A. F., 1988).

Remnants of the water structures were found in Börükçü Location. And also reconstructed structures were revealed which were belonged to Ottaman period. archaic period's fountain which is at the roadside of the holy way proves that this way was quite old. A natural inclination was converted to a terrace so this revised field was used to place the buildings in Börükçü. In some of the terraces only graves was found. Ways formed with steps and ramp formed paths revealed in this sloping field. Börükçü Location has an apperance as industrial and manufacturing spaces and cemeteries. Certain professional groups was collected in

Fig. 3. 06BM05 numbered graves, with a north-south direction, lattice-type boats have been built in the inside 1.40 m in length. Hocker style of the skeleton was buried east-west and head north toward where it was deposited were identified. Bone anatomy and morphology after examining the molecular work, dated to the Classical period in the 10-12 age burial belongs to a daughter.

certain areas. Most revealed archeaological finds are olive oil workshops. Also organic, glass, metal finds revealed too (Mansel A. F., 1988). Graves that belongs to interval from geometric period to Roman period were revealed in Börükçü Location. Many of them were from archaic and classical period. Hellenistic and Roman graves were revealed too. Many skeletons were revealed during the excavations of graves (Güleç E. et.al., 2006).

Fig. 4. 06BM09 numbered north-south direction in the grave with oyğu boat, 0.05 m at a depth of skull and skeleton belonging to the same 0:20 m. were uncovered at a depth of body. Skeletal north-south direction have been credited with extending the right arm from the elbow was extended slightly broken. The skull is looking toward the southwest.

2. Genetic analysis of aged skeletal material

2.1 Material and methods

2.1.1 Collection of samples

DNA isolation 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. Existing techniques were refined by targeted primer design focusing on a DNA fragment shorter than 200 bp, an approach allowing us to identify up to all bone samples at the same time. In order to allow

Fig. 5. 06BM13 grave number, type and north-south direction are **oyğu boat**. A part of the east and west walls of the tomb wall **oygu soil,** other aspects are carved into the rock. One regular burial in tombs and 11 skulls were recovered. However, the irregular parts and skeleton skull burial uncovered determination could be made.

ratings on individual bones, a new staging system was developed at Archeometry-Biotechnology Laboratory in Selcuk University, Science Faculty, and assigned as period or era each bone based on visual inspection for the DNA study. The bone samples of more than 100 individuals were chosen to study the genetics of this skeletal population. In addition to numerous human skeletons, the cave contains bones from some autochthonous animal species. All skeletal material was wrapped in aluminum foil, placed in plastic bags, and labeled. Earlier analysis showed that the state of DNA preservation in the bones is excellent, mainly due to the low temperature prevailing in the cave since prehistoric times (Burger et al., 1999). Eleven animal bone samples were chosen for aDNA analysis.

2.1.2 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 (Herrmann and Hummel 1994). 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 pipets, and disposable laboratory ware were used throughout the analyses. Benches and equipment were

Fig. 6a-6b. Geometric period is dated 06BM18 numbered graves. Type of boat was built in marble tomb. Been left entirely as a rough floor sculpture made of marble in a single block. In the eastern side of the head of the skeleton in the form of slight increase has been given the bag.

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 (Sarkar and Sommer, 1990). 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.

2.1.3 Decalsification of bone and genomic DNA extraction

Extraction of DNA was carried out using the laboratory handling and cleaning protocol (Römpler 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 calsification 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 1 U 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 °C until assay for the amelogenin was performed (Figure 7).

2.1.4 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 ^{o}C. A standard phenol/chloroform organic extraction was

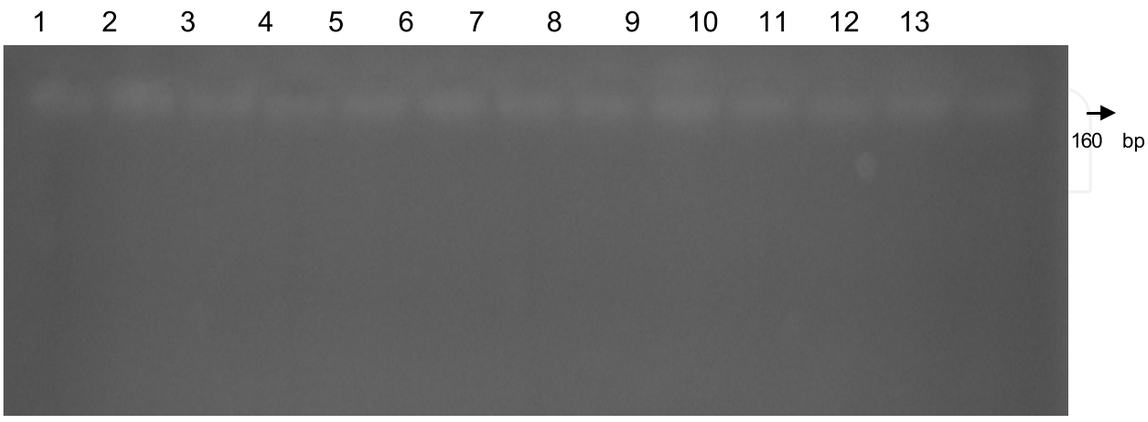

Fig. 7. Genomic DNA was isolaled from fossil bone tissue remains, respectively, Lane 1, 2, 3-13 with Bio Robot EZ1. aDNA samples submitted to electrophoresis in 1% agarose gel. Sample codes, respectively, 05BM13, 05BM22, 06BM09, 05BM29, 06BM40, 05BM21, 07BM05, 05BM23, 06BM39, 07BM13, 05BM64, 05BM30, 05BM106 illustrated in the table 1.

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 ^{o}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.

2.1.5 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.

2.2 PCR amplıfıcatıon

2.2.1 Polymerase chain reaction (PCR) amplification of species determination

Molecular archaeology is an emergent field in archaeology that has been brought about by the advancements of the recognition and understanding of DNA. This new developing branch of archaeology focuses on the acquisition of either DNA or mtDNA (mitochondrial DNA) and being able to determine species of natural archaeological finds as well as determine blood lines and/or sex of animal or human remains. These DNA. As our technology advances as well as our knowledge of the DNA itself our understanding of ancient peoples, plants, and animals, will allow us a biological window into their lives.

A 200 bp segment of the mitochondrial cytochrome b gene was amplified using the primers;

CB7u: 5'- GCGTACGCAATCTTACGATCAA-'3 and

CB7l: 5'-CTGGCCTCCAATTCATGTGAG-'3.

The PCRs were carried out in 50 ml of 60 mM KCl; 12 mM TrisHCl; 2.5 mM $MgCl_2$; 150 mM dNTPs; 0.18 mM each primer; and 2U AmpliTaq Gold (Applied Biosystems), and 0.2

microliter BSA. The temperature profile was 95 °C for 4 min, 95 °C for 30 s, 54 °C for 1 min, and 72 °C for 30 s, for 40 cycles and 72 °C 5 min.

2.2.2 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 (Faerman M., et.al., 1995). In this study, the primers for PCR amplifications used are as follows:

Sequence Amel-A (5'- CCCTGGGCTCTGTAAAGAATAGTG -3')

Sequence Amel-B (5'- ATCAGAGCTTAAACTGGGAAGCTG -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 °C for 10 min, 50 amplification cycles with denaturation at 94 °C for 30 s, annealing at 60 °C for 10 min and extension at 72 °C for 1 min in a thermocycler (Biorad, Germany). PCR blank reactions did not show spot contamination during the collection of the data. As as result, sex gender of ancient human bones was determined related with DNA fragments with different length of base pair as male and female. 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 8).

2.2.3 Negative control amplification

An increase of PCR cycle may increase the risk of minute amount of modern DNA contamination in the resulting in DNA amplification. In this study, potential modern DNA contamination was assessed based on the possible amplification produced in the negative

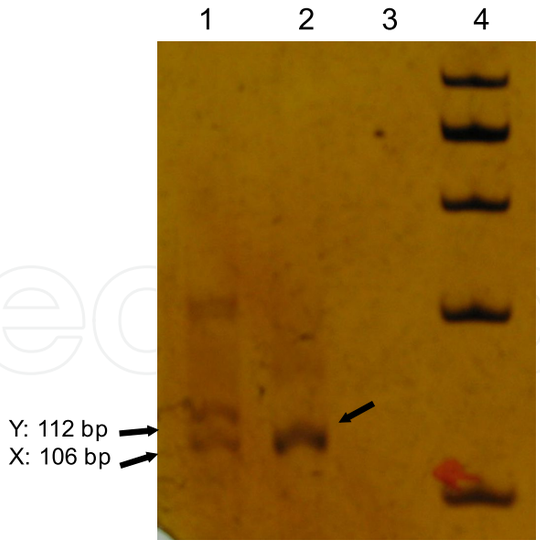

Fig. 8. Polyacrilamide gel electrophoresis of PCR products in male and female fossil DNA templates or 106/112 bp amelogenin gene PCR products. Respectively, Lanes 1-4, Lane 1 : amelogenin male sample 106/112 bp, and lane 2: amelogenin female PCR fossil sample 106 bp, Lane 3 negative control water blank or none DNA, Lane 4, 100 bp ladder size standard marker.

control extraction. The negative control extraction is a sample that contains everything used during DNA extractions procedure followed by PCR amplifications except the powdered ancient bone of the respective sample was substituted with deionized water (Yang et al., 2003; Yang et al., 1998; Pääbo S., 1985; Pääbo S., 1989; Pääbo S., et.al., 2004). An indication that a very low level or non-existence of modern DNA contamination as well as specificity of the primers and sensitivity of PCR amplifications procedures that had been utilized in this study.

2.2.4 Gel electrophoresis of PCR amplicons

PCR product was separated by electrophoresis on 2 % agarose gel in 1XTAE buffer (45 Mm Tris, 1 mM 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. 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. 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

2.2.5 Determining using RT-QPCR of aDNA quality

2 µl of DNA and 3 µl primer mix was used in a final volume of 20 µl according to the manufacturer's directions. LightCycler amplification and Real-Time QPCR detection with

SYBR Green was done as described by the supplier using the Fast Start DNA Master SYBR Green I (Roche Applied Science) with 8 mM $MgCl_2$ in the reaction mixture. Amplification conditions were 95°C for 10 min and 45 cycles, each cycle at 95 °C for 10 s, 56 °C for 10 s and 72 °C for 20 s. The LightCycler amplification and Real-Time PCR detection with fluorescence labeled hybridisation probes was done following the protocol provided either for the LightCycler. Positive and neagtive controls were included in all reactions (Figure 9).

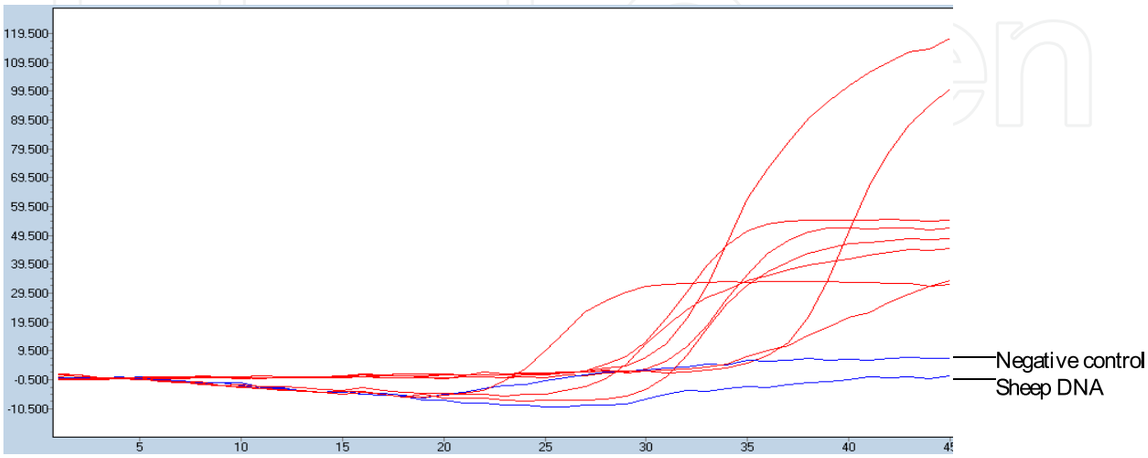

Fig. 9. Determination of species and quantification in ancient DNA isolated from burial remanis using RT-QPCR

2.2.6 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 Voegtly 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 vs. DNA quantity was examined using a t-test with equal variance assumed. The effect of PCR inhibition during QPCR on spiked samples with and without anti-inhibitory addition (BSA or commercial enhancer) was examined using a two-tailed paired t-test. In all cases results were considered significant at $p<0.05$.

3. Result and discussion

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 (Hagelberg et.al., 1989 and Hagelberg et.al., 1991). 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 (Delgado S. et.al., 2001).

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 (Delgado S., et. al., 2005). 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 our present 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 (Mitchell R.J., et.al., 2006). 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.

DNA originating from individuals from major phyla of vertebrates was isolated by the organic method from various specimens. Extracted DNA was subjected to PCR and direct cycle sequencing using a universal pair of primers. In order to evaluate the utility of this gene for discrimination of fosil bone remains as well as for exploring their phylogenetic relationships. These data show that the Cyt b gene is useful for phylogenetic study of fosil bone remains (human or animal materials) (Gill P., et.al., 1994).

Real-time PCR is now a common method for measuring gene expression, it is increasingly important for users to be aware of the numerous choices available in all aspects of this technology. Unlike traditional PCR, there are many complexities with real-time PCR that can affect overall results. However, with a well-designed experiment performed with the proper controls, real-time PCR can be one of the most sensitive, efficient, fast, and reproducible methods of measuring gene expression and DNA quantification. LightCycler Real-Time PCR using SYBR Green for detection was applied to quantify the actual amount of the prepared DNA. For every sample, a primer pair amplifying a single copy region of the genome was designed amelogenin primers. The specificity of the PCR reaction was tested after every run by determining the melting point of the respective product. All reaction products showed single peaks and the product size was verified to be in the expected range by gel electrophoresis.

As our technology advances as well as our knowledge of the DNA itself our understanding of ancient peoples, plants, and animals, will allow us a biological window into their lives. Molecular archaeology can in time, as our knowledge and technology increases, provide us with the ability to learn more about the life of ancient individuals. It can be seen how modern humans may differ from our ancestors or what plants and animals may have existed at the time and been utilized by them, which can be found by exploring what their tools and clothing or other artifacts were constructed out of. Not every area of the world is accessible to this technology due to the variety of climates, but in those areas where suitable DNA samples may be taken a whole new knowledge of the ancient culture under examination may be gained.

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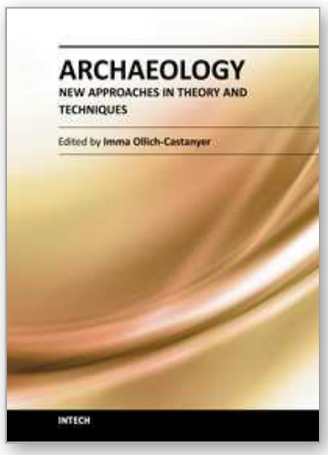

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