A SHORT FRAGMENT OF ANCIENT DNA AND ITS USE IN DETERMINATION OF SHEEP MITOCHONDRIAL DNA HAPLOGROUPS IN SOUTHEAST ANATOLIA

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submitted by **NİHAN DİLŞAD DAĞTAŞ** in partial fulfillment of the requirements for the degree of **Master of Science in Biology Department, Middle East Technical University** by,

Prof. Dr. Canan Özgen _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Gülay Özcengiz _____
Head of Department, **Biology**

Prof. Dr. İnci Togan _____
Supervisor, **Biology Dept., METU**

Examining Committee Members:

Assoc. Prof. Dr. Cemal Can Bilgin _____
Biology Dept., METU

Prof. Dr. İnci Togan _____
Biology Dept., METU

Prof. Dr. Erksin Savaş Güleç _____
Anthropology Dept., Ankara University

Assis. Prof. Dr. Mehmet Somel _____
Biology Dept., METU

Assis. Prof. Dr. Evangelia Pişkin _____
Settlement Archaeology Dept., METU

Date: 19.12.2013

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Nihan Dilşad Dağtaş

Signature :

ABSTRACT

A SHORT FRAGMENT OF ANCIENT DNA AND ITS USE IN DETERMINATION OF SHEEP MITOCHONDRIAL DNA HAPLOGROUPS IN SOUTHEAST ANATOLIA

Dağtaş, Nihan Dilşad

M.Sc., Department of Biology

Supervisor: Prof. Dr. İnci Togan

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Recent archaeozoological studies indicated that perhaps the oldest (11,000 years before present) and may be the only sheep domestication center was in Southeast Anatolia. In this study, to contribute to the understanding of sheep domestication history, ancient DNA derived from skeletal remains of sheep unearthed from archaeological sites in Turkey mainly from Oylum Höyük in Kilis were examined.

187 ancient metapodia and mandible samples, dating between 1,800-30 BCE were brought from Oylum Höyük to the dedicated aDNA laboratory which was established at the Middle East Technical University in 2012. Similarly, samples already identified as sheep (n=63), dating between 6,700-5,800 BCE from Tepecik-Çiftlik, Niğde were brought to the same aDNA laboratory. Ancient DNA extraction was performed using the samples identified as sheep by the archaeozoologists (n=57 for Oylum Höyük, n=13 for Tepecik-Çiftlik). Then, a 144 bp long mitochondrial DNA fragment which was shown to identify all five of the modern domestic sheep haplogroups A-E, was amplified.

Success rates in extractions and amplifications were 65% for Oylum Höyük samples and 92.3% for Tepecik-Çiftlik samples. For 30 out of 37 samples from Oylum Höyük, aDNA amplifications were replicated. Postmortem nucleotide changes

(misincorporations) and indels were inferred on the basis of replicated aDNA sequences and reference sequences from modern sheep samples. As the main result, aDNA sequences were used to estimate HPGs of sheep samples. The observed percentage of each haplogroup was: HPG A=50%, HPG B= 35.3%, HPG C=5.9%, HPG D=5.9%, HPG E=2.9% for Oylum Höyük and B= 83%, E= 17% for Tepecik-Çiftlik samples. When HPG distributions were examined comparatively with the accumulated data in the literature, it was observed that HPG B might be the dominating type around the sheep domestication center in the early days of domestication and HPG C frequency increased after the Hellenistic period around Kilis region. The present study being the first study from the sheep domestication center, besides contributing to the understanding of evolutionary history of domestic sheep in South/Southeast Anatolia, also shows that a short mtDNA fragment is useful to determine the HPGs of ancient sheep with respect to the HPGs observed in modern domestic sheep.

Keywords: Ancient DNA, sheep, Oylum Höyük, Tepecik-Çiftlik, mtDNA haplogroup

ÖZ

KISA BİR ANTİK DNA BÖLGESİ VE BU BÖLGENİN GÜNEYDOĞU ANADOLU'DAKİ KOYUNLARIN MİTOKONDRİYAL DNA HAPLOGRUPLARININ BELİRLENMESİNDE KULLANIMI

Dağtaş, Nihan Dilşad
Yüksek Lisans, Biyoloji Bölümü
Tez Yöneticisi: Prof. Dr. İnci Togan

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Son dönemde yapılan arkeozoolojik çalışmalar, en eski (günümüzden 11,000 yıl önce) ve belki de tek koyun evcilleştirme merkezinin Güneydoğu Anadolu'da olduğunu göstermektedir. Bu çalışmada koyun evcilleştirmesinin tarihinin anlaşılmasına katkıda bulunmak amacıyla, Türkiye'deki çeşitli kazılardan ağırlıklı olarak da Kilis ilinde bulunan Oylum Höyük'ten çıkarılan koyun örneklerinin antik DNA'ları incelenmiştir.

Oylum Höyük'ten alınan, M.Ö. 1,800-30 yılları arasına tarihlendirilen, 187 antik metapodya ve mandibula örneği Orta Doğu Teknik Üniversitesi'nde 2012 yılında kurulan antik DNA laboratuvarına getirilmiştir. Aynı şekilde, Niğde ilindeki Tepecik-Çiftlik'ten M.Ö. 6,700-5,800 yılları arasına tarihlendirilen koyun örnekleri de (n=63) aynı laboratuvara getirilmiştir. aDNA eldesi koyun olarak tanı koyulan örnekler (n=57 Oylum Höyük, n=13 Tepecik-Çiftlik) kullanılarak yapılmıştır. Daha sonra, modern evcil koyunda beş mitokondriyal DNA haplogrubunu da (A-E) ayırabildiği gözlenen 144 bç uzunluğundaki mtDNA bölgesi yükseltgenmiştir.

DNA ekstraksiyonu ve yükseltgenmesindeki başarı oranı Oylum Höyük örnekleri için %65, Tepecik-Çiftlik örnekleri için %92.3'tür. Oylum Höyük örneklerinin 37

tanesinden 30'u için aDNA yükseltgemesi tekrar edilmiştir. Canlı öldükten sonra gerçekleşen nükleotid değişimleri (hatalı baz yerleştirme) ve indeller tekrarlanan aDNA dizileri ve modern koyunlara ait referans diziler karşılaştırmalı olarak çalışılarak tahmini olarak belirlenmiştir. Yükseltgenen aDNA dizileri koyun örneklerinin HPG'larını belirlemede kullanılmıştır. Her bir haplogrubun görülme yüzdesi: Oylum Höyük örnekleri için HPG A=%50, HPG B= %35.3, HPG C=%5.9, HPG D=%5.9, HPG E=%2.9 ve Tepecik-Çiftlik örnekleri için B= %83, E= %17'dir. HPG dağılımları mevcut literatürdeki veriler ile karşılaştırıldığında, HPG B'nin evcilleştirmenin ilk dönemlerinde koyun evcilleştirme merkezi çevresinde baskın tip olduğu ve HPG C'nin frekansının Kilis bölgesinde Helenistik dönemden sonra arttığı gözlemlenmiştir. Koyun evcilleştirme merkezinden yapılan bu ilk çalışma, Güney/Güneydoğu Anadolu'daki evcil koyunun evrimsel tarihine katkıda bulunmanın yanısıra, mtDNA'nın küçük bir bölgesinin modern evcil koyunlarda gözlenen HPG'lara göre antik koyunların HPG'larını belirlemede yardımcı olduğunu göstermiştir.

Anahtar Kelimeler: Antik DNA, koyun, Oylum Höyük, Tepecik-Çiftlik, mtDNA haplogrup

To My Family and Beloved Ones,

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AD: *Anno Domini* or Common Era

aDNA: ancient DNA

BCE: Before Common Era

BLAST: Basic Local Alignment Search Tool

bp: base pair

BP: Before Present

cal: calibrated

CR: control region

DNA: deoxyribonucleic acid

dNTP: deoxyribonucleotide triphosphates

g: gravitational acceleration

HPG: haplogroup

min: minute

ml: milliliter

mm: millimeter

mtDNA: mitochondrial DNA

Myr: million years

PCR: Polymerase Chain Reaction

rpm: revolutions per minute

s: second

$tRNA^{Pro}$: transfer RNA proline gene

µl: microliter

CHAPTER 1

INTRODUCTION

1.1 Sheep domestication and mitochondrial DNA haplogroups

Plant and animal domestications and the transition from hunter gatherer life style to farming are accepted as the start of a new era in the history of humanity, known as the Neolithic Era. The origin of Neolithic transition was in the "Fertile Crescent", harboring Southeast Anatolia, the Levant region of eastern Mediterranean and Mesopotamia. This transition is approximately dated back to 10,000 years Before Common Era (BCE).

Archaeological findings indicate that sheep was first managed around 9,000 BCE in the north of Fertile Crescent (Clutton-Brock, 1999; Peters et al., 2005; Zeder et al., 2005). The area of domestication was stretching from Central Anatolia to the north of Zagros Mountains, covering mainly Southeast of Anatolia (Zeder, 2008).

To start the domestication of an animal, as an *a priori* condition, wild form of the animal must exist in the area. On the basis of molecular studies, among the wild sheep species *Ovis orientalis*, referred to as *Ovis gmelinii* by recent nomenclature (IUCN/SSC Caprine Specialist Group, 2000; Festa-Bianchet, 2000), was suggested as the ancestor of domestic sheep, rather than *Ovis vignei* (the urial) or *Ovis ammon* (the argali) (Hiendleder et al., 1998b; Bruford and Townsend, 2006; Meadows et al., 2007). The native distribution of *Ovis gmelinii* was covering the suggested domestication center of sheep as seen in Figure 1 approximately from the Middle Palaeolithic to Byzantine period.

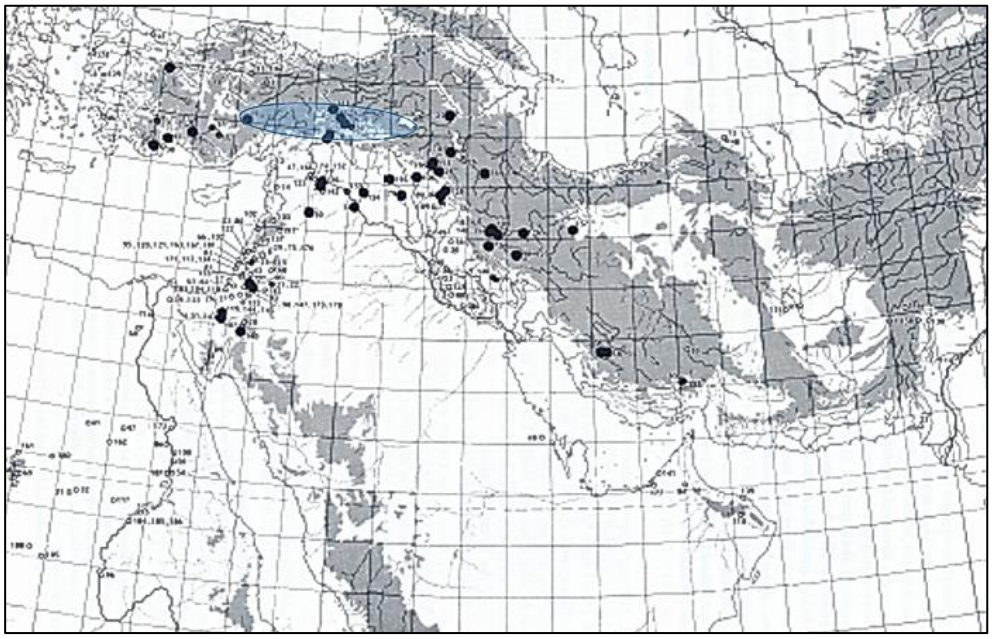


Figure 1. Ancient distribution of *Ovis gmelinii* (adopted from Uerpmann, 1987). Dots and numbers indicate the archaeological sites (in Turkey, Iran, Iraq, Syria, Israel and Jordan) that have produced finds of *Ovis gmelinii*. Blue area inside Anatolia shows the sheep domestication center (Zeder, 2008).

In 1998 and later in 2002, it was suggested that two mitochondrial DNA (mtDNA) haplogroups (HPGs) gave rise to modern sheep breeds of the world (Hiendleder et al., 1998b; Hiendleder et al., 2002). These were the Asian type (HPG A) and European type (HPG B) HPGs. HPG A and HPG B are the two most common HPGs among the modern breeds of the world today. Although, they were encountered among the sheep of almost all regions, still HPG B is the predominant type in Europe and HPG A is the predominant type in Asia (Figure 2). HPG C is less frequent compared to HPGs A and B. It was first observed among Turkish and Chinese breeds by two different studies (Pedrosa et al., 2005; Guo et al., 2005, respectively). It has been now observed in the Fertile Crescent, around the Caspian Sea and the Iberian Peninsula (Guo et al., 2005; Pedrosa et al., 2005; Meadows et al., 2005, Tapio et al., 2006, Pereira et al., 2006). HPG C was believed to have a different evolutionary history than HPGs A and B (Guo et al., 2005). HPGs D and E were observed relatively recently, with a very low frequency (<0.01%), only among the sheep in Caucasus and Turkey (Meadows et al., 2005; Tapio et al., 2006). A study by

Meadows et al. (2007) confirmed the presence of all five phylogenetically divergent mtDNA HPGs (A-E) among the contemporary domestic sheep of Turkey. The mtDNA HPG distribution of sheep over the spectrum of Eurasia was described in the form of pie charts in Figure 2.

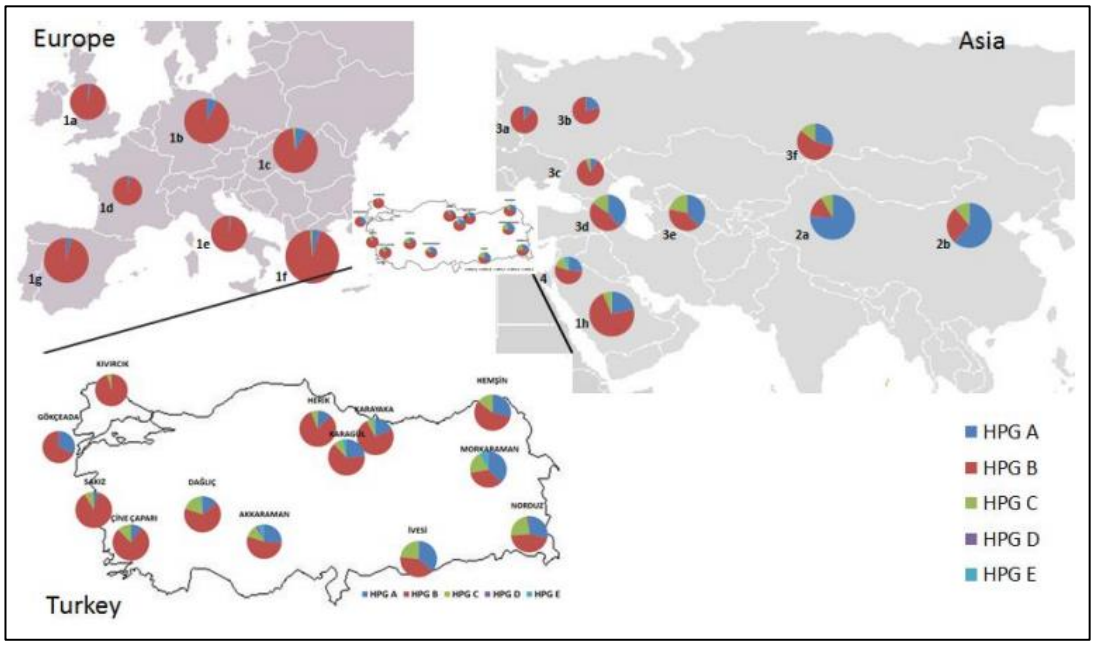


Figure 1. Spatial distribution of mtDNA HPGs on the map of Eurasia (with a special emphasis on Turkey). The pie charts on the map of Turkey were located in accordance with the sample collection sites (from Demirci, 2012).

In a recent study (Meadows et al., 2011), the topology of the tree constructed by the mitogenomes of sheep representing all five HPGs indicated that mtDNA control region (CR) is the most powerful region in reproducing the topology.

When retrovirus integration sites and their relative frequencies were considered in sheep, their frequency distributions over the Eurasia indicated that there was a second population expansion of sheep after first domestication, presumably sheep with better wool quality, with a possible expansion date of 3,000 BCE from the region of modern Iraq and Iran (Chessa et. al., 2009). Results of this study suggested that mtDNA HPGs might have differed before and after 3,000 BCE in Anatolia.

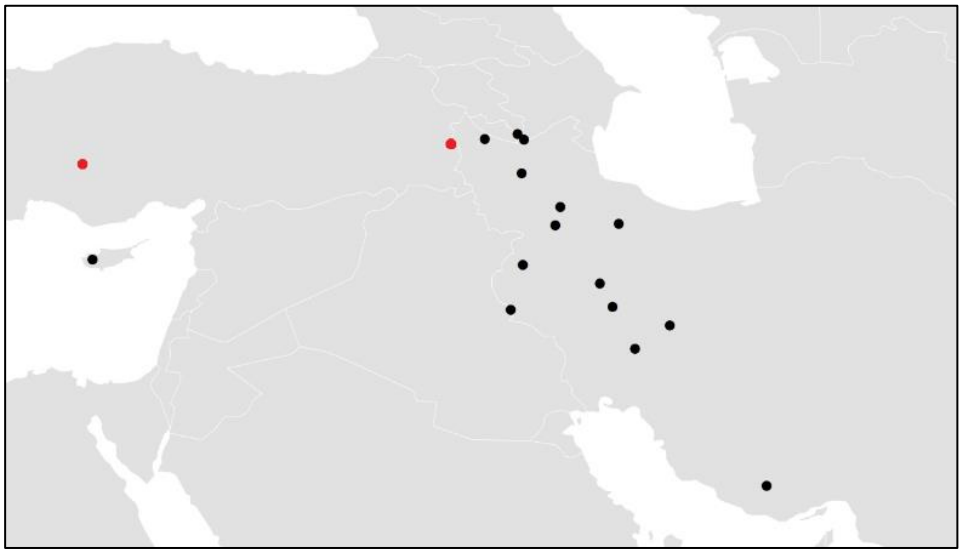


Figure 2. The distribution of *Ovis gmelinii* subspecies. Red dots represent the populations in Anatolia.

Figure 3 depicts the currently observed wild *Ovis gmelinii* individuals (reported by Rezaei et al., 2010; illustrated by Demirci et al., 2013). Today, the Anatolian wild sheep *Ovis gmelinii* exist in Turkey as subpopulations (red dots in Figure 3) which are known to be isolated for a long time. As the proposed ancestor of HPG B type domestic sheep, *Ovis gmelinii* had a wider geographic range before 1900s (Danford and Alston, 1877; Turan, 1984; Kaya et al., 2004). However, the population started to decline due to anthropological and environmental factors, and finally was confined to an area near Konya Bozdağ region (Arıhan, 2000; Sezen, 2000), where it is currently known as *Ovis gmelinii anatolica*. The population, with as few as 35 founders in the past, recently increased in number with the help of the establishment of a wildlife protection area in 1966 (Sezen, 2000). The second subpopulation (referred to as Armenian mouflon) lives on the easternmost side of Turkey and migrates seasonally to Northwest Iran (Sezen, 2000). The replacement of the corridor between central Anatolia and western Iran by deciduous forest or woodland around 6,000 Before Present (BP) was suggested to be the cause of isolation between these two subpopulations (Arıhan and Bilgin, 2002).

Regarding genetics of the modern wild sheep of Anatolia, it was reported that (Demirci, 2012) HPG A was dominant (22/30) among *Ovis gmelinii*. This study also

revealed a novel type of haplogroup, HPG X (8/30), which appears related to HPGs C and E on the neighbour-joining tree.

In a recent study, for the first time mtDNA HPGs observed (on the basis of partial cytochrome *b* sequences) among the modern domestic sheep and wild *Ovis gmelinii* individuals were plotted on the same reference frame shown in Figure 4 (Demirci et al., 2013).

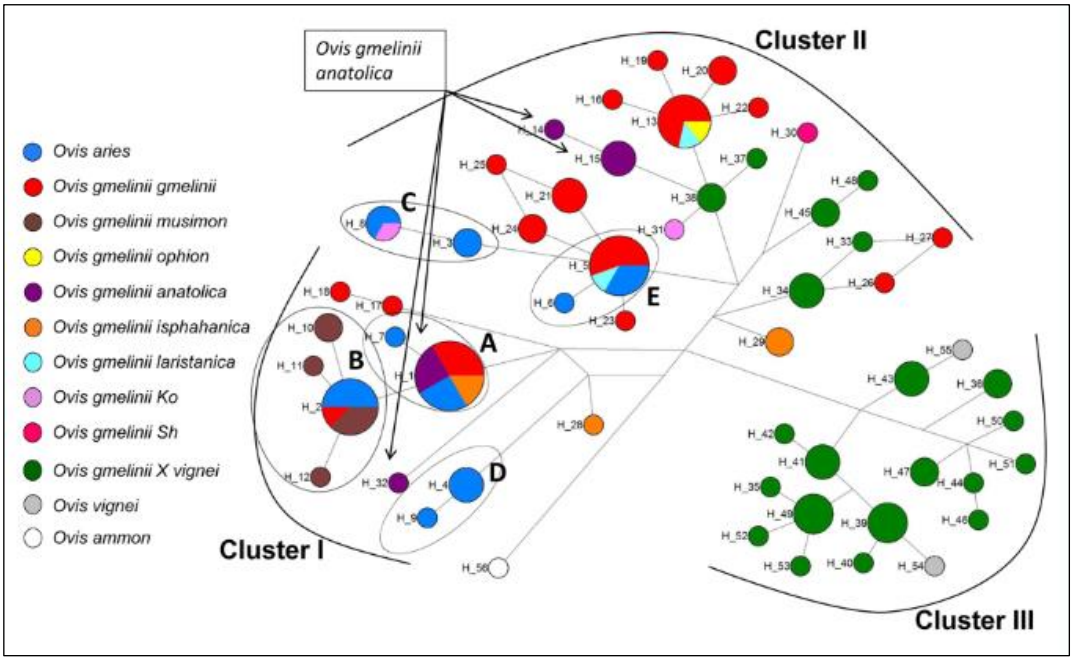


Figure 3. Median-joining network constructed with the partial mtDNA cytochrome *b* sequences of domestic (dark blue) and wild (the rest of the colors) *Ovis* species (Demirci et al., 2013). A-E stand for the mtDNA HPGs.

Meanwhile, questions such as "What was the mtDNA composition of wild sheep at the onset of domestication?", "How did the domestic sheep evolve spatially and temporally, on the basis of mtDNA HPGs?" remain to be elucidated by the ancient DNA (aDNA) studies.

The present study, by employing aDNA from sheep, aims to contribute to sheep domestication history. It is the first of a series of studies addressing the details of evolutionary history of domestic sheep from the start to the modern days. It focuses on the sites which are believed to be important in terms of sheep domestication.

1.2 Ancient DNA and the brief history of ancient DNA studies

Ancient DNA is defined as the DNA retrieved from museum specimens, archaeological findings such as bones, seeds, coprolites, fossil remains as well as other unusual sources like eggshells, feathers, parchments (Pääbo et al., 2004). As an emerging field of research, aDNA studies contribute to the understanding of population histories by revealing the unknown past; in the meantime to the knowledge of phylogeny and phylogeography. Molecular evolution, which the phylogenies are mainly based on, is a historic process through which genes accumulate changes due to stochastic events as well as selective processes (Pääbo et al., 1989). Without revealing the steps of this historic process, the conclusions drawn about the evolutionary history of the species or populations on the basis of genetic diversity observed today would be deficient or erroneous. An example of this, related with the migration of domestic pigs to Europe (Ottoni et al., 2013) is provided below. Furthermore, aDNA research may also contribute to the conservation studies of populations. With the help of statistical inference methods being developed, it will probably be possible to deduce the fate of extant populations for future and to direct the plans on their conservation in a more efficient way.

Research on aDNA has begun nearly 30 years ago with Higuchi et al.'s (1984) study on an extinct cousin of horse, quagga (*Equus quagga*). They used dried muscle as the source from which DNA was extracted and cloned. This first study covering only short fragments (229 base pairs) of cloned mtDNA of one individual, has revealed that extinct quagga sequences differ from the corresponding sequences of an extant mountain zebra by 12 nucleotide substitutions and they shared a common ancestor 3-4 Myr ago in accordance with the fossil record. A following study (Pääbo, 1985) covered 3.4 kilobase nuclear DNA sequence of a 2400-year-old Egyptian mummy. It was reported that one of the clones contained two members of a human repetitive DNA sequence family, *"Alu"*. Later the "mummy sequence" was suspected to be a modern human contaminant. One of the reasons that may cast doubt on the results was finding no or little postmortem modifications on the DNA fragments that were extracted from the mummy. This observation was contradictory to the results of two studies (Hansen et al., 2001; Hofreiter et al., 2001) which showed that for the cloned sequences of an ancient specimen the probability of observing postmortem

nucleotide differences between the clones is higher than it is between the clones and the modern sequences. Nevertheless, this work on mummy's DNA is still recognized as one of the first leading studies in aDNA research.

The real driving power that accelerated the research in this new field is the development of DNA Polymerase Chain Reaction (PCR) technique by Kary Mullis in 1983. This new technique allowed the amplification of a single DNA molecule by several million fold (White et al., 1989), making it easier to study DNA from archaeological and museum specimens without cloning. On the other hand, this discovery also led to studies such as the one claiming that they had amplified mtDNA cytochrome *b* gene from remnants of a 80-million-year-old dinosaur (Woodward et al., 1994). In addition, studies employing "aDNA of millions of years old insects embedded in amber" (DeSalle et al., 1992; Cano et al., 1993) were published. Today, the authenticity of the acquired DNA in those studies is considered questionable owing to the age of the samples (Shapiro and Hofreiter, 2012) and they are believed to be the false results governed mainly by some sort of contamination. Back then, with the additive impact of high-profile journals in which these papers were published, this new field of study fell into disrepute which has taken many years to recover (Shapiro and Hofreiter, 2012).

Thomas et al.'s (1990) study on Panamint kangaroo rat populations tried to detect temporal changes in genetic diversity by utilizing 43 museum samples collected for 78 years. Later, they compared those results with the results from 63 modern samples collected from the same localities. This study demonstrated the importance of museum samples in phylogenetic studies. Two years later, the first study revealing the phylogenetic position of New Zealand's extinct flightless bird moa was published (Cooper et al., 1992). The field saw more progress with the publication of first Pleistocene-age DNA sequences from mammoth (Hagelberg et al., 1994) and cave bears (Hänni et al., 1994). In 1995, the time period that was covered extended to 11,000 BP in the context of a population genetics study (Hardy et al., 1995) on the European rabbit. For the following 10 years, the length of the studied fragments, number and type of samples used have been further improved. First complete mitochondrial genome of moa was published by two independent groups showing

that, albeit the fragmented nature of old DNA, it is still possible to retrieve full genomes of some extinct species (Cooper et al., 2001; Haddrath and Baker, 2001). A population level study on Beringian brown bears (Barnes et al., 2002) showed that there has been some genetic structure observed over the course of last 60,000 years and most of the changes in phylogeography occurred before Last Glacial Maximum; little change was identified after that time. Through this study it was also shown how complicated the history of one species can be, as local extinctions, reinvasions and interspecies competition were all deduced from the data.

There was also a steady increase in the age of samples covered in aDNA studies. Before the PCR was invented, the oldest sample from which aDNA could be retrieved was quagga (140-year-old). With the employment of PCR technique, the oldest aDNA sequence was 40,000 years old by the year of 1994 (Hänni et al., 1994).

The type of ancient materials used for extracting DNA, as well, was subjected to change over time. The first studies used soft tissue, assuming that soft tissues such as muscle tissue should contain more DNA as is the case of living organisms. However, this assumption was proved to be wrong as bone remains started to be used as a DNA source (Hagelberg et al., 1989). Ancient bones indeed retain relatively more DNA than ancient soft tissue. As a reason, it is claimed that DNA molecules may stick to the bone mineral (a modified form of hydroxylapatite) and they can even be prevented from fragmentation inside those hydroxylapatite crystals (Shapiro and Hofreiter, 2012). The researchers utilized mostly bone samples for the following years, as bone samples are relatively abundant among archaeological remains. Later, Poinar et al. (Poinar et al., 1998) conducted an aDNA study by performing experiments on fossilized faeces (coprolite) that mainly found in caves in dry places. Thereafter different sources of aDNA followed; hair (Bonnichsen et al., 2001), sediment (Willerslev et al., 2003), feather (Rawlence et al., 2009) and eggshells (Oskam et al., 2010). As materials that sound unsuitable for DNA extraction yielded successful results, it is not unrealistic to expect that novel sources of aDNA are yet to be discovered.

The above mentioned studies were made possible with the PCR technique and the modifications leading to its improvement in the past three decades. However, in 2005

the era for Next Generation Sequencing (NGS) technology has begun. The first set of NGS machines have an approximately 300-fold higher sequence throughput compared to traditional Sanger sequencing machines. Not surprisingly, technologies continued to be improved and the DNA sequence throughput has increased by another 4 orders of magnitude by now. The first studies utilizing NGS technology were published in 2006 (Poinar et al., 2006 on woolly mammoth; Green et al., 2006 on Neanderthal). Two years later the first draft genome of woolly mammoth was released (Miller et al., 2008). In 2010, the first ancient human genome of a Palaeo-Eskimo was published (Rasmussen et al., 2010). This was followed by studies covering Neanderthal genome (Green et al., 2010), the genome of an Archaic Denisovan individual (Meyer et al., 2012) and more recently the mitochondrial genome of a 400,000 year old hominin (Meyer et al., 2013). To date, by the improvement of DNA purification and sequencing techniques the oldest successful aDNA retrieval was from an approximately 700,000 year old horse sample (Orlando et al., 2013). It is very likely that, with the rapidly changing technology, studies with older samples will follow in future.

As a final example, interesting observations made on the basis of aDNA studies about the domestication and migration of wild boars is given below.

In the study by Ottoni et al. (2013), approximately 800 base pair (bp) long fragments of mtDNA were obtained for 39 modern wild boar and 393 ancient wild and domestic pigs. The results were summarized in Figure 5.

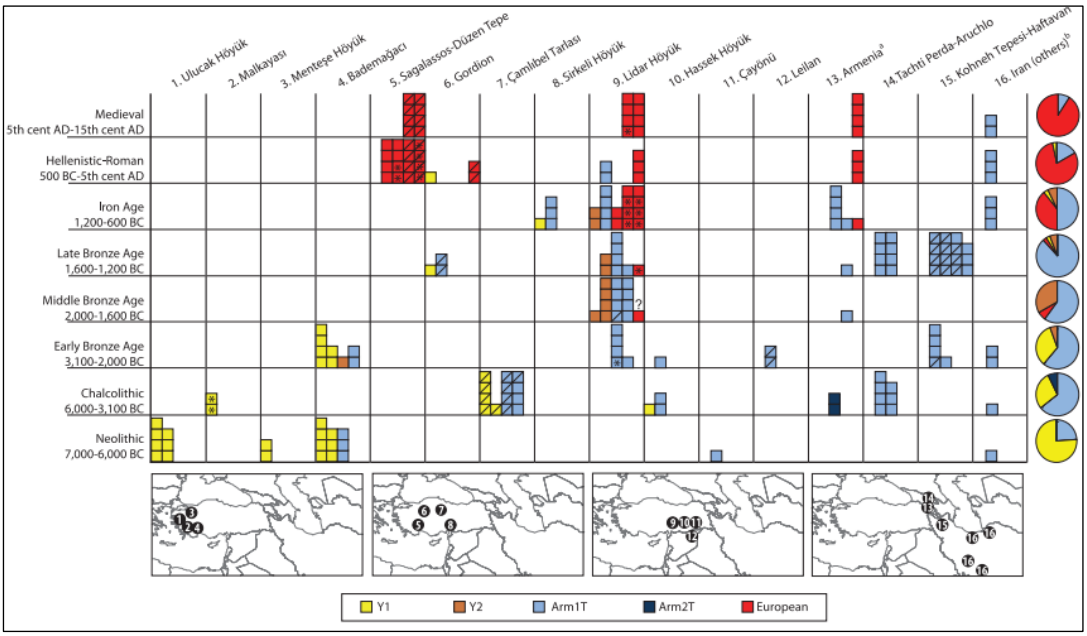


Figure 4. A depiction of pig haplotypes (Ottoni et al., 2013) with respect to sample collection sites (columns) and observed chronological periods (rows). Small maps show the approximate locations of archaeological sites. Asterisks refer to directly AMS carbon-14 dated samples, whereas question mark indicates lack of AMS carbon-14 dating. Slashed boxes depict the samples on which geometric morphometry analyses were performed. To the right of each row, pie charts exhibit the haplotype frequencies for each chronological period across all sites.

Based on these observations it was possible to propose that the wild boar, harboring haplotype Arm1T (depicted with blue color in Figure 5) in eastern Anatolia, was domesticated perhaps near to the Southeast Anatolia and later moved on to the western part, most probably through Mediterranean coast. In the western Anatolia, probably they mated and mixed with the wild boars, especially with the females belonging to haplotype Y1 (depicted with yellow color in Figure 5). Then, the domestic pigs harboring Y1 haplotype were taken to Europe during Neolithization, along with the technology of animal domestication. Eventually, European local boar populations were domesticated and those that were brought from Anatolia disappeared. Those domesticated boars having the European haplotype (depicted with red color in Figure 5) brought back to Anatolia during Middle-Late Bronze Age which later dominated the Anatolian pig haplotypes. This study shows that in the

absence of aDNA-based results, it would be impossible to understand the complex history of pig domestication and the evolutionary history of pigs.

1.3 Criteria for eliminating contamination resulted from modern samples

For most of the aDNA studies published so far, there are certain criteria to be met in order to minimize contamination in the results. These compiled criteria are;

1) The negative extraction and amplification controls should always be free of contamination.
2) The results should be repeatable and reproducible.
3) The profiles of the ancient individuals should be different from those of the laboratory researchers (for studies on human remains).
4) All pre-PCR steps should be performed in a positive pressure laboratory dedicated to aDNA.
5) Different rooms must be used for sample preparation, DNA extraction, and setting up PCR.
6) Post-PCR procedures should be carried out in a different building.
7) Surfaces should be cleaned regularly with a 10% sodium hypochlorite solution and exposed to UV light (254 nm). Full-body protective clothing, facemasks and gloves must be worn before entering the aDNA laboratory. Gloves must be changed frequently.
8) All consumables should be purchased as DNA-free, while reagents must be irradiated with UV light (for at least 20 min).
9) Every PCR assay should include extraction and amplification controls.
10) To identify potential contamination from laboratory personnel, the profiles of all staff in the project should be obtained (for studies on human remains).
11) To check for reproducibility, the experiments should be performed in parallel using duplicate sample (teeth etc.) of each individual.

1.4 Postmortem changes, differentiating endogenous DNA from recent and ancient contaminant DNA

DNA molecules start to become fragmented immediately after the death of an organism (Pääbo, 1989). Moreover, some postmortem modifications on aDNA

nucleotides start to occur. These modifications may result from nuclease activities, hydrolytic processes, oxidative processes and microbial activities (Hofreiter et al., 2001). Nucleotide transitions (changing of one purine nucleotide to another purine nucleotide or one pyrimidine nucleotide to another pyrimidine nucleotide) were classified into two as Type 1 and Type 2, by Hansen et al. (2001). Type 1 transitions are thought to be PCR artifacts, whereas Type 2 transitions are considered as an indication of authenticity in ancient samples (Olivieri et al., 2010). For example, when a cytosine molecule loses its amine group, it changes into a uracil. In the subsequent PCR steps, the presence of uracil causes erroneous addition (misincorporation) of thymine instead of cytosine by the enzyme *Taq* polymerase. This is called C to T transition, one kind of Type 2 transitions. In addition to transitions, there are also transversions (changing of one pyrimidine nucleotide to a purine nucleotide or vice versa) and indels that can be observed on ancient sequences.

In relation to aDNA sequences, short fragment length (Pääbo, 1989), increased presence of purines (A and G) before strand breaks (Briggs et al., 2007) and increased C to T substitutions close to the ends of the fragments (Briggs et al., 2007; Brotherton et al., 2007) are regarded as the indications of sequence authenticity. However, it is not possible to detect these characteristics of the obtained ancient sequences with Sanger sequencing technology used in the present study. Instead, the authenticity can be supported with the help of observing differences between the misincorporation types and rates in ancient and modern sequences.

In addition to the contamination that may have resulted from modern DNA, there is also a possibility that the sample might have been contaminated back in time. However, since it is hard to differentiate between an ancient genuine DNA from an ancient contaminant DNA, it was assumed that there was no ancient contamination in the results.

1.5 Ancient DNA studies on sheep

Cai et al.'s (2007) study, with 8 samples from 2,100-1,800 BCE's China, revealed that around 2,000 BCE sheep from northern China had the mtDNA HPG A, which might indicate that sheep belonging to other HPGs have been moved there later in

time. Another study by the same group (Cai et al., 2011) with 22 Bronze Age samples from China demonstrated that among those sheep samples there were 95.5% HPG A and 4.5% B.

In another study from South Africa (Horsburgh and Rhines, 2010), DNA was extracted from 15 out of 22 samples and 506 bp fragment of mtDNA CR was amplified. The samples were dating back to 2,000 BP. Additionally, 148 bp fragment was also amplified from 5 samples which made a total of 20 sequences that were all assigned to HPG B, which is known as the most common HPG among modern sheep.

Another study by Campana et al. (2010) examined the parchments, a common source of historical information made from animal skin. They analyzed English parchments belonging to 18^{th} and 19^{th} centuries, amplified cytochrome *b* gene and mtDNA CR of DNA's extracted from these samples. They found that a parchment's DNA content can be quite complex and it can be made up of multiple individuals even of multiple species. The sequences that were assigned to sheep all grouped with sequences in clade (HPG) B.

Brandt et al. (2011) conducted a very detailed study on sheep wool, one of the most common sources for aDNA analysis, investigating how different treatment types as well as the characteristics of wool itself affect the DNA retrieval. They concluded that, except from the ones treated with some kind of dye fixer, sheep wool is a valuable DNA source to be used in future studies.

A study by Olivieri et al. (2012) presented the phylogenetic position of a Copper Age (5,350-5,100 BP) sheep based on whole mtDNA CR and cytochrome *b* gene for the first time. This sample, from Europe, was obtained from fur garments and one of the leggings of the well-known Tyrolean Iceman or Ötzi (Hollemeyer et al., 2008). The study represents a large median joining network drawn together with 335 database sequences and the Copper Age sheep sequence, showing five different (A-E) mtDNA HPGs among which HPG B includes the Copper Age sheep sequence.

In a study on Finnish ancient sheep populations spanning Iron, Medieval and Post-Medieval periods by Niemi et al. (2013), it has been found that HPGs A and B were

present, with HPG B being the most frequent. This result indicates that HPG B has been dominating Europe since at least Iron Age times.

The studies on historical genetic diversity of sheep, by far, have ascertained only two types of HPGs among ancient populations based on mtDNA (Table 1).

Table 1. Summary of genetic studies on ancient sheep populations

Study	Year	Sample size	Origin of samples	Dating of samples	Determined HPG
Cai et al.	2007	8	China	2100-1800 BCE	A
Campana et al.	2010	13	UK	300-200 BP	B
Horsburgh&Rhines	2010	22	South Africa	2000-1365 BP	B
Cai et al.	2011	14	China	2500-1500 BCE	A,B
Olivieri et al.	2012	1	Austria-Italy	5350-5100 BP	B
Niemi et al.	2013	26	Finland	1124-275 BP	A,B

None of these studies have covered all of the five mtDNA HPGs observed today. Founder effects, existence of genetic bottlenecks or genetic drifts may explain these results. However, it is also possible that the HPG diversity was lost with increasing distance from the center of domestication.

1.6 Archaeological sites from which the samples were obtained

1.6.1 Oylum Höyük

In the southern part of Turkey, Kilis province is surrounded by some limestone hills from west and the Resulosman and Avar Mountains from north (Kesici, 1995). Being enclosed by the oldest centers of civilization such as Anatolia, Mesopotamia, northern Syria and Egypt, Kilis is believed to have been occupied by mankind from the Lower Palaeolithic period (Kökten, 1952; Çiner, 1958). Albeit not as important

as agriculture, today livestock farming is common in the area as a way of living (Kesici, 1995).

Oylum Höyük, from where the samples of this study were collected, is located five kilometers away from the city center of Kilis. It is one of the largest mounds in southern Anatolia and the director of the excavation is Dr. Atilla Engin from Sivas Cumhuriyet University. The mound has a length of 460 m and a width of 370 m; is 22 m in height at the northern tip and 37 m at the southern tip. First archaeological survey was initiated in 1985 and continued ever since. After three years of excavation, it was discovered that the mound has been inhabited between 3,000 BCE and 17th century Common Era (AD) (Özgen and Carter, 1989). The approximate location of the study sites Oylum Höyük and Tepecik-Çiftlik are compared to the sheep domestication center as illustrated by Zeder (2008) in Figure 6.

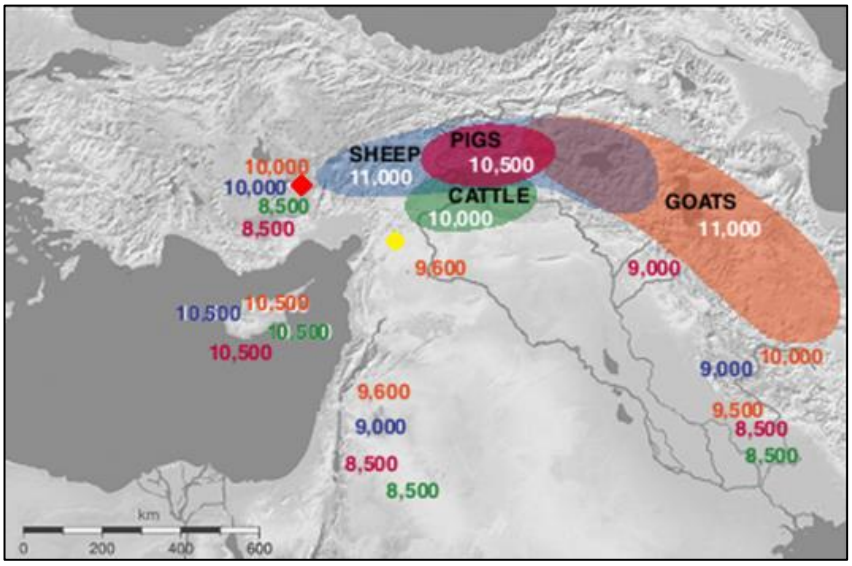


Figure 5. The map showing the domestication center for sheep with blue shade (Zeder, 2008) and the approximate locations of Oylum Höyük (yellow diamond) and Tepecik-Çiftlik (red diamond). Blue numbers outside the domestication center indicate the approximate date that domestic sheep was observed in a region (all dates in cal. years BP).

1.6.2 Tepecik-Çiftlik

The Central Anatolian plateau spans an area over 150,000 km^2 and includes the Cappadocian volcanic area on the east. The plateau has a continental climate, with cold winters and dry summers. There are more than 20 Neolithic sites discovered in Cappadocia, all of which located on the eastern volcanic area. One of these sites, Tepecik-Çiftlik inside the borders of Niğde province, has been excavated since the year 2000 by Dr. Erhan Bıçakçı from İstanbul University. According to the data retrieved in 2011 from CANeW ^{14}C database (www.canew.org), the site is dated between 6,350-6,000 cal. BCE. Tepecik-Çiftlik was a farming village with fully domestic animals and crops. It was founded in the center of the Melendiz plain on alluvium that was formed during the Pleistocene-early Holocene. This plain is bounded by the Melendiz Mountains on the south and Göllüdağ on the north. The excavations revealed five levels, 5^{th} level representing Ceramic Neolithic and 4^{th} level the transition from Neolithic to Chalcolithic (Bıçakçı, 2001; Bıçakçı et al., 2007). The ideal conditions for agriculture, strategic importance of its location enabling communication with other areas as well as management of resources and main routes of exchange make Tepecik-Çiftlik an important Neolithic site.

In the well-known Çatalhöyük, located close to Tepecik-Çiftlik, domesticated sheep and goats were revealed to be the dominant meat sources around 7^{th} millennium BCE in addition to hunted game species and a variety of fish and birds (Özbaşaran, 2011).

1.7 Objectives of the study

After the establishment of a laboratory dedicated to aDNA research, the main objectives of this study are;

1) To sequence a 144 bp fragment of sheep ancient mtDNA, so that its HPG can be determined.
2) To understand the temporal HPG composition of sheep that lived in Oylum Höyük around 1,800-330 BCE and in Tepecik-Çiftlik around 6,700-5,800 BCE.
3) To contribute to the understanding of sheep domestication history and to the history of domestic sheep in Anatolia via comparative analyses.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Collection and storage of samples

For the collection and storage of samples, the guideline provided by Dr. Licia Colli from Universita Cattolica del Sacro Cuore was followed with some minor changes. Our procedure is given below:

1) The treatments normally used by archaeologists, including washing, brushing, use of consolidants or chemicals, were avoided. The samples were kept as they are after excavation with the exception of removal of the soil by physical means (with a toothpick during identification). Otherwise, the correct classification of the samples may be hindered by the presence of soil.
2) Samples were selected among different types of archaeological remains that may yield the highest amount of DNA. These are long bones (unbroken if possible), phalanges, ribs and teeth. However, the optimal choice lies between the needs of the geneticist (high yield of DNA) and the archaeozoologist (correct species identification). Mandibles with teeth and metapodia were collected as the source material (Figures 7 and 8, respectively), because they possess relatively more DNA (as discussed in Section 1.2 for bones). Moreover, it is relatively easy to differentiate sheep from goat on the basis of these specimen types. For the identification, multiple criteria are defined in the literature for the above mentioned types of specimen. When used together with additional osteometric criteria described for metapodia, the identification is more reliable. Humerus and phalanges (n=6) from Tepecik-Çiftlik, that were identified as sheep by

archaeozoologist Dr. Can Yümni Gündem from Batman University Department of History of Art, were also subjected to DNA extraction.

Figure 6. Mandible with teeth

Figure 7.Metapodia

3) Whenever an appropriate piece of material was unearthed, it was immediately taken to a shady location by wearing gloves and face mask to avoid contamination with environmental DNA. The number of people who have access to the material was kept to a minimum (maximum of 4 to 5). The best is to allow one sample to be handled by one person.

4) The presence of live animals on the site was avoided.

5) The samples were allowed to dry for 2-3 hours under shade to remove the moisture present inside their pores. It is very important to avoid direct sunlight and excessive heating as it may cause further DNA damage.

6) After drying, each sample was put in a single-use, sealed plastic bag to avoid cross contamination. Vacuum was preferable but it was not available during our sample collection.

7) Finally, each plastic bag was labeled with the details such as collection date, type of specimen, context, coordinates etc. This step is very important since the archaeological information will be helpful when interpreting the genetic data.

The samples directly collected from the trench were kept in a cooling box to avoid summer heat, thus further DNA degradation was prevented. Following the

classification step, current protocols recommend samples to be stored in the refrigerator (+4^{o}C) for pre and post study periods.

In the present study, samples gathered from the repository were carried in a regular box to the laboratory. After their examination by Dr. Evangelia Pişkin from METU Department of Settlement Archaeology, they were also kept at +4^{o}C.

As the material of this study, of the 57 samples collected from Oylum Höyük 13 samples were from field which were collected by sterile methods and 44 samples were obtained from the repository. Six of the 13 Tepecik-Çiftlik samples were collected from the field by sterile methods and 7 were obtained from the repository. All of these samples were used for DNA extraction. Nine metapodia (metacarpal and metatarsal) and 28 mandibles from Oylum Höyük; 5 metapodia, 1 mandible, 4 humerus and 2 phalanges from Tepecik-Çiftlik yielded amplifiable DNA. One sample (OY088-2) was eliminated from analysis as there was an incompatibility regarding its archaeological context. The samples used for DNA extraction and some other related information can be seen in Tables 2 and 3.

Table 2. Summary of Oylum Höyük samples, their sample identification numbers (#), sample type, if they were collected from the field or repository and if they yielded amplifiable DNA. The sample that yielded amplifiable DNA but excluded from further study is indicated by an asterisk (*).

#	Sample type	Field/Repository	Yielded amplifiable DNA
OY003-1	Metapodium	Field	Yes
OY006-1	Metapodium	Field	No
OY007-1	Metapodium	Field	No
OY010-1	Metapodium	Field	Yes
OY012-1	Metapodium	Field	No
OY018-1	Mandible	Field	Yes
OY019-2	Metapodium	Repository	Yes
OY020-1	Mandible	Field	Yes
OY020-2	Metapodium	Repository	No
OY021-1	Mandible	Field	No
OY021-2	Metapodium	Repository	Yes
OY022-2	Metapodium	Repository	No

Table 2 (continued)

OY024-1	Mandible	Field	Yes
OY025-1	Mandible	Field	Yes
OY025-2	Metapodium	Repository	Yes
OY026-1	Mandible	Field	No
OY027-1	Mandible	Field	No
OY028-1	Mandible	Field	No
OY042-2	Metapodium	Repository	Yes
OY044-2	Metapodium	Repository	Yes
OY056-2	Metapodium	Repository	No
OY059-2	Metapodium	Repository	Yes
OY061-2	Metapodium	Repository	Yes
OY065-2	Mandible	Repository	Yes
OY066-2	Mandible	Repository	Yes
OY067-2	Mandible	Repository	Yes
OY068-2	Mandible	Repository	No
OY069-2	Mandible	Repository	No
OY070-2	Mandible	Repository	Yes
OY072-2	Mandible	Repository	Yes
OY078-2	Mandible	Repository	Yes
OY081-2	Mandible	Repository	Yes
OY082-2	Mandible	Repository	Yes
OY086-2	Mandible	Repository	Yes
OY088-2	Mandible	Repository	Yes*
OY089-2	Mandible	Repository	Yes
OY090-2	Mandible	Repository	Yes
OY091-2	Mandible	Repository	Yes
OY104-2	Mandible	Repository	No
OY105-2	Mandible	Repository	Yes
OY108-2	Mandible	Repository	No
OY109-2	Mandible	Repository	No
OY110-2	Mandible	Repository	Yes
OY113-2	Mandible	Repository	No
OY114-2	Mandible	Repository	Yes
OY122-2	Mandible	Repository	No
OY123-2	Mandible	Repository	Yes
OY130-2	Mandible	Repository	Yes
OY131-2	Mandible	Repository	Yes
OY132-2	Mandible	Repository	No

Table 2 (continued)

OY133-2	Mandible	Repository	Yes
OY134-2	Mandible	Repository	Yes
OY135-2	Mandible	Repository	No
OY136-2	Mandible	Repository	No
OY137-2	Mandible	Repository	Yes
OY138-2	Mandible	Repository	Yes
OY142-2	Mandible	Repository	Yes

Table 3. Table summarizing Tepecik-Çiftlik samples, their sample identification numbers (#), sample type, if they were collected from the field or repository and if they yielded amplifiable DNA.

#	Sample type	Field/Repository	Yielded amplifiable DNA
TC007-3	Metapodium	Field	Yes
TC008-3	Metapodium	Field	Yes
TC009-3	Mandible	Field	No
TC041-3	Humerus	Field	Yes
TC043-3	Metapodium	Field	Yes
TC045-3	Mandible	Field	Yes
TC048-3	Metapodium	Repository	Yes
TC049-3	Humerus	Repository	Yes
TC052-3	Phalanx	Repository	Yes
TC053-3	Metapodium	Repository	Yes
TC056-3	Phalanx	Repository	Yes
TC057-3	Humerus	Repository	Yes
TC058-3	Humerus	Repository	Yes

2.2 Methods

2.2.1 Species identification

After bringing the samples to the laboratory, species were identified by the archaeozoologist Dr. Pişkin based on morphological and morphometric criteria. The samples were first examined by eye and then characteristics such as their side (left/right), type of element, dental age were recorded.

Further along the examination, 4 morphological criteria by Boessneck (1969; evaluated by Zeder and Lapham, 2010) on distal epiphyses and distal shaft (latter for metatarsal) of metapodia were applied. The morphological criteria were confirmed by using two osteometric methods; first one proposed by Boessneck (1969) and the

second one proposed by Payne (1969). Studies by Payne (1985) and Halstead et al. (2002) were used to identify sheep mandibles. Moreover, a discussion by Gillis et al. (2011) about the criteria on mandibles was also taken into account.

The criteria used are shown in Table 4 and 5 for some of the samples. Of the criteria, D1: the ratio between the peripheral part and the axial part of trochlear condyle, D2: the shape of the peripheral part of trochlear condyle, D3: the orientation of the axial part of trochlear condyle, D4: (only for metatarsals) the shape of the junction between the 3rd and 4th metatarsals, Gl: greatest length, Bd: distal breath, Lcl: lateral condyle large measurement, Lcs: lateral condyle small measurement, Mcl: medial condyle large measurement, Mcs: medial condyle small measurement are seen in Table 4; P: permanent premolars, M: molars, M D1: presence of foramen on mandible, M D2: presence of hollow on mandible are seen in Table 5. Each criterion was evaluated by eye and recorded as either sheep (S), goat (G), unidentified (?) or not applicable (N.A.). The necessary measurements were made in mm. To identify the sample as sheep and select it for DNA extraction, at least three of the criteria had to be met and no criterion on the same sample should be scored as goat.

Table 4. The morphological and morphometric criteria (Payne, 1969; Boessneck, 1969) used to identify metapodia samples.

ID	D1	D2	D3	D4	Gl	Bd	Lcl	Lcs	Mcl	Mcs	Left/Right	Notes
5	G	G	G	G	1202	258	164	106	157	100	Left	
6	?	S	S	?	N.A.	N.A.	175	122	165	111	Left	
7	G	?	G	G	N.A.	N.A.	171	107	184	118	?	
8	?	S	?	S	N.A.	N.A.	173	117	162	101	Left	
9	S	S	S	S	N.A.	N.A.	173	121	N.A.	N.A.	Right	
10	S	S	?	?	N.A.	N.A.	152	102	N.A.	N.A.	Right	
11	S	S	?	S	N.A.	228	152	95	142	87	?	
12	S	?	S	S	N.A.	N.A.	172	118	164	99	Right	
13	S	S	S	S	N.A.	N.A.	190	132	181	120	Right	
14	S	S	S	S	N.A.	N.A.	181	126	N.A.	N.A.	Right	
15	S	S	S	S	N.A.	N.A.	184	124	180	117	Left	broken+erosion

Table 5. The morphological criteria (Halstead et al., 2002) used to identify mandible samples.

ID	D 3.1	D 3.2	D 3.3	D 3.4	D 4.1	D 4.2	D 4.3	YM 1.1	YM 1.2	P 3.1	P 3.2	P 3.3	P 4.1	P 4.2	P 4.3	M 1.1	M 1.2	M 1.3	M 3.1	M 3.2	M 3.3	M 3.4	M 3.5	M 3.6	M S1	M S2	M S3	M D1	M D2	L/R
63	S	N.A.	N.A.	N.A.	S	S	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	N.A.	LEFT
64	S	S	?	?	S	S	S	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S?	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	ABSENT	N.A.	LEFT
65	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	S	?	S	?	N.A.	S	?	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	N.A.	LEFT
66	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	?	S	S	?	N.A.	S	?	N.A.	S	S	?	?	?	N.A.	N.A.	N.A.	N.A.	S	N.A.	RIGHT
67	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	S	S	S	?	N.A.	S	S	N.A.	S	S	N.A.	N.A.	N.A.	S	N.A.	N.A.	N.A.	S	N.A.	RIGHT
68	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	S	N.A.	S	S	S	S	N.A.	N.A.	N.A.	N.A.	N.A.	S	N.A.	RIGHT
69	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	S	N.A.	S	?	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	N.A.	LEFT
70	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	S	?	?	N.A.	N.A.	S	?	N.A.	S	S	N.A.	S	N.A.	N.A.	N.A.	N.A.	N.A.	ABSENT	N.A.	RIGHT
71	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	?	?	S	?	N.A.	S	G?	N.A.	S	N.A.	N.A.	S	N.A.	N.A.	N.A.	N.A.	N.A.	S	S	LEFT
72	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	S	S	S	?	N.A.	N.A.	?	S	S	S	S	S	N.A.	N.A.	N.A.	N.A.	N.A.	ABSENT	S	RIGHT
73	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	?	?	?	G?	N.A.	N.A.	N.A.	N.A.	S	S	S	?	N.A.	N.A.	N.A.	N.A.	N.A.	S	?	RIGHT
74	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	S	N.A.	S	G	N.A.	S	S	S	S	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	LEFT
75	S	S	S	?	S	?	S	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	S	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	ABSENT	N.A.	LEFT
76	S	S	S	N.A.	S	N.A.	S	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	S	S	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	ABSENT	N.A.	RIGHT
77	S	S	?	?	S	S	S	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	?	S	S	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	N.A.	RIGHT
78	N.A.	S	S	S	S	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	S	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	N.A.	RIGHT
79	N.A.	N.A.	S	N.A.	S	S	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	S	N.A.	RIGHT

2.2.2 Contamination precautions taken in the aDNA laboratory

All of the pre-extraction, extraction and PCR preparation steps were performed in a dedicated aDNA laboratory at METU where modern DNA has never been studied or introduced. Before going into the laboratory the personnel put on single-use laboratory coats, face masks, bonnets, double pair of gloves and laboratory slippers to avoid possible contamination via the DNA carried from outside to the sterile room (Figure 9). The samples were not touched even with gloves whenever possible. If touched, gloves were changed between different samples. Clean mortar and pestles were used for powdering each sample. When the powdering step is finished, mortar and pestles were embedded in water diluted sodium hypochlorite solution (6/1) overnight and next day cleaned under running water by robust brushing. Then, they were UV sterilized for at least 12 hours to damage any remaining DNA that may cause cross contamination. The surfaces were frequently wiped with bleach solution before they were UV sterilized overnight.

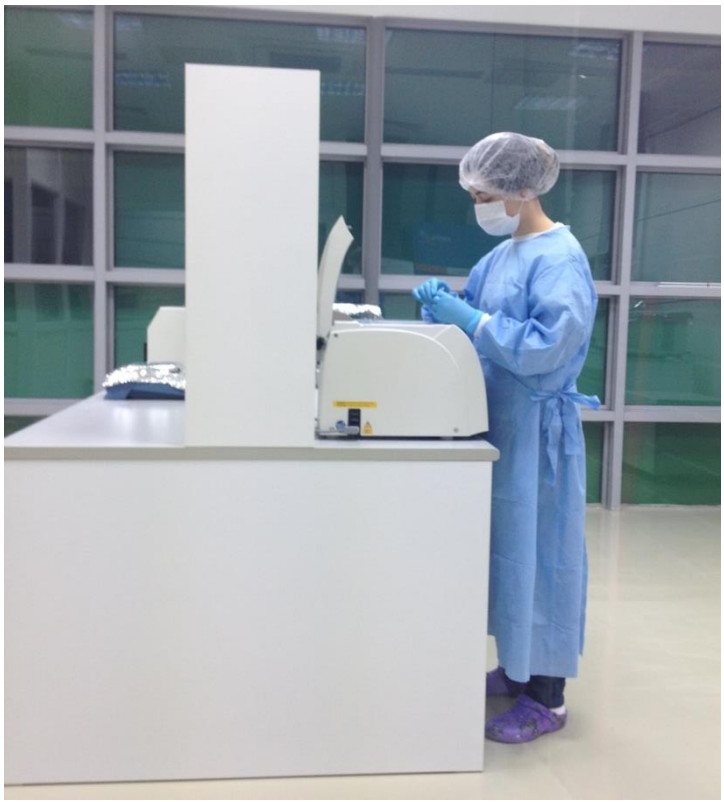


Figure 8. Working in the aDNA laboratory

In every DNA extraction and PCR preparation, there was an order of samples which was followed unidirectionally throughout the procedure. Two blank controls, one at the beginning one at the end, were also included to check any possible contamination or cross-contamination, respectively. All of the buffer solutions that are not sensitive to UV irradiation (e.g. proteinase K was sensitive) were UV-radiated for 30 min to avoid the contamination risk. Throughout all the procedures DNA-free reagents and single-use materials were used. PCR and post-PCR steps were carried out in a different laboratory so as amplified PCR products not to be introduced into the aDNA laboratory. The only missing criterion was replication of the results in a different laboratory. However, some previous studies such as Olivieri et al. (2012) also did not perform the experiments in another laboratory, but still got reliable results.

2.2.3 Pre-extraction

The preparation of the sample for extraction was carried out in a Class 2A Biosafety cabinet (MetiSafe) located in the pre-extraction room of aDNA laboratory. Inside the cabinet, the materials and equipment used were cleaned with a tissue soaked with dH_2O and UV irradiated (254 nm) for minimum of 15 min to avoid contamination. Separate pieces of aluminum foil laid inside the safety cabinet were used for each different sample to lower the risk of contamination. Before cutting a piece of sample to be used for DNA extraction, the surface layer of samples was removed by using a drill (Proxxon Micromot 40/E) with single-use cutting discs (Proxxon, catalog numbers: NO28812 and NO28830). Then, a piece of the sample was cut and put in a mortar to grind the freshly uncovered part as fine as possible with the help of a pestle. One should keep in mind that the finest powder does not yield the best result. Therefore, the piece of sample was grinded in a way that allows enough DNA to be extracted not causing further DNA damage by physical means. The same holds true for cutting the sample. The minimum speed of the drill was used to prevent excessive heating. 230-250 mg of bone powder was used for each extraction, which was later put into the tubes labeled with sample ID, amount of bone powder and the date of extraction.

2.2.4 DNA Extraction

For DNA extraction, the column-based extraction method explained in Rohland et al.'s (2009) study was followed with a few minor changes. The procedure is as follows;

Five ml extraction buffer was added to each bone powder. The tubes were sealed with parafilm and rotated overnight (16-20 hours) at room temperature in an incubator (FinePCR Combi-SV12). Later, they were spun in a table-top centrifuge (Thermo Scientific SL16) for 2 min at 5000g. New tubes were labeled and 2.5 ml binding buffer was added to each new tube. The supernatants were transferred into these tubes containing binding buffer (the pellet was kept at -20^{o}C in case the sample is not sufficient). Silica buffer was vortexed for 10 s and 100 µl silica buffer was added to the tubes. The tubes were sealed again with parafilm and rotated for 3h. Then they were spun under the conditions given above. The supernatants were discarded (first by pouring and later with the help of the pipette). 400 µl binding buffer was added to the pellets and they were resuspended by gently shaking (or by pipetting up and down). The samples were transferred into newly labelled 2 ml tubes containing filter (Whatman GF/B 1.0 µm) assembled columns (MoBiTec GmbH, Goettingen, Germany; catalog number: M1002S) and they were spun for 2 min at 13.300g in a microcentrifuge (Thermo Scientific MicroCL17). The supernatants were discarded and 450 µl washing buffer was added to each tube. They were spun for 2 min at 13.300g and the supernatants were discarded. The washing step was repeated once more. After discarding the supernatants the empty columns were spun for 2 min at 13.300g to remove remaining buffer. Then 50 µl TE buffer was added to the tubes and waited for 10 min with closed lids. The tubes were spun for 2 min at 13.300g and the columns were discarded. One µl of %10 Tween (BioShop Life Science Products, Ontario, Canada; catalog number: TWN511) was added to prevent DNA from adhering to the surface of the tube. The DNA extracts were frozen (at -20^{o}C) if not going to be used immediately, otherwise kept at +4^{o}C.

2.2.5 DNA Amplification

A pair of primers designed by Cai et al. (2007), using the mtDNA sequence of one individual with the GenBank accession number AF010406.1 (Hiendleder et al., 1998a) as a reference, was used to amplify a 144 bp fragment of mtDNA. This fragment partially encompasses $tRNA^{Pro}$ and the CR of mitochondrial genome (Figure 10).

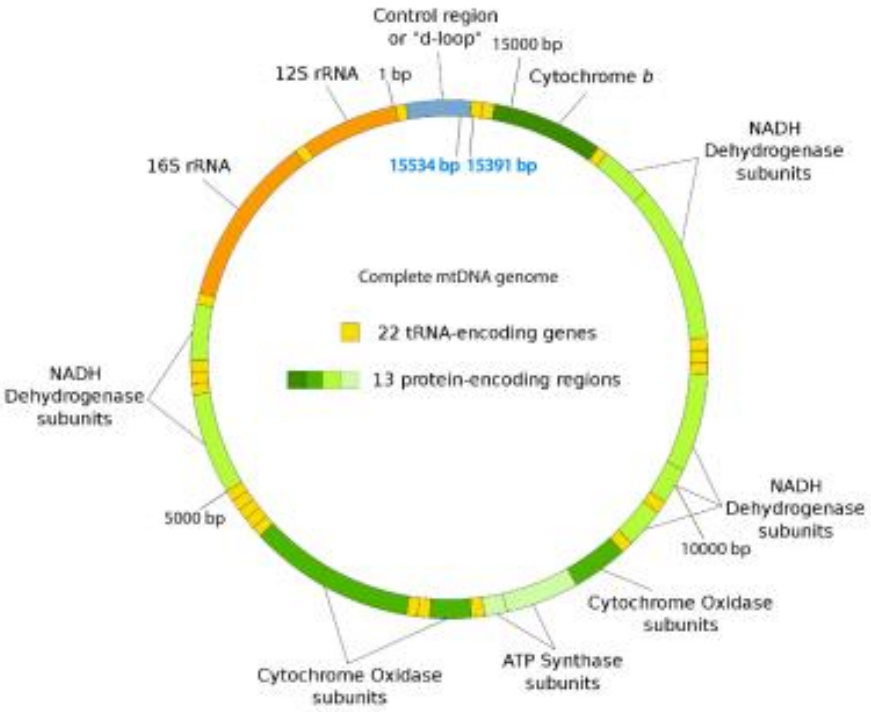


Figure 9. The mitochondrion genome showing important coding and noncoding sites (taken from Bollvåg, 2010). The position of the fragment amplified in this study is shown between blue numbers.

Sequences of the primer pair are shown in the Figure 11 below.

mtDNA site	Primer	Primer sequence	Fragment length (bp)
$tRNA^{Pro}$ - HVRI	L15391[a]	5′-CCACTATCAACACCCAAAG-3′	144
	H15534	5′-AAGTCCGTGTTGTATGTTTG-3′	

Figure 10. The forward and reverse primer sequences (L15391-H15534), their mtDNA sites and fragment lengths. Figure was taken from Cai et al.'s (2007) study.

On the onset of the experiments, the second pair of primers from Cai et al.'s study (2007) was also included to amplify another 166 bp mtDNA CR fragment. Later, it was abandoned due to unsuccessful amplifications.

Amplifications were performed in 20 µL (5 µL template DNA added to 15 µL PCR mix) reaction volumes with the reagents detailed in Table 6.

Table 6. Reagents for PCR amplification

HPLC grade H_2O	7.4 µL
Buffer (10x)	2.0 µL
Mg^{+2} (25 mM)	1.6 µL
Primer F (10 µM)	1.5 µL
Primer R (10 µM)	1.5 µL
AmpliTaq Gold 360 (5 U/µL)	0.6 µL
dNTP (25 mM)	0.2 µL
BSA (10 mg/mL)	0.2 µL

One of the most important reagents, the enzyme DNA polymerase "AmpliTaq Gold 360" (Applied Biosystems Inc., Foster City, CA, USA; catalog number: 4398823) is provided in an inactive state to make sure that unspecific primer binding will not occur before all DNA strands are denatured at high temperatures. The amplifications were performed using Techne Techgene Thermal Cycler FTGENE2D (Bibby Scientific Limited, Staffordshire, UK) by following the reaction conditions described in Table 7.

Table 7. PCR conditions

Initial denaturation	94°C	10 min	
Denaturation	94°C	30 s	60 cycles
Primer Annealing	53°C	45 s	
Extension	72°C	45 s	
Final extension	72°C	5 min	
Base temperature	10°C	∞	

2.2.6 Gel Electrophoresis

The products were separated by electrophoresis on 2.5% agarose gel prepared with 0.5x TBE buffer and 0.4 µl SYBR Safe (Life Technologies Corporation, Carlsbad, CA, USA). Five µL of each PCR product was mixed with 1 µL 6x Blue/Orange loading dye (Promega Corporation, Madison, WI, USA) before it was applied to one of the wells. The gel was run for 75 min at 85 V and visualized under UV light by utilizing Vilber Lourmat CN-3000WL. By using 50 bp DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA) as the reference, products were checked if their sizes were 144 bp long. Reference DNA ladder is composed of DNA fragments with known sizes (50, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900, 1000 bps).

2.2.7 Purification and Sequencing

Successfully amplified samples were purified with GeneJET PCR Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) to remove any excess dNTPs, salts, primers and enzymes. Following the manufacturer's protocol, the procedure is as follows.

1) Binding buffer (1:1 volume) was added to completed PCR product (i.e., for every 15 µL of reaction mixture remained after electrophoresis, 15 µL of binding buffer was added) and mixed thoroughly. The color of the solution was checked. A yellow color was observed in each case, which indicates an optimal pH for DNA binding.
2) Since the DNA fragment is ≤500 bp, 1:2 volume of 100% isopropanol was added (i.e., 15 µL of isopropanol was added to 15 µL of PCR product combined with 15 µL of binding buffer) and mixed thoroughly. If the fragment was longer, this step would be neglected.
3) The solution from step 2 (45 µL) was transferred to the purification column, which was supplied with the kit, and centrifuged for 1 min. The flow-through was discarded right after centrifugation.
4) Wash buffer (700 µL) was added to the purification column and centrifuged for 1 min. The flow-through was discarded and the purification column was placed back into the collection tube.

5) The empty purification column was centrifuged for an additional 1 min to completely remove any residual wash buffer since the presence of ethanol may inhibit subsequent reactions.
6) The purification column was transferred to a UV-sterilized 1.5 mL centrifuge tube. Twenty µL of elution buffer was added to the center of the column membrane and the tube was centrifuged for 1 min after 10 min waiting time to elute as many DNA molecules as possible. Elution volume was reduced to 20 µL rather than 50 µL to increase DNA concentration.
7) The purification column was discarded and the purified products were kept at +4^{o}C until they are sent for sequencing.

All purification steps were carried out at room temperature. For all the centrifugation steps Hettich table-top minicentrifuge was used at 15000 rpm. Following the purification, these samples were sent to a company (RefGen, http://www.refgen.com/) for direct sequencing with ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA) by using the same primers which were used for the PCR. The raw data was provided to us in the form of DNA chromatograms which were analyzed to obtain the consensus sequences.

2.3 Analyses

2.3.1 Editing and alignment of the sequences

The acquired sequences, amplified by forward and reverse primers, were edited by using Geneious 7.0.5 software (created by Biomatters, http://www.geneious.com). First, the raw chromatograms of forward and reverse sequences were loaded separately to the software and the primer binding sites at the beginning together with the last nucleotides that were not successfully read were trimmed to increase quality of the further alignment. Then, the two sequences of the same sample were assembled and the inconsistencies between them were corrected manually by checking the peaks found in chromatograms. After the first attempt, forward and reverse sequences that could not be assembled successfully were aligned to the reference sequence (AF010406.1 from Hiendleder et al., 1998a). Following this alignment, it was possible to check the inconsistent points that caused the assemblage to fail. At the end, all of the obtained consensus sequences were compared to the

sequences in GenBank database by using BLAST application incorporated in the software to confirm that the sequences belong to sheep. All of the sequences included in the analyses were assigned to sheep. Finally, the consensus sequences were exported for the multiple alignment.

Sequences representing 1 Merinolandschaf breed (GenBank accession number: AF010406.1 from Hiendleder et al., 1998a), 13 Turkish breeds (AKK: Akkaraman (n=21), HEM: Hemşin (n=17), IVE: İvesi (n=25), HER: Herik (n=14), KGR: Karagül (n=20), MRK: Morkaraman (n=24), DAG: Dağlıç (n=22), GOK: Gökçeada (n=11), CIC: Çineçaparı (n=16), KIV: Kıvırcık (n=8), KRY: Karayaka (n=15), NOR: Norduz (n=22), SAK: Sakız (n=18); GenBank accession numbers: KF677024-KF677114, KF677116-KF677124, KF677126-KF677176, KF677178-KF677212, KF677214-KF677236, KF677238-KF677241, KF677243-KF677245, KF677247-KF677263 from Demirci et al., 2013) and 1 wild population of *Ovis gmelinii* (n=29) *anatolica* (GenBank accession numbers: KF677264-KF677282, KF677284-KF677293 from Demirci et al., 2013), in total 263 sequences, were used as references for the multiple alignment by Consensus Alignment method (with default parameters) in Geneious 7.0.5. The resulting alignment was checked for any erroneous nucleotides incorporated or any unnecessary insertion-deletions (indels). After ensuring that the nucleotides differing from the majority on some positions are indeed correct, the mtDNA HPGs were assigned to each sequence.

2.3.2 Determination of haplogroups

Mitochondrial DNA HPGs of 263 reference sequences were known from previous studies (Hiendleder et al., 1998a; Demirci et al., 2013). HPG designations (A-E) were performed on the basis of nucleotide differences at certain nucleotide positions along partial mtDNA CR. While this study covers a 144 bp region partially encompassing both $tRNA^{Pro}$ and CR on mtDNA, the positions that are key points in determining mtDNA HPGs are all found on CR. Therefore, Table 8 covers five nucleotide positions on CR (15459^{th}, 15476^{th}, 15484^{th}, 15509^{th} and 15512^{nd} nucleotides) to make it easier to compare. The reference sequences in the table were chosen randomly from 263 modern sequences used in the present study.

Table 8. The positions important for HPG determination (A similar table was included in the paper written by Demirci et al. (2013)).

HPG	Reference Sequence	HPG determining positions on AF010406.1				
		15459	15476	15484	15509	15512
HPG A	KF677026	T	T	A	A	T
HPG B	AF010406.1	C	T	G	A	T
HPG C	KF677208	C	T	G	G	T
HPG D	KF677056	C	T	G	A	C
HPG E	KF677144	C	C	G	G	T

2.3.3 Calculations of nucleotide misincorporations

As DNA is subjected to chemical change after the death of an organism, it is expected that certain amount of misincorporated nucleotides as transitions, transversions or indels are present. The positions of these misincorporated nucleotides were determined by comparing with the reference sequences, whenever there is a difference between samples and the references. Then, the probability of observing each nucleotide at a given position was calculated on the basis of 263 reference sequences. That way, it was possible to understand if the observation was likely or unlikely for that particular position (suggesting if there is a misincorporation).

2.3.4 Calculations of per nucleotide transition rates

For the two chronological groups of Oylum Höyük, the rates of transitions per nucleotide sequenced per sample were calculated as a mean. The number of transitions observed for one sample was divided by the number of nucleotides sequenced for that particular sample. After calculating the rate for all of the samples in one group, the mean of the rates was calculated by first adding the rates together and then dividing the total value by the total number of samples.

2.3.5 Significance test

For some of the results, the test of significance was performed by utilizing Fisher's exact test in R statistical software version 3.0.2 (http://www.r-project.org/).

CHAPTER 3

RESULTS

During the archaeological season of 2011, Oylum Höyük was visited by the author to collect samples from the field. The following year, the author and Eren Yüncü obtained samples from the field and the repository; the latter was to increase the sample size. In 2013, the site was visited again by the author and 10 samples were collected from the repository. Additionally, another archaeological site, Tepecik-Çiftlik was visited by Elçin Ekşi to bring the samples to the laboratory.

The identification of Oylum Höyük samples was performed by Dr. Pişkin. 96 out of 187 were classified as sheep. For the Tepecik-Çiftlik samples, identification was done by Dr. Gündem. Sixty three sheep samples were stored in the aDNA laboratory.

The treatment of samples before DNA extraction was performed as described in Section 2.1. Moreover, all of the criteria mentioned in Section 1.3 related to the procedure were met.

3.1 DNA Amplification

Ancient DNA from 49 out of 70 samples (37/57=65% from Oylum Höyük, 12/13=92.3% from Tepecik-Çiftlik) was successfully extracted. DNA extractions and especially the amplifications were repeated for some samples to see the reproducibility of the HPG identifications. For this reason, randomly chosen samples (n=2) were subjected to DNA extraction at two different occasions and the extracts were amplified several times. The rest of the samples (n=47) were extracted once and some randomly chosen samples were amplified once (n=18), twice (n=23) or three times (n=6) (Table 9 in Section 3.3). Due to time and budget constraints and since

there was no difference between the replicates in terms of HPG assignment, not all of the samples were amplified more than once.

Before the sequencing step, the amplicons were checked to see whether they are approximately 144 bp in length. Figure 12 shows the results of successful amplifications observed on 2.5% agarose gel.

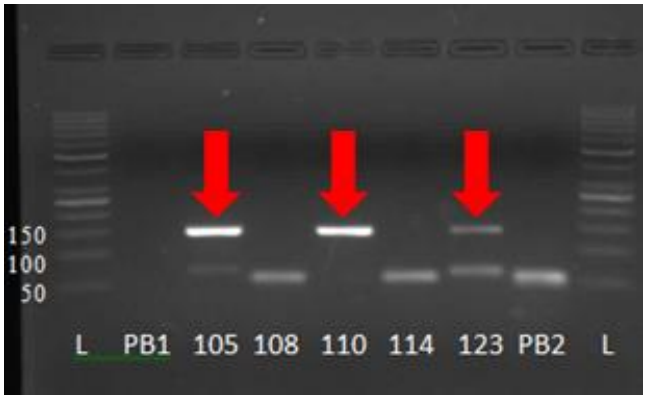


Figure 11. An example of an agarose gel showing successfully amplified (indicated by arrows) samples. Wells indicated by 108 and 114 represent failed amplification attempts. The bands showing a lower molecular weight than the expected band are primer artifacts. Symbols represent the following: L: reference ladder, PB1: PCR blank 1, Samples: OY105-2, OY108-2, OY110-2, OY114-2, OY123-2, PB2: PCR blank 2.

It can be seen that samples OY105-2, OY110-2 and OY123-2 (with a faint band) have the expected fragment (144 bp) when compared to the size of the PCR products with the reference ladder (L).

Figure 13 shows the amplification with the first and the second pair of primers, the latter was also included in the study on the onset of the experiments. However, the second pair did not amplify the target region although the first pair was successful for the sample OY003-1 (the band as the same size of 150 bp band of the reference ladder). Thus, the second pair was not used in the study.

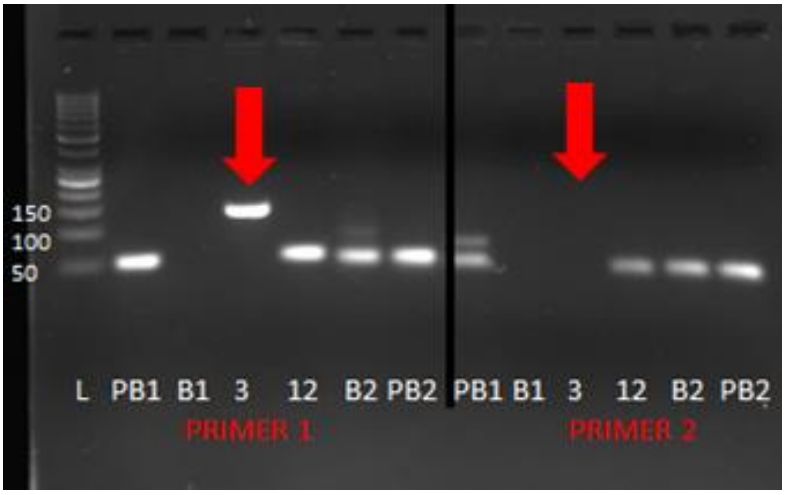


Figure 12. An example of an agarose gel showing two samples (OY003-1 and OY012-1) amplified by using two pairs of primers described in Cai et al. (2007). Red arrows indicate the successful (left) and unsuccessful (right) amplifications by utilizing first and second primer pairs, respectively. The bands showing a lower molecular weight than the expected band are primer artifacts. Symbols represent the following: L: reference ladder, PB1: PCR blank 1, B1: extraction blank 1, Samples: OY003-1, OY012-1, B2: extraction blank 2, PB2: PCR blank 2.

Figure 14 below shows another 2.5% agarose gel, on which a contamination in sample OY026-1 was detected. In fact, in such cases the subsequent sequencing was not performed to save the limited funds. Here, however, the contaminated sample was sequenced to see the source species of contamination.

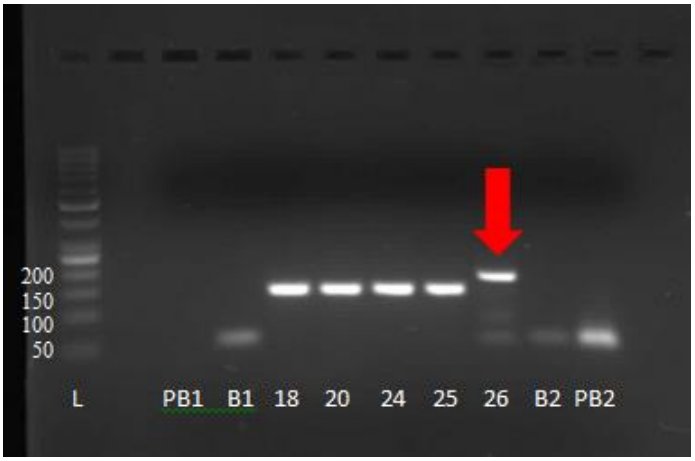


Figure 13. An example of an agarose gel showing a contamination detected (indicated by the red arrow). The bands showing a lower molecular weight than the expected band are primer artifacts. Symbols represent the following: L: reference ladder, PB1: PCR blank 1, B1: extraction blank 1, Samples: OY018-1, OY020-1, OY024-1, OY025-1, OY026-1, B2: extraction blank 2, PB2: PCR blank 2.

In Figure 14, while the expected bands are on the same level with 150 bp band of the reference ladder (L), sample OY026-1 has a band near 200 bp. After a BLAST search, its sequence gave a 88% hit with a soil bacterium, *Ralstonia syzygii*.

All of the successfully amplified samples were purified and then sent out for sequencing.

3.2 Sequence editing and alignment

A raw file generated by the ABI PRISM® 3100 Genetic Analyzer is given in Figure 15 when opened with the software Geneious 7.0.5 (created by Biomatters, http://www.geneious.com). Each sample had both a forward and a reverse sequence. Figure 15 shows the forward sequence of sample OY019-2 with a screenshot from the software before the low quality peaks were trimmed. The low quality peaks can be seen at the opposite ends of the sequence.

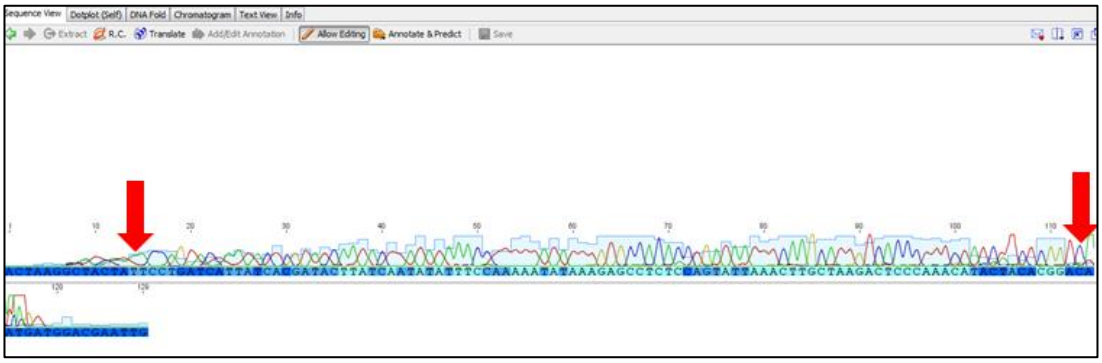


Figure 14. Raw data generated by ABI PRISM® 3100 Genetic Analyzer which belongs to the forward sequence of OY019-2 shown in Geneious 7.0.5. Red arrows point out the low quality DNA sequences (to the left from the first arrow and to the right from the second) that were trimmed from the ends.

Another output from the software can be seen in Figure 16. This figure shows assembled forward and reverse sequences of the sample OY003-1, with pink horizontal bars indicating the trimmed regions from the ends of 144 bp fragment. The consensus sequences were formed with the good quality peaks observed from both sequences, enabling to decide about the type of the nucleotide at a given DNA position without ambiguity.

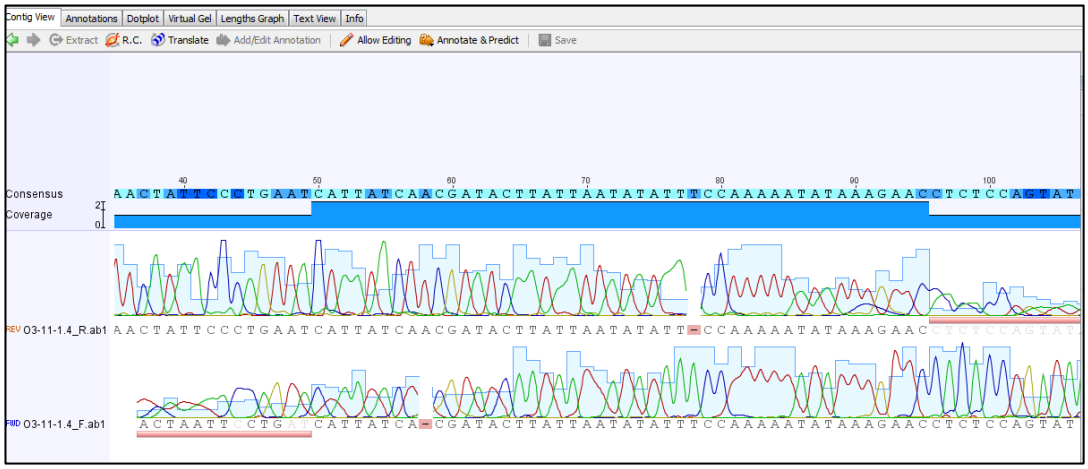


Figure 15. A screenshot from the software Geneious 7.0.5, showing the chromatograms belonging to the sequences amplified by forward (lower) and reverse (upper) primers (sample OY003-1). At the very top, the consensus sequence with the letters indicating 4 different nucleotides (A, T, G, C) is shown.

After all of the consensus sequences were exported, the resulted list of consensuses is created (Figure 17) for the multiple alignment.

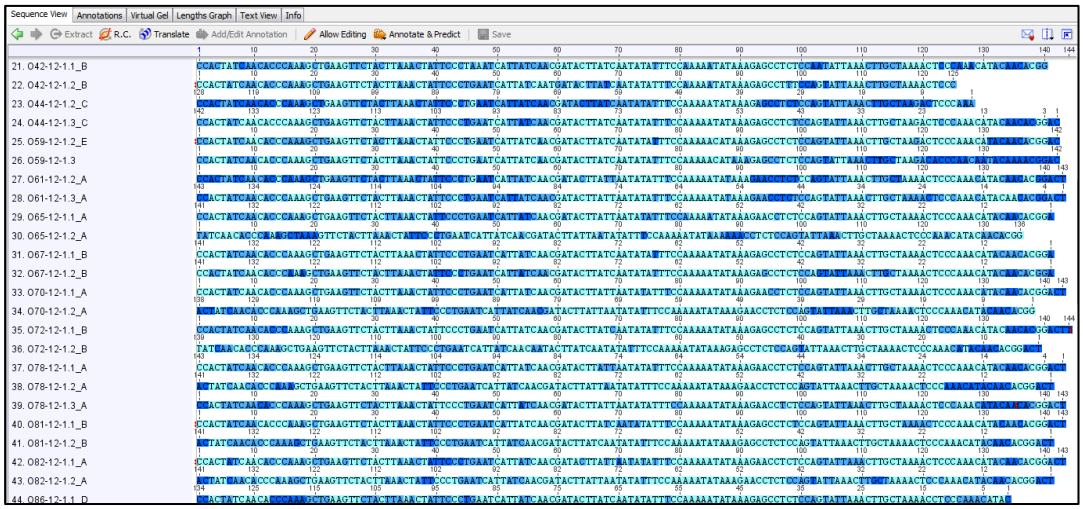


Figure 16. A screenshot from the software Geneious 7.0.5, which displays the consensus sequences that were ready for the multiple alignment.

Figure 18 below shows the aligned sequences covering some of the Oylum Höyük samples and references retrieved from GenBank, detailed in Section 2.3.1.

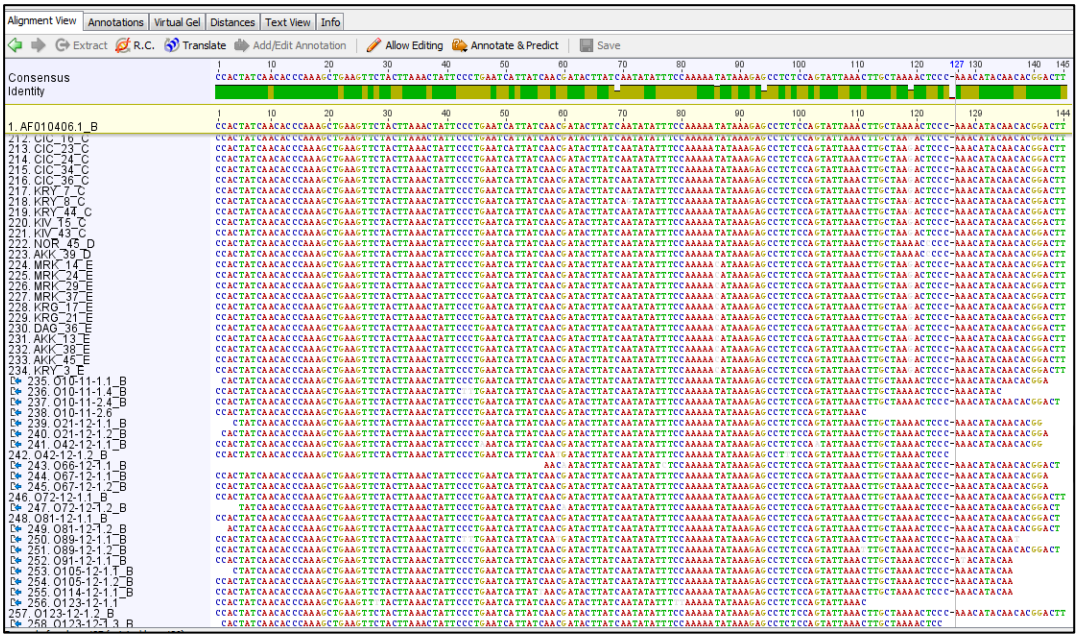


Figure 17. Some of the Oylum Höyük consensus sequences and the references that were aligned with Geneious 7.0.5 software. Each color represents one of the four different nucleotides (blue: C, red: A, green: T, yellow: G).

After the differences on the alignment seen in Figure 18 were checked, the final corrected alignment was used to determine HPGs.

3.3 Haplogroup assignment based on partial mtDNA CR

The mtDNA fragment as short as 144 bp was useful for differentiating the HPGs since each HPG is composed of a different combination of nucleotides on the HPG. The samples that were successfully sequenced and assigned to HPGs were given in Tables 9 and 10. Dating was based on archaeological context, except the samples OY042-2, OY044-2 and OY019-2 which were sent for Accelerated Mass Spectrometry (AMS) carbon-14 dating (to Beta Analytic Inc. Laboratory, Miami, FL, USA).

Table 9. All sequenced Oylum Höyük samples and their identified HPGs. Number of DNA extractions and PCR amplifications were recorded next to each sample. A similar table was depicted in the study by Demirci et al. (2013). Asterisks (*) indicate the results of AMS carbon-14 dating.

Sample code (DNA extractions/PCR amplifications)	**Dating by context**	**Haplogroup**
OY003-1 (2/6)	1800-1700 BCE	A
OY018-1 (1/2)	1800-1700 BCE	A
OY020-1 (1/2)	1800-1700 BCE	A
OY024-1 (1/2)	1800-1700 BCE	A
OY025-1 (1/2)	1800-1700 BCE	A
OY065-2 (1/2)	1800-1700 BCE	A
OY010-1 (2/4)	1800-1700 BCE	B
OY042-2 (1/2)	1800-1700 BCE (1880 -1690 BCE)*	B
OY131-2 (1/1)	1800-1600 BCE	A
OY137-2 (1/1)	1800-1600 BCE	A
OY134-2 (1/3)	1800-1600 BCE	B
OY086-2 (1/2)	1800-1600 BCE	D
OY130-2 (1/2)	1800-1600 BCE	D
OY044-2 (1/3)	1800-1600 BCE (1880 – 1680BCE)*	C
OY133-2 (1/2)	1600-1200 BCE	A
OY066-2 (1/1)	1600-1200 BCE	B
OY105-2 (1/2)	1600-1200 BCE	B

Table 9 (continued)

OY067-2 (1/2)	1600-1200 BCE	B
OY070-2 (1/2)	1200-900 BCE	A
OY061-2 (1/3)	1200-900 BCE	A
OY059-2 (1/3)	1200-900 BCE	E
OY090-2 (1/1)	1200-330 BCE	A
OY123-2 (1/3)	1200-330 BCE	B
OY072-2 (1/2)	1200-330 BCE	B
OY019-2 (1/2)	900-700 BCE (1000 – 840 BCE)*	C
OY078-2 (1/3)	700-330 BCE	A
OY082-2 (1/2)	700-330 BCE	A
OY110-2 (1/2)	700-330 BCE	A
OY025-2 (1/2)	700-330 BCE	A
OY142-2 (1/2)	700-330 BCE	A
OY089-2 (1/2)	700-330 BCE	B
OY091-2 (1/1)	700-330 BCE	B
OY081-2 (1/2)	700-330 BCE	B
OY138-2 (1/2)	700-330 BCE	B
OY021-2 (1/2)	330-30 BCE	B
OY114-2 (1/1)	330-30 BCE	B

In addition to the samples in Table 9, the sample OY088-2 that was excluded from further analyses was assigned to HPG A.

Table 10. Summary of Tepecik-Çiftlik samples sequenced and their identified HPGs. Number of DNA extractions and PCR amplifications were recorded next to each sample.

Sample code (DNA extractions/PCR amplifications)	**Dating by context**	**Haplogroup**
TC007-3 (1/1)	6000-5800 BCE	B
TC008-3 (1/1)	6000-5800 BCE	B
TC009-3 (1/1)	6000-5800 BCE	B
TC041-3 (1/1)	6000-5800 BCE	B
TC043-3 (1/1)	6000-5800 BCE	B
TC045-3 (1/1)	6000-5800 BCE	B
TC048-3 (1/1)	6000-5800 BCE	E

Table 10 (continued)

TC049-3 (1/1)	6000-5800 BCE	B
TC052-3 (1/1)	6700-6500 BCE	B
TC053-3 (1/1)	6700-6500 BCE	B
TC056-3 (1/1)	6700 BCE	E
TC057-3 (1/1)	6700 BCE	B
TC058-3 (1/1)	6700 BCE	B

Table 11 below shows the overall alignment of all the sequences from the present study together with randomly chosen reference sequences (n=14).

Table 11. The overall alignment of all the sequences. Red boxes indicate five critical points that help to differentiate HPGs. First 14 samples are the randomly selected reference sequences, while the rest are the samples from the present study. The replicates that belong to the same sample were indicated as a group of alternating colored-uncolored sequences in the second column of the table. (Nucleotide misincorporations: blue: A, orange: T, green: C, yellow: G, pink: Y; dark grey boxes: deletions and nucleotides at the end of sequences that could not be identified because of poor sequencing quality; alternating colored/uncolored sequences represent the replicates of each sample; dots: same nucleotide with the reference sequence).

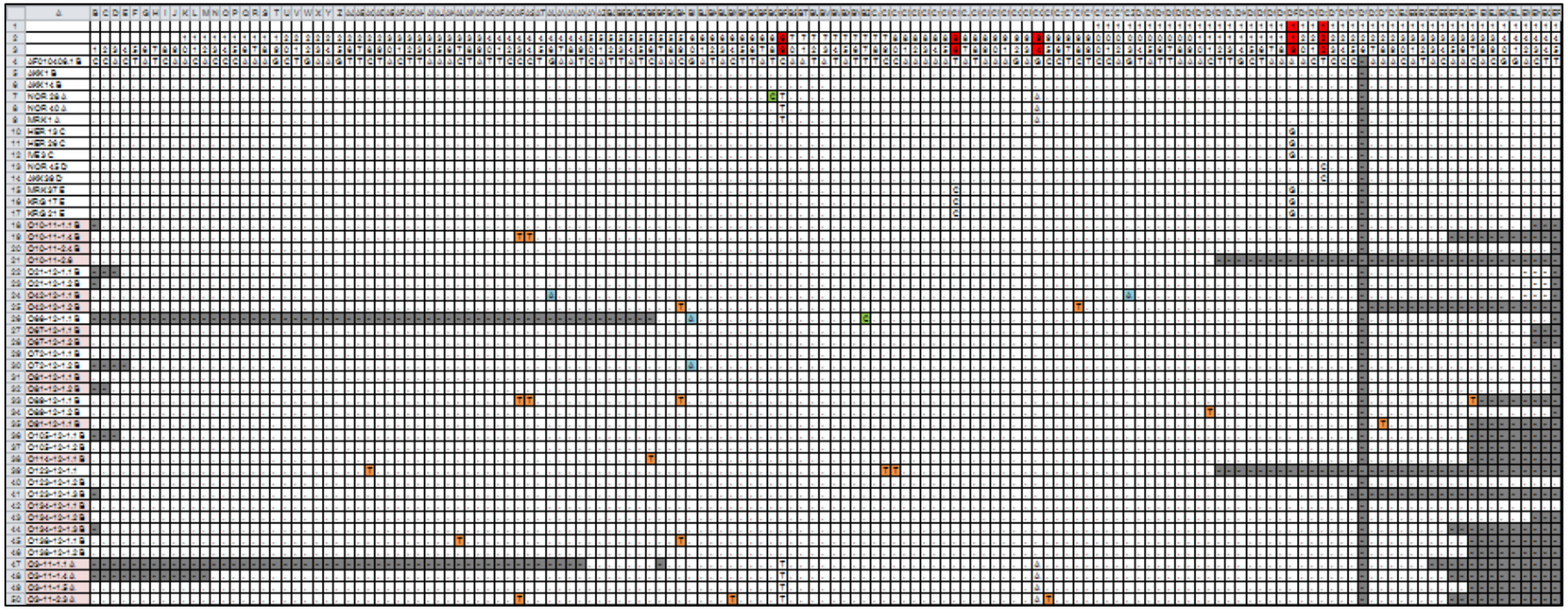


Table 11 (continued)

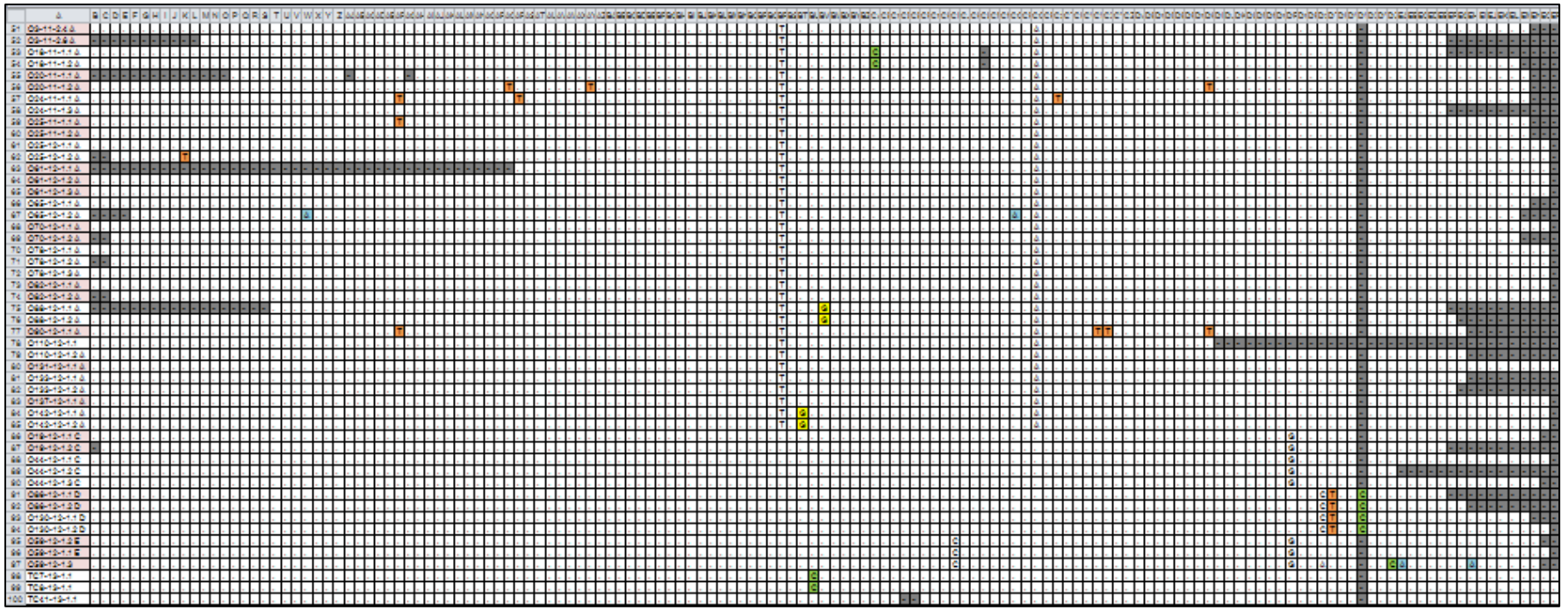
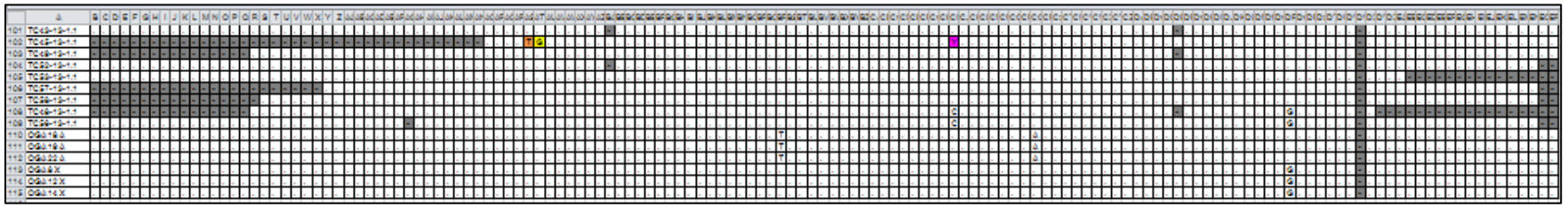


Table 11 (continued)



Samples OY066-2, OY091-2, OY114-2, OY090-2, OY131-2, OY137-2 and all of the samples from Tepecik-Çiftlik have one sequence, lacking replicates to check for the reliability of nucleotide misincorporations. Thus, for these samples HPGs were assigned but could not be confirmed.

When the wild *Ovis gmelinii anatolica* sequences which have the mtDNA HPG of X (Demirci, 2012) are compared to the sequences of studied samples, it is observed that the 144 bp mtDNA fragment was unable to differentiate between X and C HPGs.

3.4 Haplogroup distributions with respect to archaeological chronology

By considering the stratigraphy of unearthed layers at Oylum Höyük (Erdem Dağ, 2004), samples were grouped into two: the second half of Bronze Age (1,800-1,200 BCE) and Iron Age (1,200-330 BCE). Moreover, the study also covers two samples from Hellenistic period (330-30) belonging to HPG B. However, as there are not enough samples dated to Hellenistic period another group was not formed. The distribution of HPGs for two chronological groups of Oylum Höyük is shown in Table 12, together with the distribution of Tepecik-Çiftlik samples spanning the period between 6,700 and 5,800 BCE.

Table 12. The HPG distribution of Oylum Höyük and Tepecik-Çiftlik samples

Archaeological site	Dating	HPG A	HPG B	HPG C	HPG D	HPG E	Total
Oylum Höyük	1800-1200 BCE	9	6	1	2	_	18
	1200-330 BCE	8	6	1	_	1	16
Tepecik-Çiftlik	6700-5800 BCE	_	10	_	_	2	12

Among 34 samples obtained from Oylum Höyük spanning the period 1,800-330 BCE, HPG A (17/34, 50%) was the most common HPG, followed by HPG B (12/34, 35.3%). HPG C has a frequency of 5.9% (2/34) which is the same as HPG D (2/34, 5.9%). Finally, HPG E is observed in 1 out of 34 samples (2.9%).

Mostly HPG B (10/12, 83%) and then HPG E (2/12, 17%) constituted HPG composition of Tepecik-Çiftlik samples as seen in Table 12.

3.5 Putative misincorporations and authenticity of aDNA sequences

To determine the putative misincorporations and indels only those samples represented with at least two amplifications were considered. Each nucleotide on the sequences was examined comparatively with respect to its state at the homologous position between the replicates of its own and reference sequences. The evaluation is explained by two examples given in Tables 13 and 14.

Table 13. A part of the two replicate sequences belonging to sample OY089-2 is given with the reference sequence (AF010406.1). (The orange boxes: nucleotide misincorporations, dots: same nucleotide with the reference sequence).

Nucleotide positions	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65
AF010406.1	T	T	C	C	C	T	G	A	A	T	C	A	T	T	A	T	C	A	A	C	G	A
OY089-2-1.1	.	.	.	T	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.
OY089-2-1.2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

The nucleotide “T” on the 43rd position of OY089-2-1.1 was “C” on the same position for OY089-2-1.2 (second sequence of the same individual in Table 13). Moreover, it is different than all the nucleotides of the reference sequences (represented by AF010406.1 in Table 13 which has the same nucleotide composition as the other reference sequences) on this position such that they all had “C”. Thus, it was considered as a nucleotide misincorporation (transition Type 2) given low probability of heteroplasmy. Another example is the nucleotide “G” observed on the 71st position of two replicates of the sample OY142-2 in Table 14. “G” is also observed among the reference sequences (represented by AF010406.1 in Table 14) with a probability of greater than 0.5% (“A” is observed with 99.2% probability), in that case it is not considered as misincorporation. However, if the probability was less than 0.5% (an arbitrarily chosen threshold value) then again it would be considered as misincorporation.

Table 14. A part of the two replicate sequences belonging to sample OY142-2 is given with the reference sequence (AF010406.1). (The yellow boxes: nucleotide misincorporations, dots: same nucleotide with the reference sequence).

Nucleotide positions	7 0	7 1	7 2	7 3	7 4	7 5	7 6	7 7	7 8	7 9	8 0
AF010406.1	A	A	T	A	T	A	T	T	T	C	C
O142-12-	.	G	.	.	.	.	.	.	.	.	.
O142-12-	.	G	.	.	.	.	.	.	.	.	.

The extended table where all the references together with the aDNA sequences (including replicates) obtained in the present study (n=92) are given in Appendix B. It is important to note that sample OY088-2 was not included in these evaluations.

It is important to emphasize that none of the possible misincorporations except one (in sample TC045-3, which is denoted as Y in Table 11) were observed for positions utilized in HPG determination. This nucleotide "Y" represents a "C" or "T" found at this position, as the forward sequence of the sample TC045-3 has "C" while the reverse sequence has "T".

The number of putative misincorporations in terms of their transition types (Type 1: A=>G, T=>C and Type 2: C=>T, G=>A), transversion types and indels that were present on the ancient sequences are summarized in Table 15. It must be noted that, since some of the nucleotides might be true nucleotide substitutions rather than misincorporations or they are the errors observed in PCR amplifications, "misincorporation" estimates may be over estimates.

Table 15. Total number of putative nucleotide misincorporations found across the ancient sequences.

	Transitions (Type 1)		Transitions (Type 2)		Transversions				Deletions
	A=>G	T=>C	C=>T	G=>A	G=>T/T=>G	A=>C/C=>A	A=>T/T=>A	C=>G/G=>C	
Putative Misincorporations	0	0	24	5	0/0	1/2	1/1	0/0	3

In Table 15, it can be seen that on the basis of the studied mtDNA region for 34 samples from Oylum Höyük Type 1 transitions or insertions were not observed. Whereas Type 2 transitions (n=29) were outnumbering the total nucleotide changes observed among the ancient samples (n=34). It must be noted that 21 out of 34 samples were found to not contain any misincorporations.

Table 16 shows the observed changes with respect to archaeological chronology at Oylum Höyük. Type 2 transitions were calculated as the mean of transitions per nucleotide sequenced per sample for that chronological group as different replicates have different lengths due to PCR or sequencing quality.

Table 16. Observed per nucleotide misincorporation rates with respect to archaeological chronology at Oylum Höyük.

Dating	Transitions (Type 1)	Transitions (Type 2)	Transversions	Deletions	Insertions
1800-1200 BCE	0	0.0031	0	3	0
1200-330 BCE	0	0.0023	5	0	0

CHAPTER 4

DISCUSSION

In the present study, to contribute to the domestication history of sheep, the mtDNA partial $tRNA^{Pro}$ and CR sequences (144 bp) of ancient samples collected from Oylum Höyük (Kilis) together with ancient samples from Tepecik-Çiftlik were obtained. The mtDNA HPG compositions of ancient sheep from Oylum Höyük and Tepecik-Çiftlik were determined.

4.1 The aDNA laboratory, sample collection and the establishment of the procedures

The very first step of the studies was establishing a dedicated aDNA laboratory which is necessary for any kind of research involving ancient samples. Without having this facility and necessary control experiments, the work may not be considered reliable. This is because modern DNA may contaminate the ancient sequences and may dominate the amplified DNA sequences giving rise to false information. For the establishment of the laboratory the guidelines given by Dr. Licia Colli and Dr. Michael Hofreiter were followed. The aDNA laboratory in MODSIMMER Building, at METU campus is now established and is operating for more than a year, since July 2012.

The second step in aDNA studies was the sample collection. First, guidelines provided by Dr. Licia Colli were taken into account. During 3-4 day visits of different archaeological sites, usually very few useful samples (max 10) were unearthed. In order to collect 50-60 samples in one excavation season, samples from the repositories were also included. This gives an advantage of quickly increasing the number of samples and studying the samples from already ended excavations such as

the archaeological sites that will be submerged by the Ilısu Dam in Southeast Turkey. Currently in our aDNA laboratory, the amount of aDNA source material is growing each year with the samples from new excavation sites. They are stored in the dedicated laboratory. These samples will be used to address new questions in the future research.

Differentiating sheep from goat remains is a challenging task for an archaeozoologist as these species have similar phenotypes and have been herded mostly together for many years. The identifications were performed by Dr. Pişkin and Dr. Gündem for Oylum Höyük and Tepecik-Çiftlik samples, respectively.

To learn the experimental procedure for aDNA extraction and preparation of the extracted aDNA for sequencing, the author visited Dr. Hofreiter's laboratory in York, UK and gained hands-on experience.

4.2 Choice of the amplification site on sheep aDNA

Mitochondrial DNA CR was proved to be the most informative region in identifying 5 mtDNA HPGs in sheep (Meadows et al., 2011).

The part of mtDNA CR region that was examined in the present study was chosen after Cai et al.'s study (2007). The region was short (< 150 bp), as required in aDNA studies because aDNA is highly fragmented. Furthermore, it was discriminating the most commonly seen three HPGs (HPGs A, B and C) of modern sheep. When all available modern domestic sheep total mtDNA CR sequences (n=234) were classified into HPGs, there was a hundred percent match between this classification and that of the classification based on the fragment suggested by Cai et al. (2007). Thus, first of all, the DNA region proved to be very reliable in identifying HPGs A-C. Moreover, in these comparisons among the modern domestic sheep it was observed that the region also identified HPGs D and E which were not detected by Luo et al. (2005). Perhaps, it was not observed in Luo et al.'s (2005) study because the rare HPGs are observed mainly among the Turkish sheep (Meadows et al, 2007; Demirci et al, 2013). However, HPG X (Demirci et al., 2013) observed among the wild *Ovis gmelinii anatolica* could not be differentiated from HPG C with the fragment of CR used in the study.

Cai et al. (2007) suggested 2 pairs of primers for the region. However, the second pair of primers did not work during first attempts (as shown in Figure 13). When tested through the databases, it was observed that the heavy strand of the second primer pair (H15661) did not match with the reference sequence (AF010406.1) which was used to design the primers. Therefore, the second pair was eliminated from the study. Although, it caused shortening of the region the sequence amplified by the 1st primer pair was still useful enough to differentiate the mtDNA HPGs. Using single set of primers rather than two, in addition to the amplification of a short segment of aDNA, helped us to further economize on resources and time.

The samples (OY066-2, OY091-2, OY114-2, OY090-2, OY131-2, OY137-2 and all of the samples from Tepecik-Çiftlik) that were represented by only one sequence are in need of another DNA extraction in order to confirm the results. This will be performed in the following study period.

4.3 Retrieval of aDNA and its authenticity

Success rate for the DNA recovery and amplification was 65% (37 out of 57 samples yielded amplifiable DNA) for Oylum Höyük samples (1,800-30 BCE), while Tepecik-Çiftlik samples (6,700-5,800 BCE) had a recovery rate of 92.3% (12 out of 13 samples). Other studies, such as Ottoni et al.'s study (2011) on Byzantine human populations of South Anatolia had 62% recovery rate whereas Cai et al. (2007) and Larson et al. (2007) studies had recovery rates of 88.8% and 46.2%, respectively. The range of recovery rates in the present study, 65-92.3%, transcends or covers the given examples' recovery rates. Better recovery rates of the present study can be attributed to the relatively small size of the targeted aDNA region. In all of the previous sheep aDNA studies (Cai et al., 2007, 2011; Campana et al., 2010; Olivieri et al., 2012; Niemi et al., 2013) the length of the target DNA region was at least 2 times longer than the target region in the present study. Therefore, three sets of primers had to amplify their regions simultaneously, only then the amplification from the sample was considered as "successful". Furthermore, the recovery rates of the present study may also resulted from the difference between the ages of the samples. For example, the samples used in Ottoni et al.'s study (2011) were at least more than 1000 years younger than Oylum Höyük samples and much younger than Tepecik-

Çiftlik samples. Nevertheless, differences in geography, number of samples studied and the method used can be mentioned as the factors that have contributed to higher recovery rates.

Sawyer et al.'s study (2012) revealed that there is a significant negative correlation between the age of a sample and the amount of endogenous DNA preserved. The results of the present study do not fit this observation due to contradicting rates of successful DNA retrieval from older Tepecik-Çiftlik samples and younger Oylum Höyük samples. It can be anticipated that environmental conditions (macro and micro climatic conditions, soil conditions) may affect the preservation rate of aDNA. When samples are from permafrost, e.g. in Orlando et al.'s study (2013) where full genome of a horse was retrieved from a fossil found in Canadian permafrost dated between 560,000-780,000 BP, it seems that aDNA can be obtained more easily. In the present case, the difference between the environmental conditions of Oylum Höyük (south of Southeast Anatolia) and Tepecik-Çiftlik (south of Central Anatolia) could be one of the reasons for obtaining different recovery rates. Figure 19 shows the different levels of predicted aDNA recovery rates from different geographic zones of Anatolia. Oylum Höyük being in the zone described as medium to poor amount of DNA yield and yet, Tepecik-Çiftlik from the one described as medium to good amount of DNA yield, may also explain for the differences in the observed aDNA success rates between two archaeological sites.

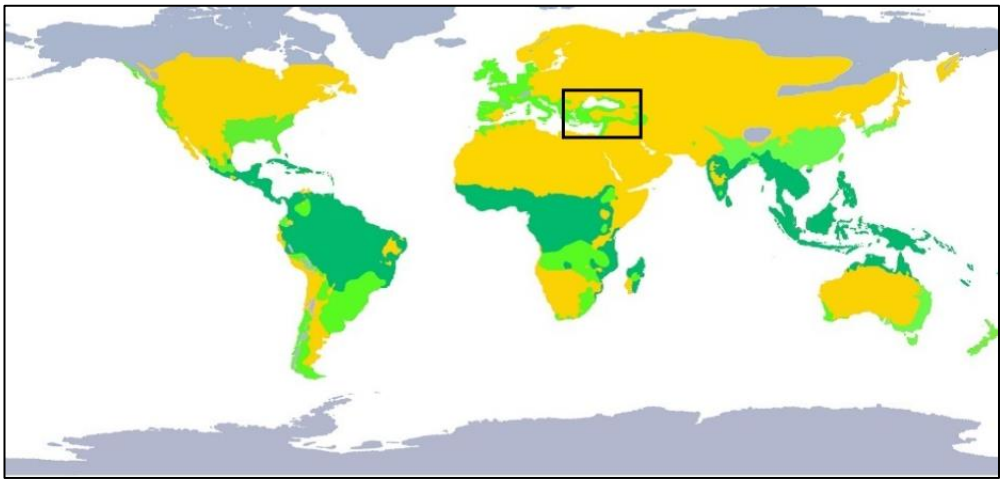


Figure 18. The map showing the suitability of Anatolia (inside the black rectangle) and the world for aDNA recovery (adopted from Haile et al., 2009). Yellow color indicates medium to good amount of DNA yield whereas green color indicates medium to poor amount of DNA yield.

The sequences of 144 bp region of mtDNA (with minor missing nucleotides possibly due to PCR or sequencing quality) for a total of 49 samples (from both archaeological sites) were successfully obtained and their mtDNA HPGs were assigned. One of these samples, OY088-2 that was among HPG A samples, was eliminated from further analysis as its archaeological context was not certain.

In ancient samples, C to T transitions were the most frequently observed and hence most commonly expected (Hofreiter et al., 2001) postmortem nucleotide changes. Transversions were recorded with a lower frequency in aDNA studies. Recently, Sawyer et al. (2012) summarized the patterns of nucleotide changes in aDNA. However, the patterns described by Sawyer et al. (2012) can be observed by next generation sequencing, not when the sequencing is by Sanger sequencing as employed in the present study.

In the present study, within the limits of estimation method and the obtained data, high number of Type 2 transitions (29/34) and especially C to T transitions (24/29) can be considered as the support of authenticity of the sequences. However, in some of the samples, the region under consideration seems to be identical to the modern references. This was expected when the short length of the region is considered. Yet,

their ratios among the ones collected from the field (1/6) and from the repositories (20/28) seem to be different (Fisher's exact test, p value=0.02137 indicating the difference is significant) suggesting that if there is contamination by modern DNA, it might be more likely for the repository samples.

4.4 On the distribution of haplogroups

During the second half of Bronze Age (1800 BC-1200 BC) the frequencies of HPG A, HPG B and HPG C are (9/18, 50%), (6/18, 33.3%) and (1/18, 5.5%), respectively. As for the samples dated back to Iron Age (1200 BC- 330 BC) HPG A (8/16, 50%), HPG B (6/16, 37.5%) and HPG C (1/16, 6.2%) have the same (for HPG A) or approximately same frequencies as Bronze Age samples (Fisher's exact test, p value=0.8069 indicating the difference is not significant). It is not possible to compare HPGs D and E, as both chronological groups lack one of these HPGs for comparison (HPG D was observed among Bronze Age samples, while HPG E was found among Iron Age samples).

The frequencies of HPGs suggest that HPG A, making 50% of the samples overall, is the most common HPG among the individuals from Oylum Höyük. HPG B follows HPG A with 35.3% as the second common group. HPG C (5.9%), HPG D (5.9%) and HPG E (2.9%) are the rare HPGs observed for this sample set, which is consistent with HPG D and HPG E frequencies of modern Turkish breeds (Demirci et al., 2013). HPG A and HPG B frequencies are similar to the observed frequencies of the same HPGs in modern counterpart breed Ivesi (37% and 41%, respectively) in Demirci et al.'s study (2013). However, for HPG C there is an inconsistency with the frequency observed today and among the sheep breeds of the region. Currently in the region, Ivesi (distribution shown in Figure 20) has a HPG C frequency of 22%. Moreover, breeds herded in close neighbourhoods Norduz and Akkaraman show 24% and 14% HPG C frequencies, respectively.

Figure 19. Contemporary distribution of Ivesi breed in Turkey (TAGEM, 2009).

Several alternative (not necessarily mutually exclusive) explanations can be provided for the observed increase in HPG C frequency in the region. First, the sample size may not be high enough to reflect the exact frequencies of the time. Therefore, in the next set of experiments new samples will be added to the study. Furthermore, new sites in the vicinity (for instance Arslantepe) will be also examined to see if parallel low frequencies of HPG C will be observed. However, since the sample size is >30, which is generally excepted as the adequate number for the statistical analysis, and since the HPG C frequency is similar in two time intervals, it can be concluded that HPG C frequency of at least before the Hellenistic period was indeed low (~6%) around Kilis.

As a second explanation, one may suggest that mtDNA, as a single uniparental haploid locus might have experienced drift and HPG C diverged from ~6% and reached to 22% recently.

As another explanation, it can be suggested that individuals (or breeds) which were rich in terms of HPG C were brought to the region more recently, apparently after 330 BC. The frequency of HPG C may have increased as a result of this migration. A migration scenario in relation to part of Oghuz Turks, known as Seljuks who were pastoral nomads, who had a long journey from the east of Aral Sea to the Southeast Anatolia around 1,000 AD (Cahen, 1968) possibly passing through the HPG C rich

regions around the Caspian Sea and Central Asia (Tapio et al., 2006). Furthermore, a similar migration route for the shepherd dog, Kangal, has already been revealed (Koban et al., 2009). This scenario suggests that sheep brought along by Seljuks to Southeast Anatolia contributed the distribution of mtDNA HPGs at least in this region.

Finally, the migration proposed by retrovirus insertion frequencies (Chessa et al. 2009) might be related with the change of HPG C in the region. Increase in HPG C is much later (after the 330 BCE) than the Chessa et.al.'s (2009) predicted time of migration (3,000 BCE) in Southeast Anatolia, which is in agreement with the suggested time of secondary products (such as wool, milk, traction) revolution by Sherratt (1981). Retrovirus insertions are on the autosomal chromosomes. If the second expansion of sheep and migration was governed by the rams and ewes' diffusion lagged the migration of the rams, it may reconcile with the increasing frequency of HPG C.

Since in the present study we observed that HPG C of modern times cannot be differentiated from HPG X of wild sheep, another point is, may be in the era before the common time the domestic sheep had HPG X and then HPG C entered and increased in frequency.

Further aDNA studies may provide the true explanation in the future.

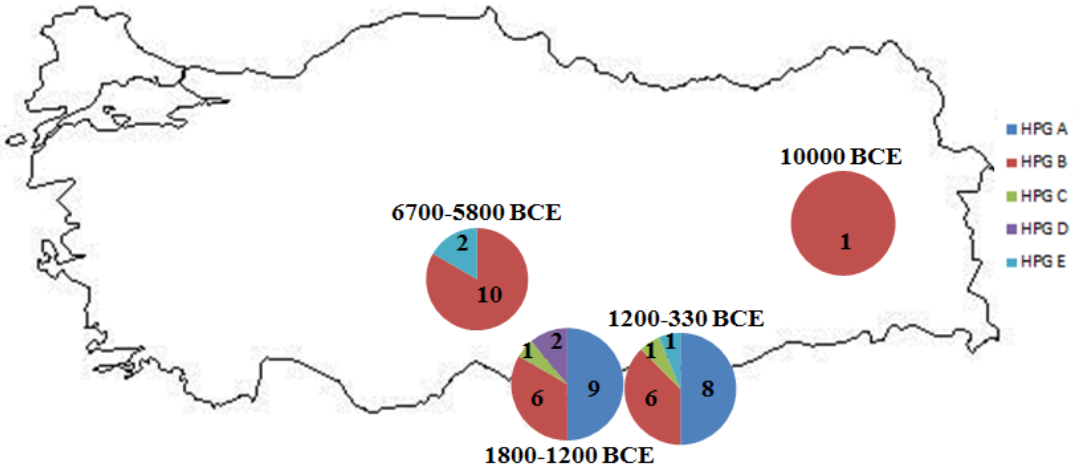


Figure 20. Spatiotemporal HPG distributions from some archaeological sites (from west to east: Tepecik-Çiftlik, two pie charts representing Oylum Höyük chronological groups and Hallan Çemi) in Anatolia. Colors are in accordance with those in Figure 2. Numbers in pie charts indicate the sample sizes.

As another finding of this study it can be confirmed that, since mainly HPG A and HPG B were observed around Kilis province of Southeast Anatolia, these two HPGs are most probably the first domesticated or dominated HPGs in the region. This is in accordance with the frequencies of these two HPGs as the two most common among modern breeds of the world. Moreover, the presence of HPG B in 10 out of 12 samples from Tepecik-Çiftlik dating back to 6,700-5,800 BCE and 1 HPG B sample from Hallan Çemi (unpublished data) dating around 10,000 BCE supports the idea that HPG B was found around the region (Figure 21), probably with a high frequency during the time of domestication and perhaps it was the first domesticated HPG.

4.5 The importance of the present study

This is the first study to show the presence of multiple mtDNA HPGs in ancient sheep of Oylum Höyük in Southeast Anatolia and contributes to the growing data and picture of sheep domestication. Despite the small number of samples, Tepecik-Çiftlik was also examined for the first time in terms of sheep mtDNA HPGs. Two different HPGs, HPG B and HPG E but mainly HPG B, were discovered among 12 samples.

This study made it easier to determine HPGs based on a short segment of mtDNA CR sequence. Previously, it was not possible to differentiate HPGs C and E among the modern sheep with the RFLP method. Furthermore, with the SSCP method which was claimed to be more robust than mtDNA CR RFLP in terms of HPG determination, HPGs D and E in modern sheep could not be identified as well (Yüncü, 2009). In order to identify the mtDNA HPGs high number of long sequencing had to be done for the total CR (Demirci, 2012). Before the present study, discrimination power of the region for all 5 HPGs was unknown. However, as it is shown in the present study, in a short time with a limited budget mtDNA HPGs of ancient sheep can be obtained.

It was recognized that some of the samples might be the parts of the same individual and it may deflect the results. However, for some samples it is highly likely that they represent different individuals as they have been collected from different trenches or they belong to the same side (for instance left side) of an archaeological element.

The present study also highlights a new dimension for the studies in archaeozoology. By utilizing aDNA from archaeological material, it is possible to infer past population changes; such as the HPG C increase in the present study would not be detected just by morphological evaluations. It can be anticipated that a synergy between archaeologists and biologists will emerge soon in Turkey.

4.6 Planned and Suggested Future Studies

In order to increase the resolution of historical genetic diversity of ancient sheep populations in Anatolia, the presented study here needs to be further expanded with new samples spanning a wider range of chronological layers and representing more geographic locations. In the scope of the aDNA projects, samples were collected from Arslantepe (Malatya), Barçın Höyük (Bursa), Maydos Kilisetepe Höyük (Çanakkale), Hallan Çemi and Çemi Alo Sırtı (Batman). With increasing number of samples distributed spatiotemporally, the conclusions will be more robust and informative. Those further genetic studies on ancient samples collected across Anatolia especially from the region for sheep domestication (Zeder, 2008) will contribute to the understanding of both the early stages of the domestication process and the history of domestic sheep.

Nevertheless, more studies based on mitogenomes and whole genomes should be carried out to comparatively study populations of the past in terms of many informative polymorphic sites and to support the results of one locus studies like the ones based on short fragments of mtDNA or Y-chromosome.

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APPENDICES

APPENDIX A

DETAILS OF SAMPLES COLLECTED DURING THE STUDY AND THEIR ARCHAEOLOGICAL CODES

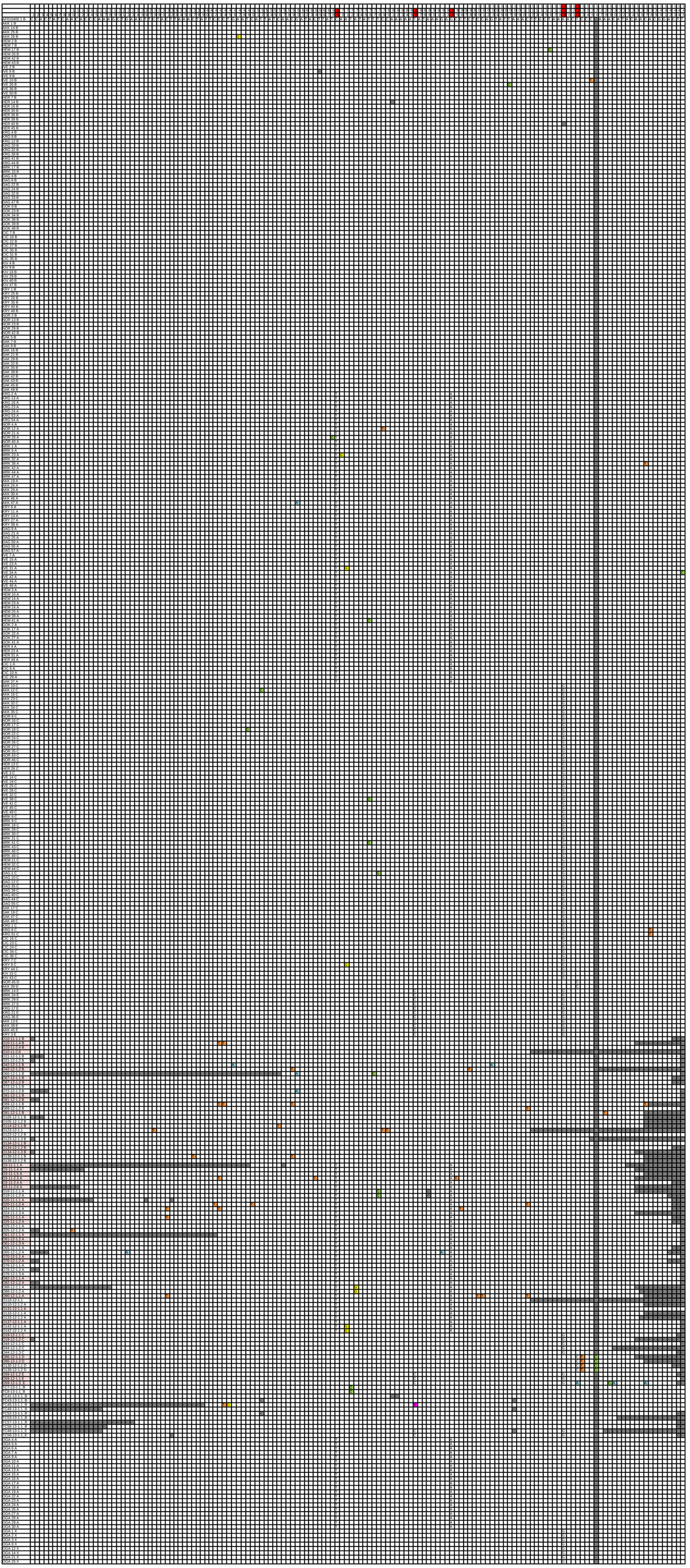
Sample code used in the study	Oylum Höyük archaeological code
OY003-1	OY 11/809, K22, 141#15
OY018-1	OY 11/814, K22, 141#17
OY020-1	OY 11/804, K22, 141#13
OY024-1	OY 11/394a, K22, 141#11
OY025-1	OY 11/623, J21, 155#21
OY065-2	OY 09/1472, J22, 131#8
OY010-1	OY 11/394c, K22, 141#11
OY042-2	OY 11/1330, K22, 146#3
OY134-2	OY 11/864a, K22, 141#20
OY086-2	OY 11/123, K21, 123#2
OY130-2	OY 11/1353a, K22, 146#4
OY044-2	OY 11/1353b, K22, 146#4
OY133-2	OY 12/3007, K21, 146#1
OY105-2	OY 12/1565, L21, 123#4
OY067-2	OY 09/1657, L22, 76#2
OY070-2	OY 11/1042, K21, 88#1
OY061-2	OY 10/1430, M22, 72#5
OY059-2	OY 10/1448, M22, 72#8

APPENDIX A (continued)

OY090-2	OY 10/798, M22, 61#7
OY123-2	OY 12/1504, L21, 61#4
OY072-2	OY 09/968, M21, 27#5
OY019-2	OY 12/3695, M23, 20#6
OY078-2	OY 12/4189a, L23, 25#1
OY082-2	OY 12/4713, K23, 31#1
OY110-2	OY 12/4687, K23, 27#1
OY025-2	OY 12/4548, K23, 13#1
OY142-2	OY 12/4069, L23, 16#1
OY089-2	OY 10/730, M22, 22#14
OY091-2	OY 09/207, L22, 24#1
OY081-2	OY 12/4189b, L23, 25#1
OY138-2	OY 12/4282, L23, 36#1
OY021-2	OY 12/4047a, L23, 10#2
OY114-2	OY 12/4047b, L23, 10#2
OY131-2	OY 11/885, K22, 141#21
OY137-2	OY 11/864b, K22, 141#20
OY066-2	OY 09/2301, L22, 77#4

APPENDIX B

THE SEQUENCES OF SAMPLES OBTAINED IN THE PRESENT STUDY AND THE REFERENCES THEY WERE COMPARED TO



APPENDIX C

THE TABLE DEPICTING NUCLEOTIDE MISINCORPORATION AND DELETION POSITIONS ON THE STUDIED MTDNA FRAGMENT (TS: TRANSITION, TV: TRANSVERSION, DEL: DELETION)

	mtDNA positions													
Replicates	10	22	26	28	31	32	37	42	43	44	46	50	57	59
O25-12-1.2	A=>T (tv)													
O65-12-1.2		G=>A (ts)												
O20-11-1.1			T=>del											
O123-12-1.1				C=>T (ts)										
O24-11-1.1					C=>T (ts)									
O25-11-1.1					C=>T (ts)									
O20-11-1.1						T=>del								
O138-12-1.1							C=>T (ts)							
O20-11-1.2								C=>T (ts)						
O10-11-1.4									C=>T (ts)					
O89-12-1.1									C=>T (ts)					
O3-11-2.3									C=>T (ts)					
O24-11-1.1									C=>T (ts)					
O89-12-1.1										C=>T (ts)				
O10-11-1.4										C=>T (ts)				
O42-12-1.1											G=>A (ts)			
O20-11-1.2												C=>T (ts)		
O3-11-1.1													A=>del	
O42-12-1.2														C=>T (ts)
O89-12-1.1														C=>T (ts)
O138-12-1.1														C=>T (ts)

APPENDIX C (continued)

	mtDNA positions													
Replicates	60	64	79	80	92	95	96	98	103	111	122	129	130	137
O72-12-1.2	G=>A (ts)													
O3-11-2.3		C=>T (ts)												
O123-12-1.1			C=>T (ts)											
O123-12-1.1				C=>T (ts)										
O65-12-1.2					G=>A (ts)									
O3-11-2.3						C=>T (ts)								
O24-11-1.1							C=>T (ts)							
O42-12-1.2								C=>T (ts)						
O42-12-1.1									G=>A (ts)					
O89-12-1.2										C=>T (ts)				
O20-11-1.2										C=>T (ts)				
O59-12-1.3											T=>A (tv)			
O59-12-1.3												A=>C (tv)		
O59-12-1.3													C=>A (tv)	
O89-12-1.1														C=>T (ts)
O59-12-1.3														C=>A (tv)