Extraction and analysis of ancient DNA from human remains of Masjede Kabood burial site

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Abstract
Extraction and analysis of DNA from ancient remains has numerous applications in archeology and molecular evolution. However, it has become obvious that ancient DNA (aDNA) can be easily contaminated with modern DNA, so it is crucial to detect contamination and to distinguish contaminant from authentic results. In the present study, we report the successful extraction and amplification of aDNA from 3000-3500 year-old human remains excavated from Masjede kabood (Tabriz, North-West of Iran) burial site. To test the authenticity of the extracted aDNA, we have developed a nested PCR/restriction enzyme digestion method for molecular sex determination of the skeletal remains, which their gender was known based on their morphology and belongings (Crown, Sword, Bracelet etc.). A simple and effective modified ethanol precipitation-based protocol was used for DNA extraction from 35 human skeletal remains. A segment of Homologous Amelogenin Gene (AMG), which has different alleles on X and Y-chromosomes, 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 showed that reliable aDNA can be extracted and amplified from archeological remains. The presented sex determination procedure could also be used as a reliable control for testing the authenticity of aDNA results.
Keywords: Ancient DNA; Amelogenin; Nested-PCR; Restriction enzyme digestion; Sex determination and Molecular archaeology.

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INTRODUCTION

Ancient DNA (aDNA) broadly refers to DNA that can be recovered and analyzed from museum specimens, fossil remains; archaeological and paleontological finds (Paabo *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 (Paabo, 1985 and Higuchi *et al*., 1984). The finding 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 (Paabo *et al*., 1989).

DNA extracted from ancient samples is in very low amount and poor quality, which is mostly degraded to small sizes often between 100 to 500bp (Paabo *et al*., 2004). The extent of the degradation, however, is primarily depends on the environmental factors and varies among different burial sites (Gutierrez and Marin, 1998; Cano, 1996). The study of ancient DNA has been revolutionized by the application of polymerase chain reaction (PCR), and interest is growing very rapidly. Fields as diverse as archeology, evolution, anthropology, medicine, and agriculture have quickly found applications in the recovery of aDNA (Paabo *et al*., 2004; Brown and Brown,1994; Montiel *et al*., 2001; Audic and Beraud-Colomb, 1997).

One aspect of molecular archeology, i.e. 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. The main problem has been that

the complete elimination of the contamination risk is seemingly unlikely (Dr. Svante Paabo, personal communication). In these kinds of studies it is therefore very important to detect contamination and to distinguish contaminants from authentic results (Paabo, 1989 and Hofreiter *et al.*, 2001).

Sex determination based on analysis of aDNA is away to know about the gender of human remains that are badly damaged (especially in skull and pelvic), children and infants that anthropometric methods are unreliable to mention their genders (Paabo *et al.*, 2004 and Stone *et al.*, 1996).

In the present study, we report the successful extraction and amplification of aDNA from 3000-3500 year-old human remains excavated from Masjede kabood burial site (Tabriz, North-West of Iran). To set up our aDNA extraction/amplification facility and also to eliminate the chance of getting false results due to the potential contamination of samples with modern DNA, we primarily aimed to determine the sex of the samples with known gender according to archaeological reports. Briefly, a segment of Homologous Amelogenin Gene (AMG), which has different alleles on X and Y-chromosomes, 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

Sample Collection and Preparation: 35 bone and tooth samples from human remains of Masjede Kabood burial site (Fig. 1) were provided by the Department of Archeology (Tarbiat Modarres University, Tehran). Most of the skeletons were intact and their degree of apparent preservation was very high, compare to similar samples excavated from Ardabil burial sites. The oldness of the samples was estimated around 3000-3500 years old. The age and gender of the remains was determined by anthropometric evaluations. The general characteristics of the bone samples are listed in table 1.

Contamination precaution: Standard authentication criteria for ancient DNA studies, including multiple extractions, and independent PCR amplification, have been followed. In order to prevent possible contamination all stages of the work were carried out under sterile conditions, using latex gloves, and mouth masks. All appliances, containers and the work areas (laminar

Figure 1: Location of Masjede Kabood in Tabriz, northwest of Iran.

airflow surface, PCR box) were cleaned and irradiated with UV light for at least 60 min. The extraction buffer (without proteinase K), Dextran Blue solution, NH_4-acetate and deionised, distilled water were irradiated for 30 min all steps (bone cutting, surface removing, powdering, extraction and amplification) were carried out in separate places.

DNA Extraction: For extraction of DNA from bone and tooth samples a very simple and effective modified ethanol precipitation-based protocol (Kalmar *et al.*, 2000) was used. Briefly the following steps carried out: 1) a surface layer of about 2 mm was removed from bones and teeth, using sterile blades and sandpapers. Then the bones were bleached and UV irradiated (for 30 min.) and powdered in a sterile mortar filled with liquid nitrogen. 2) 500-700 mg bone powder was added to 1.6 ml extraction buffer consisting of 0.1 M EDTA, 0.5% N-laurylsarcosine-Na salt and 100 μg/ml Proteinase K. After vortexing the tube, the samples incubated overnight at 37°C. 3) Phase separation was done by centrifugation at room temperature at maximum speed (11269×g) for 10 min. 250 μl supernatant was transferred to a 1.5 ml eppendorf tube and 3.5 μl Dextran Blue (1 μg/μl) as a carrier for removing the PCR inhibitors, 250 μl NH_4-acetate (4M) and 500 μl ethanol 96% were added and mixed by vortexing. 4) The DNA was precipitated at -70°C for 7 min and centrifuged at maximum speed (11269×g)at 4°C for 15 min. 5). The pellet was dried in room temperature at about 30 min and re-dissolved in 20-30 μl deionised, distilled water and stored at -20°C until required for testing.

DNA Amplification: Approximately 500 ng of DNA extract was used for amplifying a segment of AMG

Mohandesan *et al.*

Table 1: Number of tomb, age and gender of 35 skeletal remains excavated from Masjede Kabood burial site.

Some samples from Masjede Kabood burial site
3000 – 3500 years B.P

No	Number Of Tomb	Sex & Age	No	Number Of Tomb	Sex & Age
1	81/6	Unknown 2-3 years old	19	80/18	Male 35-45 years old
2	81/1	Female 45-50 years old	20	80/12	Male 20-25 years old
3	79/24	Unknown 1-2 years old	21	80/13	Female 14-15 years old
4	79/7	Female 40-45 years old	22	80/25	Unknown 2.5-3 years old
5	79/12	Female 12-13 yeas old	23	80/23	Unknown 2-2.5 years old
6	79/11	Male 35-40 years old	24	80/17	Unknown 3-4 years old
7	79/10	Female 55-60 years old	25	80/19	Female 50-55 years old
8	79/8	Male 30-35 years old	26	80/11	Male 35-40 years old
9	79/18	Female 40-45 years old	27	81/5	Male Unknown
10	79/14	Male 35-40 years old	28	80/21	Female 20-22 years old
11	79/1	Female 30-40 years old	29	80/24	Male 20-25 years old
12	79/22	Male 25-30 years old	30	80/27	Male 30-35 years old
13	79/21	Unknown 2-3.5 years old	31	80/2	Unknown
14	81/4	Male 25-35 years old	32	82/21	Female Unknown
15	81/3	Female 18-20 years old	33	82/12	Female Unknown
16	80/15	Male 25-30 years old	34	80/9	Female Unknown
17	80/10	Male 45-50 years old	35	80/27	Male 30-35 years old
18	80/14	Female 18-22 years old			

gene. PCR reaction was consisted of 1X Buffer, 1.5 mM $MgCl_2$, 200 µM Mix dNTP, 1.2 µM each of primers (Table 2) (MWG-Biotech AG, Germany), 160 µg/ml BSA (Biolabs, USA), 1U *Taq* polymerase (Cinagen, Iran) and water up to 50 µl total volume.

In order to compensate the less amount of DNA, usually found in archaeological specimens, and to increase the specificity of the PCR, primers were designed as external and internal (nested) ones on exon 3 and intron 3-4 (Fig. 2). The primers amplified a segment of AMG, located on parts of exon 3 and intron 3-4, with the predicted PCR product sizes of 258/252 bp for the first round of PCR and 112/106 bp for the second (nested) round for X and Y copy, respectively.

An initial denaturation step was performed at 94°C for 5 min. Each of the 40 amplification cycles consisted of: 1 min denaturation at 94°C, 1 min annealing at 57.5°C (for AML Primers) and 60°C (for British Primers, Sullivan *et al*., 1993) and 1 min elongation at 72°C. The last cycle was followed by an extra-extension at 72°C for 10 min. PCR products were electrophoresed on 2% agarose gel and/or 12% polyacrylamide gel.

DNA Digestion: In order to have a better distinguish between product sizes of AMG gene, the PCR products were digested by *BsmF I* [BioLab,USA] restriction enzyme. A typical digestion reaction contained 1X Buffer, 1X BSA and 1U *BsmFI* enzyme, 1 µg PCR product and deionized distilled water up to 50 µl. The

Table 2: Sequence of primers used to amplify AMG gene. Also, the sizes of the amplified products as well as the references from which the sequence of primers had been acquired are listed.

Name	Sequence	Size of Products
AML Forward	5` acctcatcctgggcaccctgg 3`	252 bp
AML Reverse X	5` tgagtcagagtggccaggcgg 3`	
AML Forward	5` acctcatcctgggcaccctgg 3`	258 bp
AML Reverse Y	5` tcaatccgaatggtcaggcag 3`	
British Forward	5` ccctgggctctgtaaagaatagtg 3`	112 bp on Y Ch.
British Reverse	5` atcagagcttaaactgggaagctg 3`	106 bp on X Ch.

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1    ctaccacctcatcctgggcaccctggttatatcaacttcagctatgaggtaattttttctc 60
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1    ctaccacctcatcctgggcaccctggttatatcaacttcagctatgaggtaattttttctc 60


61   tttactaattttgaccattgtttgcgttaacaatgccctgggctctgtaaagaatagtgt 120
     |||||||||||||| || |||||||| ||| || | |||||||||||||||||||||||||
61   tttactaattttgatcactgtttgcattagcagtcccctgggctctgtaaagaatagtgg 120


121  gttgattctttatcccagat------gtttctcaagtggtcctgattttacagttcctac 174
     || |||||||| |||||| ||      |||||||||||||| |||||||||||||||||||
121  gtggattcttcatcccaaataaagtggtttctcaagtggtcccaattttacagttcctac 180


175  caccagcttcccagtttaagctctgatggttggcctcaagcctgtgtcgtcccagcagcc 234
     || ||||||||||||||||||||||||||||||||||||||||||||||| | |||||| ||
181  catcagcttcccagtttaagctctgatggttggcctcaagcctgtgttgctccagcaccc 240


235  tcccgcctggccactctgactcagtctgtcctcctaaatatggccgta 282
     ||| ||||| ||| || || | | ||| |||||||||||||||||| |||
241  tcctgcctgaccattcggattgactctttcctcctaaatatggctgta 288
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Figure 2: Position of external (Blue) and nested (Red) primers on human AMG gene. The top and bottom lines are showing the sequence of X and Y copy of AMG alleles, respectively. Green letters indicate the six extra base pairs in Y copy of AMG.

reaction was incubated at 65°C for 1-3h. Finally, the 49 and 53 bp digested products from Y allele and the 106 bp non-digested products from X allele were detected by 2% agarose gel electrophoresis.

RESULTS

Successful extraction of amplifiable aDNA from ancient skeletons: We employed a simple, effective and modified "ethanol precipitation method" (Kalmar *et al*., 2000) to extract sufficiently pure aDNA required for successful PCR. The results confirm that aDNA can be extracted and amplified in ancient skeletal remains as old as 3500 years ago.

The quantity and purity of extracted aDNA was evaluated by means of agarose gel electrophoresis and UV spectrophotometry. As expected, there was no clear band or smear of DNA corresponding to genomic DNA (data not shown). Also, the light absorption of samples at 260/280-wave length was far from the ones of the modern DNA (1-1.6 and 1.8-2 respectively). Our findings indicate the low quantity and quality for retrieved aDNA.

239   Mohandesan *et al*.

Figure 3: The skeletal remains of a male (A) and a female (B) excavated from Masjede Kabood burial site. In addition to the morphological appearances of the remains there are some artifacts (sword in A, crown, bracelet and rings in B, as indicated by arrows), which used to clearly determine the gender of cases.

Sex determination on ancient human remains: To determine the gender of samples, we amplified a small piece of AMG gene, which has two variable alleles on X and Y-chromosomes. We selected the skeletal remains, which their gender was known based on their morphology and belongings such as crown, sword, bracelet etc. (Fig. 3A and 3B, male and female, respectively). At the first round of PCR (with 40 cycles), we couldn't find any signal at the expected size (258 bp for Y copy and 252 bp for X copy) on agarose gel (data not shown). However, in the second round of PCR signals with the expected sizes of (112 bp for Y copy and 106 bp for X copy) were detected on agarose gel (Fig. 4A).

Confirming the molecularly determined sex of the samples by means of restriction enzyme digestion: The size difference between the amplified segments of X and Y copy of AMG (6 extra base pairs in Y copy, as indicated in figure 2) was not big enough to be detected clearly on agarose or polyacrylamide gel electrophoresis (PAGE). For that reason, we searched the list of commercial restriction enzymes and find a new enzyme capable of recognizing and cleaving the PCR

product for Y copy of AMG, but not the X copy. The cleavage generated 2 smaller bands on the Y copy of AMG (49 and 53 bp), which was easily distinguishable from the 106 bp band amplified on the X copy by means of agarose gel electrophoresis (Fig. 4B).

A total of 15 samples with obvious and confirmed gender were selected from the total list of collected samples. The molecular determination of gender based on AMG PCR/Restriction enzyme digestion was compared with anthropometric reports. At the beginning stages of the project the molecular sex determination was both different from anthropometric reports and also not reproducible. However, after optimizing the procedure and setting guidelines to eliminate the risk of contamination we were able to have reliable and reproducible molecular sex determination (there was only one error in the reproducing of the results in the last effort to confirm the gender of all 15 samples).

DISCUSSION

In the present study we have successfully extracted and amplified aDNA from human remains excavated
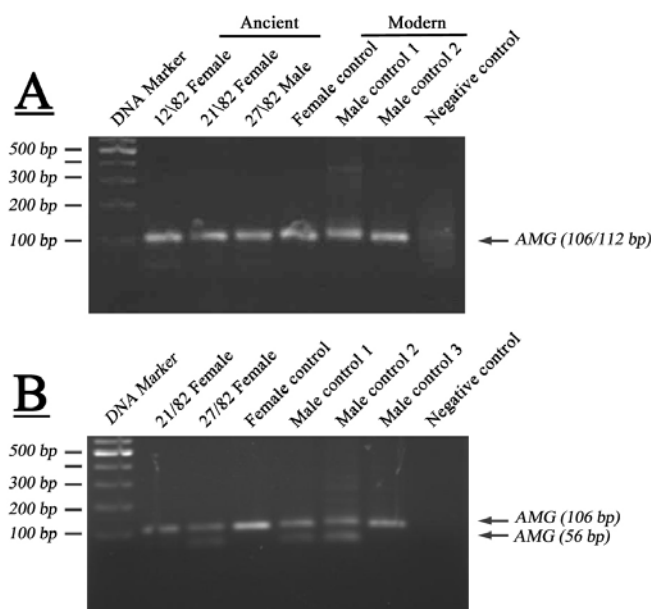
Figure 4: (A) Nested-PCR analysis of AMG gene in ancient (lanes 2-4) and modern samples (lanes 5-7), (B) Digestion products of AMG gene by *Bsm*FI enzyme, The size of the products on X chromosome is 106bp and on Y chromosome is 53/49bp, in male control 1 just the Y chromosome was amplified, in male control 2 the X and Y chromosome was both amplified and in male control 3 just the X chromosome was amplified.

from Masjede Kabood burial site. The site is one of the many burial sites scattered throughout Iran and contains numerous artifacts, which can provide interesting information about the ancient culture and art. However, there is other important information that is hidden within the DNA sequences of ancient human, animal and plant remains. DNA extracted from plant and animal remains can provide valuable information about the agriculture and animal husbandry in ancient times (Paabo *et al.,* 2004; Troy *et al.*, 2001; Bailey *et al.*, 1996; Vila *et al.*, 2001; Matsuoka *et al.*, 2002 and Piperno and Flannery, 2001). On the other hand, the aDNA retrieved from human skeletal remains could provide important data on the gender, relationship, kinship, race and other features of the excavated remains (Paabo *et al.*, 2004; Krings *et al.*, 1997 and 1999; Ovchinnikov *et al.*, 2000; Schmitz *et al.*, 2002; Krings *et al.*, 2000; Gill *et al.*, 1994 and Kaestle and Horsburgh, 2002).

There are various ways to successfully extract DNA from ancient source material such as Phenol-chloroform, Sodium-acetate-isopropanol and Silica Guanidinium Thiocyanate based methods. We used all of the mentioned techniques to extract and amplify aDNA from our samples. However, neither of tech-

niques was able to extract amplifiable aDNA from the ancient bone samples. As a very last effort, a simple and effective modified ethanol precipitation-based protocol was employed for the preparation of aDNA. The method is fast and requires neither hazardous chemicals nor special devices. The later method has been successfully used previously by others (Kalmar *et al.*, 2000) and it seems that the presence of dextran blue in the protocol is responsible for the removal of the PCR inhibitor agents within the samples (Kalmar *et al.*, 2000).

In addition to the low quality of the extracted aDNA, the extraction procedure also yielded a very low amount of aDNA. Consequently, we failed to detect any PCR product band for AMG on agaroe gel, even by using 40 cycles of amplification. This observation was in contrast with our PCR results when we used the same conditions for fresh tissues. In order to compensate for the very low amounts of retrieved aDNA, we designed a nested PCR reaction to increase the specificity and sensitivity of the technique.

Despite the potential usefulness of aDNA, the problem of verifying results by reproduction is still a major concern. One reason for this is technical and derives from the low copy number and damaged state of ancient DNA molecules. The other reason is the risk of contamination of ancient samples with the modern DNA (14).

Contamination is a very serious problem in ancient DNA research, especially when the samples are from human remains. For that reason, it is necessary to have accurate controls, throughout the work procedure (Hofreiter *et al.*, 2001; Handt *et al.*, 1994; Lindahl 1993, Cooper and Poinar, 2000).

While the main aim of the current research hasn't been the sex determination of excavated skeletal remains, we decided to do so for the following reasons: 1) To set up our aDNA extraction and amplification facility 2) To test the authenticity of extracted aDNA. 3) To monitor and eliminate any potential contamination of aDNA with modern DNA.

For that reason, we developed a novel nested-PCR/restriction enzyme digestion method for DNA-based sex identification that uses nucleotide differences between the X and Y copies of the AMG. The procedure applied successfully in approximately 35 individuals with known gender. At the beginning, the results of molecularly sex determination were neither reproducible nor identical to the anthropometric reports. However, by following the proposed guidelines (Paabo *et al.*, 2004) to eliminate potential contamination from authentic aDNA, especially by sepa-

Mohandesan *et al.*

rating the extraction and amplification steps in separate spaces, we were able to produce reproducible and reliable results.

Our results demonstrate that despite the well-known problems of poor DNA preservation and the ever-present possibility of contamination with modern DNA, reliable aDNA can be obtained and used as a reliable source of new information into ancient populations.

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