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Full Length Research Paper

Species determination of ancient bone DNA from fossil skeletal remains of Turkey using molecular techniques

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The use of genetic technology in forensic science and archaeometry is applied primarily to distinguish between individials who may be the source of biological material associated with archeological remains. DNA sequences from ancient fossils have great potential for studies of phylogeny, biogeography and molecular evolution. DNA from fossils also facilitates the rigorous testing and calibration of mutation rates among related taxa, sex test and molecular divergence time (Cano et al., 1993; Burger et al., 1999). In this study, a rapid and quantitative ancient DNA extaction methods from human skeletal remains was developed for application of forensic science and archaeometry. For that reason, DNA was extracted from ancient human bones from Mugla in Turkey. Furthermore, all the bone samples which are obtained from burial place are subjected to DNA isolation and then interspecific sequence polymorphisms in the mitochondrial cytochrome b gene were analyzed by PCR to determine the species origin of Bronze Age animal and human skeletal remains. Existing techniques were refined by targeted primer design focusing on a DNA fragment shorter than 200 bp, an approach allowing us to identify up to all bone samples at the same time. For routine applications in archaeometry, food or material analyses, PCR may thus provide a simple alternative to sequencing of PCR products, allowing discrimination between species, even if the template DNA is degraded or contains traces of DNA from various species.

Key words: Ancient DNA, species determination, cytochrome b gene, mtDNA.

INTRODUCTION

An important goal of any forensic investigation involving unidentified human fossil remains is positive identification. Forensic specialists including anthropologists and odontologists may evaluate the remains, estimating the individual's sex, stature, age at death, origin, species determination and ancestry. Any identifiable characteristics such as fingerprints, if flesh is still intact, unique skeletal features, and dental arrangement are also noted. However, when antemortem reference records are not available for comparison or remains are fragmented or otherwise in a state in which definitive conclusions cannot be made as to the person's identity or sex determination. DNA sequences were also soon extracted from species that became extinct so long ago that they are only found by archaeologists, and speleologists. The first results achieved were from the extinct moas (Cooper et al., 1992).

Determination of the species origin of prehistoric objects is one of the common tasks of ancient DNA (aDNA) analysis (Burger et al., 2000). DNA analysis may be required. Indeed, identification through analysis of DNA from human skeletal remains has been used in numerous cases, beginning in 1989 (Pääbo et al., 1989). Advancements in techniques and applications occurred in the 1990's (Boles et al., 1995; Hagelberg et al., 1991; Primorac et al., 1996; O'Rourke et al., 2000), and in 1991 the Armed Forces DNA Identification Laboratory was established for the identification of the remains of

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Abbreviations: aDNA, ancient DNA; **mtDNA,** mitochondrial DNA.

U.S. military personnel (Holland and Parsons, 1999). Given the type of skeletal material (long bone, flat bone, tooth, etc.) and its level of weathering, valuable predictions could be made about the probable amount of obtainable DNA and its level of degradation, thus leading the scientist to the best genetic loci and polymerase chain reaction (PCR) primers for analysis. DNA fragment length isolated from ancient DNA is typically small (O'Rourke et al., 2000). To more exactly pinpoint useful size ranges for analysis, PCR can be used to determine the largest size class of DNA existing in each sample by targeting a series of amplification products.

Ancient DNA (aDNA) broadly refers to DNA that can be recovered and analyzed from museum specimens, fossil remains; archaeological and paleontological findings (Pääbo et al., 2004). The first successful attempts to retrieve aDNA carried out two decades ago by extracting and molecular cloning of DNA from a 140 year-old quagga remain (a type of zebra) and a 2400 year-old Egyptian mummy (Pääbo, 1985; Higuchi et al., 1984). The findings that DNA can persist in ancient remains, especially in bone and tooth samples, opened a new and exciting field of research known as molecular archeology (Pääbo et al., 1989). Determination of the species origin of prehistoric objects is one of the common tasks of ancient DNA (aDNA) analysis. Usually, short fragments of conserved regions of mitochondrial DNA (mtDNA) are PCR amplified and then sequenced (Loreille et al., 1997). If a more detailed phylogenetic analysis of the obtained sequence is required or if the specimen contains extremely degraded DNA, the PCR products must be cloned before sequencing (Handt et al., 1996).

Methods that allow specific sequences of interest to be retrieved from such tagged libraries (Briggs et al, 2009; Gnirke et al., 2009) make it possible to quickly analyse many sequences of interest from such libraries. Criteria of authenticity that are currently successfully applied to PCR-based studies of ancient DNA, such as reproduction of results from an independent extraction from the same bone, will then be useful just as they have been hitherto in PCR-based studies. In contrast, these criteria are not easily applicable to high-throughput shot-gun sequencing of entire ancient genomes. This is a particular problem for the Neandertal genome but applies also to other ancient genomes, such as mammoths (Miller et al., 2008), because all mammals including humans share conserved DNA sequence elements that may confuse results. They have therefore developed a time-saving and less expensive PCR amplification method is used which allows reliable species identification from prehistoric animal and human bone samples, even those containing only degraded DNA.

One aspect of molecular archeology, that is, extraction and successful PCR amplification of aDNA from human remains, has great importance but is particularly difficult because of the risk of contamination with modern human DNA. In the present study, the authors report the successful extraction and amplification of aDNA from 3000 - 3500 year old human remains excavated from Mugla in Turkey. To set up their aDNA extraction/ amplification facility and also to eliminate the chance of getting false results due to the potential contamination of samples with modern DNA, they primarily aimed to determine the species origins of the samples with which is known gender or species origin and human and animal bone remains with animal blood and the human modern DNA for positive control of species according to archaeological reports. Briefly, a segment of CB7u and CB71 was amplified and analyzed. The obtained data were compared with anthropometric reports as a control for the rate of precision in aDNA analysis. The results demonstrate that reliable aDNA can be extracted and amplified from archeological remains.

MATERIALS AND METHODS

Collection of samples

DNA isolation a subset of 100 bones from the total set obtained from Mugla in Turkey was analyzed in this study. Upon recovery of the skeletal remains, the bones were described in terms of sex, estimated age, and some of the skeletal weathering stages. Existing techniques were refined by targeted primer design focusing on a DNA fragment shorter than 200 bp, an approach allowing us to identify up to all bone samples at the same time. In order to allow ratings on individual bones, a new staging system was developed at Archeometry Laboratory in Selcuk University, Arts and Science Faculty, and assigned as period or era of each bone based on visual inspection for the DNA study (Table 1). The bone samples of more than 100 individuals were chosen to study the genetics of this skeletal population. In addition to numerous human skeletons, the cave contains bones from some autochthonous animal species. Earlier analysis showed that the state of DNA preservation in the bones is excellent, mainly due to the low temperature prevailing in the cave since prehistoric times (Burger et al., 1999). Eleven animal bone samples were chosen for aDNA analysis.

Contamination controls

All DNA extractions and PCR setups were carried out in a dedicated ancient DNA laboratory following the suggested protocols for contamination controls and detections (Herrmann and Hummel, 1994). All bone samples and extraction reagents were exposed to UV irradiation. Furthermore, All post-extraction manipulations were conducted by Vural. Disposable laboratory coats, gloves, fitler tips, dedicated pipetmen, and disposable laboratory ware were used throughout the analyses. Benches and equipment were frequently treated with a 20% bleach solution. Sterile water was aliquoted and irradiated by placing the tubes directly on a light source of 254 nm for 30 min (Sarkar and Sommer, 1990). Two extractions were prepared for each bone sample by two researchers to test reproducibility and aDNA quality. The amount of contaminant DNA in this study was probably not significant.

Sample preparation and DNA isolation

Approximately 1 cm^3 of bone was cut from the source section using a Dremel MultiPro tool and was collected in a tube. Samples were

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

Number of bone samples	Codes	Period	Location site	Excavation region
2	05BM13	ROMA	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM22	GEÇ HELLENİSTİK-ERKEN ROMA	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM09	GEÇ KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM29	HELLENİSTİK-4.YY	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM40	HELLENİSTİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM21	GEÇ HELLENİSTİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM05	GEÇ GEOMETRİK-M.Ö.730-680	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM23	HELLENİSTİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM05	GEÇ KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM40	HELLENİSTİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM13	ROMA	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM37	ROMA	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM85	HELLENİSTİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM118	GEOMETRİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM13	GEÇ HELLENİSTİK-ERKEN ROMA	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM39	GEÇ KLASİK-M.Ö.377	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM27	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM01	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM14	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM41	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM22	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM02	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM17	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM64	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM29	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM37	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM31	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM01	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM42	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM26	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM30	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM58	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM95	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM106	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM18	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	05BM100	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM10	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM02	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM11	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM25	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM05	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM55	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM29	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	07BM13	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM42	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM39	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM25	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM23	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA
2	06BM45	KLASİK	BÖRÜKÇÜ	YATAĞAN/MUĞLA

Figure 1. The pictures of fossil human, animal and human teeth bone.

then immersed in filter-sterilized wash buffer (1% SDS, 25 mM EDTA) and 0.1 mg/ml proteinase K, and incubated for one hour at room temperature. Following the incubation, the wash buffer was poured off and each sample was washed with 1 ml of sterile dH_2O for six consecutive times. Samples were allowed to air dry. Bone powder from the dried bone samples was collected in one of two ways. Bone was either ground to powder drilled using the Dremel tool both fitted with 1/16 microfuge tube and weighed. Four hundred microliters of digestion buffer (20 mM Tris, 100 mM EDTA, 0.1% SDS) and 0.4 mg/ml proteinase K was added to each ground bone sample and incubated overnight at 56˚C.

A standard phenol/chloroform organic extraction was performed on each of the samples. The DNA were precipitated using 3 M sodium acetate and 95% ethanol, vacuum dried, and resuspended in TE buffer (10 mM Tris, 1 mM EDTA) based on the original mass of the bone powder. Furthermore, after addition of proteinase K, solution of bone was incubated at 37˚C. Genomic DNA from supernatant was extracted automatically by using EZ1 Automatic Nucleic Acid Isolation System (Qiagen, Germany) with investigator kit (Qiagen, Ilden, Germany) from ancient bones. Amount and purity of extracted DNA from ancient bones were measured by spectrophotometer (Shimadzu, Kyoto, Japan). In addition to spectrophotometric measurement, extracted DNA was applied to 1% agarose gel, stained and imaged under ultraviolet (UV) irradiation. As a result, 50 ng pure DNA was extracted from ancient bones. Several precautions were taken to prevent contamination during the experiments. Grinders and drills used to generate bone powder were washed with 70% EtOH and 10% bleach, and UV irradiated between each sample preparation. Pre-amplification and post-amplification steps were carried out in separate rooms. Finally, negative controls and reagent blanks were included in all experiments (Figure 1).

Ancient DNA quantity

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

Polymerase chain reaction

A 200 bp segment of the mitochondrial cytochrome b *gene* was amplified using the primers; CB7u: 5’-GCGTACGCAATCTTACGATCAA-‘3 and CB7l: 5’-CTGGCCTCCAATTCATGTGAG-‘3. The PCRs were carried out in 50 ml of 60 mM KCl; 12 mM TrisHCl; 2.5 mM $MgCl_2$; 150 mM dNTPs; 0.18 mM each primer; and 2U AmpliTaq Gold (Applied Biosystems), and 0.2 microliter BSA. The temperature profile was 95˚C for 4 min, 95˚C for 30 s, 54˚C for 1 min, and 72˚C for 30 s, for 40 cycles and 72˚C 5 min.

DNA sequencing

Sequencing of the PCR products was performed using both the forward and reverse primers of PCR amplification and the BigDye Terminator kit (ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit). The cycle sequencing products were separated with a POP-7TM polymer (Applied Biosystems) and detected by an ABI 3730 DNA Analyzer. Sequences were compared with those registered in the EMBL databank by the FASTA program accessed through the website: www.ebi.ac.uk/fasta33/nucleotide.html.

Negative control amplification

An increase of PCR cycle may increase the risk of minute amount of modern DNA contamination in the resulting in DNA amplification. In this study, potential modern DNA contamination was assessed based on the possible amplification produced in the negative control extraction. The negative control extraction is a sample that contains everything used during DNA extractions procedure followed by PCR amplifications except the powdered ancient bone of the respective sample was substituted with deionized water (Yang et al., 2003; Yang et al., 1998; Eshleman and Smith, 2001; Kalmar et al., 2000). An indication that a very low level or non-existence of modern DNA contamination as well as specificity of the primers and sensitivity of PCR amplifications procedures that had been utilized in this study.

RESULTS AND DISCUSSION

Molecular archaeology is an emergent field in archaeology that has been brought about by the advancements of the recognition and understanding of DNA. This new developing branch of archaeology focuses on the acquisition of either DNA or mtDNA (mitochondrial DNA) and being able to determine species of natural archaeological findings as well as determine blood lines and/or sex of animal or human remains. As their technology advances, their knowledge of the DNA itself and their understanding of ancient peoples, plants and animals, will allow the study get an access of a biological window into their lives. Molecular archaeology came in time, as their knowledge and technology increases, and this provides the study with the ability to learn more about the life of ancient individuals. It can be seen as, how modern humans may differ from their ancestors or how plants and animals may have existed at the time and what things were utilized by them, which can be found by exploring what their tools and clothing or other artifacts were constructed from. Not every area of the world is accessible to this technology due to the variety of climates, but in those areas where suitable DNA samples may have be taken, a whole new knowledge of the ancient culture under examination may be gained.

Identifying the species of origin, their data clearly show that it is possible to identify the species of origin from circa 3000-year-old bone specimens by using RFLPs of a 200 bp PCR amplified fragment and at the same time to exclude possible (human) contamination. If the locus and the restriction enzyme are chosen properly, this procedure can be applied to other species or populations as well. Certainly, the principle of PCR-RFLP is not new and is widely used, for example in the field of food analysis (e.g. Meyer et al., 1995; Plath et al., 1997; Carrera et al., 2000). However, fulfilling each of the four criteria mentioned in Materials and Methods shown (presence of a RFLP specific for the considered species, fragment length of less than 200 bp, exclusion of contaminating sequences, and a restriction site within at least one of the primers) optimizes and refines existing methods, making them applicable to analysis of truly old and highly degraded DNA, but at the same time makes the experimental design slightly more laborious. Analyses of the aDNA sequences from this study were carried out together with all published cyt *b* haplotypes from extinct and extant elephantid taxa available from GenBank. Population aggregation analysis (Davis and Nikon, 1992) was used to identify diagnostic sites (e.g. fixed difference between predetermined lineages) for the two lineages (*Elephas* and *Mammuthus*) and to assign each aDNA sequence to a given taxon.

Two types of sequences from each of 8 individual chamois have been obtained by standard PCR-sequencing protocol of the cytb *gene*. The sequences were inferred to be a functional mitochondrial *gene* and a nuclear pseudogene from selective extraction of mtDNA and study of substitution patterns. The results add to the reports of unwanted amplification of nuclear pseudogenes in phylogenetic studies of close related species (Lo´pez et al., 1997; Kim et al., 2006). Phylogenetic inferences will be seriously distorted if nuclear sequences are included in the analysis, and it is therefore mandatory to follow recommendations given elsewhere (Thalmann et al., 2004) to avoid their unnoticed inclusion. On the other hand, numt sequences represent molecular ‘‘fossils’’ and are potentially useful for phylogenetic studies to be used as outgroups.

The Cytochrome b (Cyt b) *gene* has proved to be useful for identification and classification of many mammals. The Cyt b *genes*, approximately 1080 base pairs, were found to be A/T rich, and their 5' terminal-editing regions were highly conserved. One of the stages of dealing with biological material submitted to forensic and archeaometry laboratories is species identification. The aim of the present work was to validate and assess the possibility of applying sequence analysis of the region coding cytochrome b as a method of species identification in the field of archaeometry. DNA originating from individuals from major phyla of vertebrates was isolated by the organic method from various specimens. Extracted DNA was subjected to PCR and direct cycle sequencing using a universal pair of primers. In order to evaluate the utility of this *gene* for discrimination of fossil bone remains as well as for exploring their phylogenetic relationships. These data show that the Cyt b *gene* is useful for phylogenetic study of fossil bone remains (human or animal materials) (Plath et al., 1997).

Species determination on ancient human remains

To determine the species origin of samples, they amplified a small piece of A 200 bp segment of the mitochondrial cytochrome b *gene*. They selected the skeletal remains, which their species was known based on their morphology and belongings such as crown, sword, bracelet etc. At the first round of PCR with 40 cycles. However, in the second round of PCR signals with the expected sizes of (200 bp band profile specific-

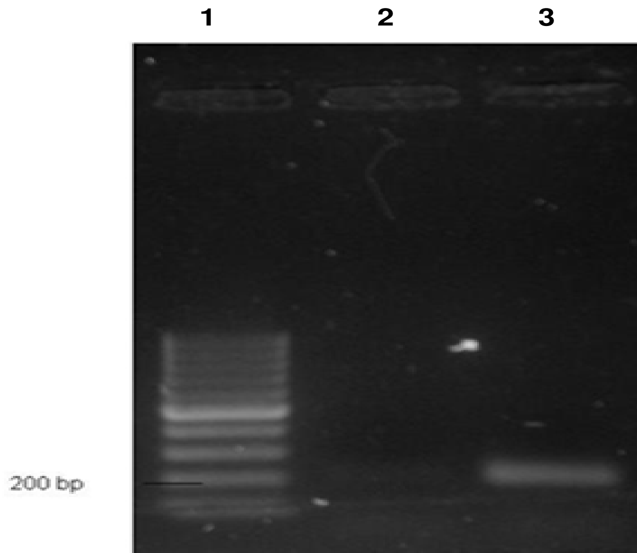

Figure 2. Identification of amplified aDNA from human ancient bone from the samples. The PCR amplification products were electrophoresed on 2% agarose gels. Forward and reverse primers specific to region coding cytochrome b as a method of species identification were used for aDNA amplification. Both amplifications showed formation of 200 bp DNA band. Lane 1 is standard molecular size makers of 100 bp ladder, Lane 2. Ancient bone sample PCR product and Lane 3. Positive control from human blood.

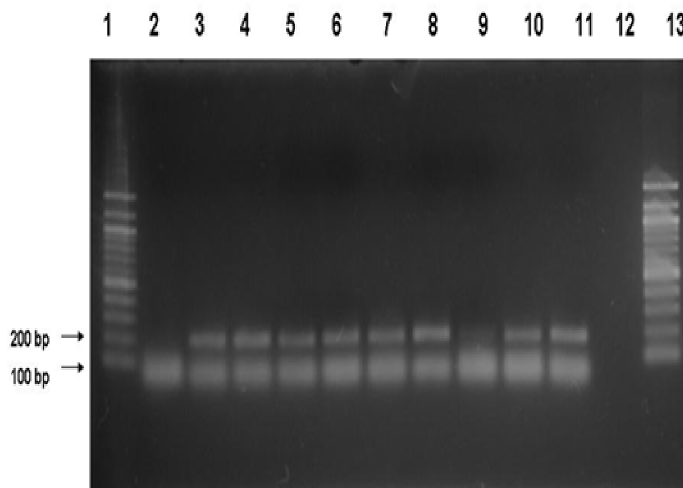

Figure 3. Shows the result of amplification of aDNA from different species with varying amounts of target DNA using primers CB7u and CB7l. Template DNA from human and animal fosil bone, whose sequences have an almost complete match with the primers, was amplified under all conditions and produced strong bands. Evidently, all species can be distinguished from one another and all specimens, including those derived from prehistoric remains. PCR products were loaded in a %2 agarose gel and separeted by electrophoresis and visualised ethidium bromide staining with transillumination. Respectively, Lane 1 and 12, 100 kb ladder size standard. Lane 2 and 11, ancient bone sample PCR products and Lane 13, Negative Control blank (None DNA or water).

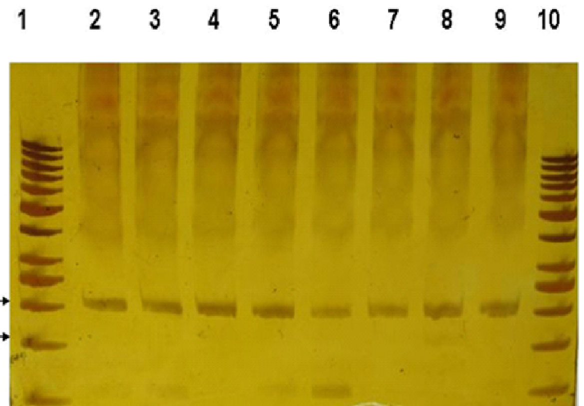

Figure 4. Screening of polyacrilamide gel electrophoresis of ancient bone DNAs amplified by PCR. Lane 1 and 10, 50 bp ladder size standart, respectively, Lane 2-9, PCR products of aDNA isolated from ancient bone with Bio Robot EZ-1. Forward and reverse primers specific to region coding *cytochrome b* as a method of species identification were used for aDNA amplification. Both amplifications showed formation of 200 bp DNA band. Lane 1 is standard molecular size makers of 100 bp ladder. The skeletal remains of all the samples excavated from Mugla burial site in Turkey. In addition to the morphological appearances of the remains there are some artifacts, which used to clearly determine the species of cases.

human fossils bone) were detected on agarose gel. Furthermore, this study showed that the absence of amplified modern DNA or modern DNA contamination during extraction procedures. That the procedure of DNA extraction is able to produce a pure DNA from ancient bones that can be amplified using PCR procedure (Figure 1, 2,3 and 4).

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