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Molecular Archeology of Ancient Bone From 400 Year Old Shipwreck

Shahrul Hisham Zainal Ariffin[1], Rohaya Megat Abdul Wahab[3], Zulkeflie Zamrod[1], Samsol Sahar[4], Mohd Fauzi Abd Razak[1], Eni Juliana Ariffin[1] And Sahidan Senafi[1].

[1]*School of Bioscience and Biotechnology, Faculty of Science and Technology, UKM, Bangi, Selangor.*
[3]*Department of Orthodontics, Faculty of Dentistry, UKM, Kuala Lumpur*
[4]*Department of Museum and Antiquities Malaysia, Kuala Lumpur.*

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Abstract. Molecular archeology is a new frontier of ancient archeology. Studies involving molecular archeology can give extra information useful to interpret our past. Ancient DNA basically consists of small DNA fragments that survive for a very long period. This DNA can be used as a template to study molecular archeology. We were able to isolate ancient mitochondrial DNA (mtDNA) from bones that were recovered by the Department of Museum and Antiquity Malaysia during their Wanli's shipwreck excavation project recently. The amount of recovered mtDNA was very small; extracted bone showed there was no DNA band after agarose electrophoresis. Amplification using PCR approach at D-loop I of human mitochondrial DNA was able to produce the amplified DNA of 239bp in size. However, only 215 nucleic acids sequences were able to be confirmed when subjected to DNA sequencing procedures. Further analysis of the DNA sequenced of this amplified DNA showed that 99% of the generated sequenced was identical with D-loop region I of human mitochondrial DNA. The results showed that our procedure of DNA extraction although produces very low amount of DNA but yields pure DNA that can be used for further analysis such as PCR amplification and DNA sequencing.

Keywords. ancient mitochondrial DNA, human bones, PCR amplification.

INTRODUCTION

Analysis of ancient DNA gives archeologists and anthropologists alternative and innovative ways to interpret and understand the past (Jones, 2003; Kaestle *et al.*, 2002; O'Rourke *et al.*, 2000.; Richards *et al.*, 1995). The first studies were done on Egyptian mummified material by Paabo and colleagues in 1985 (Paabo, 1985). Molecular archeology has generated many new insights on important archeological and anthropological questions, encompassing human evolution, population affinities (Eshleman *et al.*, 2003; Krings *et al.*, 1997; Ovchinnikov *et al.*, 2004; Stone *et al.*, 1993) to domestication of animal and plant species (Brown *et al.*, 1998; Vila *et al.*, 2001).

Two major hurdles in molecular archeology are the degradation of DNA over time and the contamination of ancient samples with modern DNA (Kaestle *et al.*, 2002; Hofreiter *et al.*, 2001; Taylor *et al.*, 1996). Physical and chemical treatment during DNA extraction can further destroy DNA molecules that exist in the ancient remains. Therefore, the analysis of such DNA was not practical until the polymerase chain reaction (PCR) became available (Paabo, 1989). The PCR technique is essential for detecting ancient DNA molecules since the technique is extremely sensitive in detecting minute amounts of specific DNA molecules and amplifying these molecules many times in a matter of hours (Innis *et al.*, 1990; Yang and Watt, 2005).

Damage of DNA templates and the presence of inhibitors may result in low efficiencies or failure of the PCR amplification (Herrmann and Hummel, 1994; Yang *et al.*, 2003). In order to overcome this problem, it is important to increase the efficiency of PCR amplification. Yang *et al.* (1998) were able to amplify ancient mitochondrial DNA (mtDNA) from 2000 year old human skeletal remains from an Imperial Roman necropolis using hypersensitive PCR amplification, i.e. with a high number of PCR cycles. However, their approach utilized special DNA polymerase called AmpliTaq Gold™ making the procedure expensive (Yang *et al.*, 1998).

The *Wanli* was a trade ship believed to have sailed from China and sank in the 1630s during an attack by another trade ship. The shipwreck was discovered by Sten Sjostrand

*Author for Correspondence.
Mailing address: School of Bioscience and Biotechnology, Faculty of Science and Technology 43600, Universiti Kebangsaan Malaysia, Selangor. Tel: 603-89213245
Email:hisham@cgat.ukm.my

in November 2002 in the waters of Dungun, Terengganu at a depth of 43 meters. Primary excavation operation by the Department of Museum and Antiquity Malaysia found a coat owned by Captain Alvaro Vilas Boas, a Portuguese armada captain who once actively sailed from China to Malacca around 1630s. The architecture of the vessel indicated that it was of European design with carpentry skill of the Chinese and made of tropical hardwood.

In this report, we describe a simple procedure for mitochondrial DNA extraction and subsequent PCR amplification using low cost *Taq* polymerase (Promega) on DNA extracted from two human bones found on *Wanli*. The DNA extraction involves a specialized dental drill to clean the outer layer of the bone followed by generation of powdered DNA using mortar and pestle. The powdered bones were subjected to DNA extraction procedures, phenol-chloroform and ethanol precipitated purification procedure followed by PCR amplification and DNA sequencing.

MATERIALS AND METHODS

Skeletal remains. Two bones were discovered from the shipwrecked *Wanli*. Both bones were presumed to be human and subjected to further molecular analysis

Contamination controls. Throughout the extraction procedure, great care was taken to minimize the contamination of modern DNA. The bone samples and disposables were treated with 1.0% domestic Chlorox followed by UV irradiation exposure in an ultraviolet chamber. On the other hand, the extraction reagent was only subjected to UV irradiated exposure without treatment with 1.0% domestic Chlorox. The UV irradiated exposure was performed overnight in order to destroy modern DNA on the outer layer of the intact or powdered bones, disposables and also DNA extraction reagents. The non-disposable equipment such as dental drill metal bur, mortar and pestle were decontaminated between samples. Decontaminated latex gloves and protective clothing were worn when handling the decontaminated bones. Extration of bone DNA was performed using dedicated laboratory chamber (Bredent, Germany) that were priory decontaminated by 1.0% domestic Cholorox followed by 70% ethanol prior to DNA extraction to minimize cross contamination with modern DNA.

Extraction of ancient bone DNA. The bone surface was ground using specialized dental micromotor (Nouvag, Switzerland) in a special dust protective chamber (Bredent, Germany) using specialized coarse plain cut straight hand piece metal bur (Nouvag, Switzerland). The bone was powdered by UV irradiated mortar and pestle. Approximately 5 grams of powdered bone were taken for each DNA extraction. In this study, due to the importance of the respective samples, we were allowed to extract the mtDNA from a total of 5 grams of powdered bone. The powdered bone was UV irradiated prior to DNA extraction. The UV irradiated bone powder was dissolved in 8 ml extraction buffer (10 mM Tris-HCl, 10 mM EDTA pH8.0, 50 mM sodium dichloride, 2.0% sodium dodecyl sulfate and 100 µg/ml proteinase K) and incubated in a shaking water bath at 56ºC followed by incubation at 37ºC for 24 hours. The extraction solution was centrifuged at 2,000g for 5 minutes. Approximately 1.5 ml aliquots of the supernatant was transferred to 1.5 ml microtube and spun for 5 minutes at 12,000 rpm using Microfuge® 18 Centrifuge (Beckman Coulter). The supernatant was transferred into another 1.5 ml microtube for two cycles of phenol-chlorofom extraction followed by one cycle of a standard 100% and 70% ethanol precipitation. The pellet produced during ethanol precipitation was dissolved in 50 µl deionized distilled water.

Amplification and sequencing of ancient bone mtDNA. The overall success of the extraction procedures was assessed based on the ability to amplify a 239 bp target sequence at hypervariable region I of mtDNA (Tibor *et al.*, 2000) using PCR amplification techniques. The following primers were used in PCR amplification: 5' - TGA TTT CAC GGA GGA TGG TG - 3' as the forward primer and 5' - AAC CCC CTC CCC ATG CTTA - 3' as the reverse primer. PCR amplification was carried out using the GeneAmp Thermocycler Model 2400 (Perkin Elmer) in a 50 µl reaction volume containing 50 mM KCl, 10 mM Tris-HCl, 2 mM MgCl, 0.2 mM dNTP, 1.5 mg/ml bovine serum albumin (BSA), 0.25 U *Taq* polymerase (Promega), 100 pmoles of each primer and 5 µl template, i.e. extracted bone's DNA. The respective mixture was subjected to PCR amplification of 35 cycles at 94ºC for 40 seconds, 53ºC for 20 seconds and 72ºC for 20 seconds. A final 3 minutes of extension at 72ºC was performed after the last cycle. Five µl of PCR product was separated by electrophoresis on 2% agarose gel in standard electrophoresis buffer. The 100 bp ladder (Promega) was used as a standard size marker. After electrophoretic separation at 90 Volts for 20 minutes, the gel was stained with ethidium bromide and photographed under UV illumination.

The PCR product was purified further using QIAquick™ PCR purification kit (QIAGEN) and re-amplification using similar PCR amplification procedures. The recovered PCR product was subjected to purification using similar PCR purification kit prior to DNA sequencing. The samples were sequenced twice with the ABI3730 automated sequencer using the forward primer. The DNA sequencing was performed at the Malaysia Genome Institute, Bangi.

RESULTS

PCR amplification. Both forward and reversed primers were design specifically to amplify 239 bp of fragment at

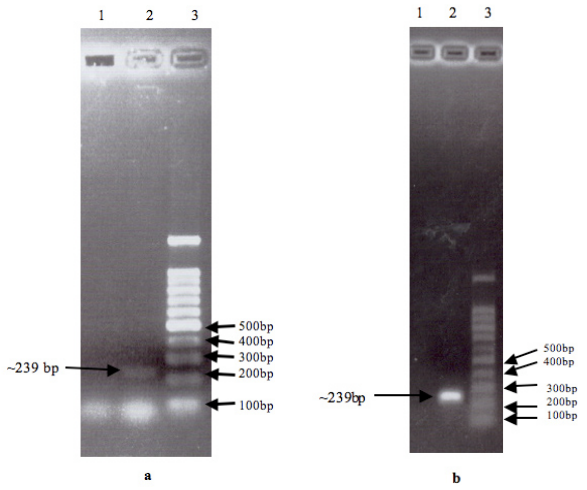

Figure 1. Identification of amplified mitochondrial DNA from human ancient bone from the Wanli shipwreck. The PCR amplification products were electrophoresed on 2.0% agarose gels. Forward and reverse primers specific to D-loop hypervariable region of mitochondria DNA were used. Lane 1; amplification (a) and re-amplification (b) of negative control extraction. Only the amplification of negative control extraction shows formation of primer-dimers. Lane 2; consists of ancient bone sample subjected to DNA extraction followed by PCR amplification (a) and re-amplification (b). Both amplifications showed formation of 239 bp DNA band. Lane 3 is standard molecular size makers of 100bp ladder (Lane 3; a and b).

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Amplified DNA     AGGGGCCCCTATCTGAGGGGGGTCATCCATGGGGACGAGAAGGGATTTGACTGTAATGTG
                  |||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human mtDNA       AGGGACCCCTATCTGAGGGGGGTCATCCATGGGGACGAGAAGGGATTTGACTGTAATGTG

Amplified DNA     CTATGTACGGTAAATGGCTTTATGTACTATGTGCTGTTAAGGGTGGGTAGGTTTGTTGGT
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human mtDNA       CTATGTACGGTAAATGGCTTTATGTACTATGTGCTGTTAAGGGTGGGTAGGTTTGTTGGT

Amplified DNA     ATCCTAGTGGGTGAGGGGTGGCTTTGGAGTTGCAGTTGATGTGTGATAGTTGAGGGTTGA
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human mtDNA       ATCCTAGTGGGTGAGGGGTGGCTTTGGAGTTGCAGTTGATGTGTGATAGTTGAGGGTTGA

Amplified DNA     TTGCTGTACTTGCTTGTAAGCATGGGGAGGGGGTT
                  |||||||||||||||||||||||||||||||||
Human mtDNA       TTGCTGTACTTGCTTGTAAGCATGGGGAGGGGGTT
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Fig. 2: DNA sequence of amplified ancient mitochondrial DNA. DNA sequencing was repeated three times using forward and reverse primers. However, only 215 nucleotides were confirmed from both generated sequences. The generated sequence was analyzed using the NCBI database. The nucleotide sequence above representing generated sequence from amplified samples whereas sequences below representing human mitochondrial D-loop region I position 16438 to 16653 (Human mtDNA) in the database. The bold letter represent nucleotides that were not identical to human D-loop region I mitochondrial DNA.

mitochondrial DNA hypervariable region I (Tibor *et al.*, 2000). A band approximately 239 bp was amplified in the samples containing ancient bone (Figure 1a, lane 2) whereas negative controls were unable to amplify the 239 bp band (Figure 1a, lane 1). However, both ancient bone and blank extraction samples showed the presence of primer-dimers (Figure 1a) during PCR amplification as expected due to the sequences of the primers (Tibor *et al.*, 2000).

The ability to amplify the 239 bp target sequence, albeit producing a faint band, is evidence of a successful amplification of DNA from an ancient bone found. The target sequence is from the 239 bp hypervariable region I of mitochondrial DNA. After 35 amplification cycles, only a single weak band were produced as shown at Figure 1a, Lane 2.

The amplified product was subjected to gel purification and re-amplified using a similar program, i.e. 35 cycles of 94ºC for 40 seconds, 53ºC for 20 seconds and 72ºC for 20 seconds with 3 minutes of final extension at 72ºC after the last cycle. Electrophoresis on 2% agarose gel showed a much cleaner amplification product (Figure 1b, Lane 2) whereas the negative control showed the absence of any PCR amplification product (Figure 1b, Lane 1). The amplified product showed similar PCR product in term of size as previous amplification (Figure 1a and 1b; Lane 2). The re-amplified band was subjected to purification and DNA sequencing.

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). The negative controls of amplified by PCR showed that only primer-dimer was present (Figure 1a; Lane 1). However, primer-dimer was not present in the re-amplification of negative control (Figure 1b; Lane 1). Both amplification and re-amplification of negative control showed no amplified product except for the existence of primer-dimer (Figure 1a and 1b; Lane 1). 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.

Sequencing PCR products. PCR products from successful amplifications were subjected to DNA sequencing. BLAST analysis (Figure 2) showed that the generated DNA sequence from amplified ancient DNA were 99% identical to the human mitochondrial D-loop sequence.

DISCUSSION

A complete and convincing genetic analysis of the bone is an expensive, time-consuming and destructive (5 grams of bone) undertaking. There is a fair amount of doubt that the amplified target sequence is contaminant-free. The integrity of the ancient DNA is questionable due to its age and condition. Furthermore, the organic content of the ancient bone is very low relative to modern bone. However, there is evidence that bones that are old are able to yield DNA that could be enzymatically amplified and sequenced (Yang *et al.*, 1998).

In order to overcome these problems, a logical solution would be to increase PCR amplification efficiency. Studies by other groups showed that a strong boost of ancient bone PCR amplification could be produced when using higher cycle, i.e. 35-60 cycles. Unfortunately, this will increased unexpected appearance of positive amplification of the negative control sample (Yang and Eng, 2003). In our study, we chose a slightly lower number of PCR cycle, i.e. 35 cycles.

In this study, negative control showed only primer-dimer amplification (Figure 1, lane 1). This showed that the absence of amplified modern DNA or modern DNA contamination during extraction procedures. Amplification of bone extraction sample (containing powdered bone) showed 239 bp of amplified product (Figure 1a and 1b; Lane 2) similar in size to ancient D-loop mitochondrial hypervariable region I amplified by Tubor *et al.* (2000). This indicates that the procedure of DNA extraction is able to produce a pure DNA from ancient bones that can be amplified using PCR procedure.

Contamination is of the utmost concern when working with ancient DNA (Yang and Watt, 2005). Contaminating human DNA can be introduced at any point during the process right from archeological excavation, extraction and finally PCR setup (Yang and Watt, 2005; Yang *et al.*, 1998). The respective researchers have to exercise strict contamination control. Some centers provide a special PCR setup or even dedicated laboratory for ancient DNA extraction. Unfortunately most of the solutions and disposables have to take place outside of the laboratory such as manufacturing chemicals, reagents, columns, tubes and buffers that can be contaminated by modern DNA (Eshleman and Smith, 2001). Therefore, contamination can be introduced to the extracted samples even in dedicated laboratory is used. In this study, we showed that with proper decontaminated procedures were able to minimize or eliminate modern DNA in the sample. There is no amplification product found in the negative control (Figure 1a). Methods to minimize contamination performed in this study included the use of a dedicated laboratory chamber. In addition, reagents and equipments for preparation of ancient DNA samples were exposed with UV irradiation. Furthermore, disinfectants such as 1.0% Chlorox were used to eliminate any surface contamination. In this study, we also performed a modified extraction method essentially the method was based on Kalmar (2000) that is without the use of Dextran Blue that can act as a PCR inhibitor (Kalmar *et al.*, 2000).

Identification of human remains by DNA analysis especially using mitochondrial DNA has proven to be a powerful tool in forensic and historical investigations (Hagelberg *et al.*, 1989). Most mammalian cells contain hundreds of mitochondria and in turn, each mitochondrion contains several copies (2-10) of mitochondrial DNA (Shuster *et al.*, 1988; Wiesner *et al.*, 1992). The high copy number of mitochondria per cell increases the likelihood of recovering amplified ancient DNA. Mitochondrial DNA analyses were performed on human remains up to 12,000 years (Hagelberg *et al.*, 1989) and surprisingly even on the Neanderthal skeletons (Krings *et al.*, 1997; Ovchinnikov *et al.*, 2000). In our study, we chose the mitochondrial D-loop as targets for PCR approaches (Figure 1a and 1b: lane 2). Thirty five cycles are needed to obtain sufficient amount of PCR product for DNA sequencing. Higher number of cycle will able to produce higher number of copies form smaller numbers of ancient DNA (Yang *et al.*, 2003; Yang *et al.*, 1998; Eshleman and Smith, 2001).

The sequence generated by the amplified ancient DNA showed 99% identical with the hypervariable region I of D-loop region of the mitochondria. The D-loop is one of two non-coding regions of mitochondrial DNA and mainly contains known regulatory elements for mitochondrial transcription and replication. This region is also known as the most variable in sequence and size among different species (Fernandez-Silva *et al.*, 2003). Any DNA amplification that produced the expected sizes of PCR product will strongly showed that the DNA originated from human. Furthermore, the amplified DNA that was subjected to DNA sequence and analysis using NCBI databases showed that the amplified DNA was from human mitochondrial DNA (Figure 2).

In summary, we managed to extract mitochondrial DNA approximately 400 year old bone recovered from *Wanli*'s shipwreck at Dungun sea coast. The aged of the 400 year bone was based on the estimated age of the shipwreck (sank around 1630s, where the respective bones were found; Samsol, personal communication). Although the amount of mitochondrial DNA was small, the DNA sequences gave 99% homology with human D-loop mitochondria DNA.

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