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Use of RAPD and PCR double amplification in the study of ancient DNA

F. Balzano*, E. Mura, A. Mura, G. Lepore, M. Zedda

Department of Animal Biology, University of Sassari, Via Vienna 2, 07100 Sassari, Italy
* frbalzano@uniss.it

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Abstract

This project analysed the DNA extracted from bones of ancient sheep which have been brought to light in Sardinian different archaeological sites. In order to better analyse this highly fragmented DNA, a double amplification technique was chosen. The first approach consisted of RAPD-PCR and the second one in classic PCR. The RAPD-PCR amplified random fragments and allowed the production of numerous amplicons. The products of RAPD amplification have been amplified, more specifically, by the second PCR using primers for a sequence of 176 bp of mitochondrial D-loop region. These DNA fragments have been sequenced and the sequence analysis has confirmed that it belonged to Ovis aries. Consequently, this procedure can be considered a valid tool to perform amplification of degraded DNA, such as ancient DNA.

Introduction

In the most innovative and promising archaeozoological studies, molecular biology techniques are applied to the analysis of remains of ancient animals. Unfortunately, the study of ancient DNA presents some problems, which are mainly related to DNA integrity and contamination caused by modern exogenous DNA [1, 2]. After cell death, DNA is subjected to a degradation process that often results in significant fragmentation of the molecule. Ancient DNA is indeed reduced in short sequences of 100-300 nucleotides. A chemical reaction, known as depurination, is responsible for DNA degradation; in the same manner, the high temperature or acidic pH values, can facilitate the breaking of DNA strands. In contrast, anoxia, dehydration and basic pH values generally promote the conservation of double-stranded molecules [3]. Ancient DNA can sometimes be contaminated by a exogenous DNA. Contamination can be caused by virus, bacteria or fungi. A common cause of contamination of ancient DNA is exogenous human DNA from operators who handle the remains such as archaeologists or molecular biologists [4, 5]. In spite of all the precautions taken to reduce exogenous contamination, ancient DNA is extracted in small quantities and the samples are generally very fragmented. To overcome these limitations, we decided to proceed with the amplification technique that produced billions of new molecules identical to the original; in particular, one of the most popular techniques developed in the mid-80^s by Kary Mullis is the polymerase chain reaction (PCR). The PCR method provides extensive application extremely valuable in archaeozoological studies [6]. Since PCR relies on intact DNA sequence, it has some limitations in the use of degraded DNA samples. Given that ancient DNA is extremely fragmented, and that the fragmentation of the DNA affects the starting of PCR amplification, we proceeded to apply a double amplification technique. The first was a variant of PCR: the random amplified polymorphic DNA (RAPD-PCR) [7], the second was a classic PCR. In the RAPD-PCR, short oligonucleotides are used to determine the amplification of random fragments of DNA [7-10]. In this way, we obtained the molecular fingerprint of the DNA tested. The RAPD allowed the production of several amplicons. They were amplified more specifically in the second PCR using primers for a 176 bp sequence of mitochondrial D-loop region. Consequently, this procedure can be considered an effective device for the amplification of degraded DNA, like ancient DNA. In this work, we have studied the bones and teeth belonging to Sardinian sheep taken from archaeological sites of different time periods. We have undertaken these studies to make a valuable contribution to our understanding of the biological history of each species, in order to fill the gaps in phylogenetic trees, as well as to update the taxonomy of animals and identify elements of biodiversity in the past [4, 11, 12].

Materials and methods

In the present study, we have examined well-preserved bones and teeth belonging to sheep taken from Sardinian archaeological settlements of different prehistoric and historic ages: the Eneolithic village of Su Coddu (Selargius), Nuragic settlements of S. Pauli (Villamassargia), S. Antine

(Torralba), Mitza Piddighi (Solarussa) and Funtana Coberta (Ballao). In addition, animal assemblages from amphorae of a Punic shipload and from a store near the ancient Karalis harbour have been examined (Fig.1), also fragments of tooth and mandible teeth of sheep have been selected from each location (Tab. 1).

Figure 1. Sardinian map.

	sample	archaeological site	
S1	tooth	Village of Su Coddu	Eneolithic Age (3300-2800 BC)
S2	tooth		
S3	tooth		
A1	tooth	Nuraghe S. Antine	Nuragic Age (2000-300 BC)
A2	tooth		
A3	mandible		
I1	tooth	Nuraghe Iloi	
I2	tooth		
I3	mandible		
P1	mandible	Mitza Pidighi	
P2	mandible		
P3	tooth		
B1	mandible	Funtana Coberta	
B2	tooth		
B3	mandible		
V1	tooth	Nuraghe S. Pauli	
V2	tooth		
V3	tooth		
G1	mandible	Punic amphorae in the S. Giusta Pond	Punic Age (VI-IV cent. BC)
G2	mandible		
G3	tooth		
K1	tooth	Karalis	Roman Age (III century)
K2	tooth		
K3	tooth		

Table 1. Archaeological samples.

The manipulation of ancient DNA has been performed in an isolated laboratory exclusively employed for ancient DNA analyses. Each extraction has been performed by adding a negative control every two samples in order to become sure of the absence of contamination. The surface of all archaeological samples has been first washed with sodium hypochlorite, then exposed to ultra violet radiation to eliminate contamination of exogenous DNA finally ground into a fine powder using a sterile mortar and placed into sterile 2 ml tubes. Extractions have been performed by using the Mammalian Genomic kit (Sigma-Aldrich) following the manifacturer's protocol. All extracted DNA have been spectrophotometrically quantified at 260 nm [13]. In order to assess DNA quality, the extracted DNA has been loaded on 3% agarose gel. DNA produced the smear typical of ancient DNA (Fig. 2), which provided also evidence that no contamination with modern DNA occurred.

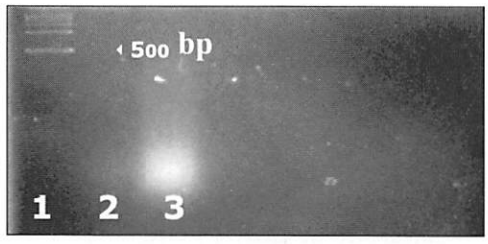

Figure 2. Ancient DNA extracted from a tooth of sheep recovered in the Village of Su Coddu (lanes 2 and 3). Lane 1 corresponds to marker Quick-Load 1 kb DNA Ladder (Bio-Lab).

Samples resulting in low quality (<1 ng/µl) have been exposed to a variant of PCR, the RAPD-PCR (Williams et *al.*, 1990) by using commercial primers (OPG 20 Operon Genomics Kit) reported in the Tab. 2. The outcome products have been first diluted 1:20, then mixed and amplified with primers specific for the region of 176 bp of mitochondrial D-loop of sheep.

Code of OPG Kit	5' to 3'	M.W.	Pmoles
OPG 4	AGCCCCTCCA	3059	5415
OPG 11	TGCCCGTCGT	2995	6161
OPG 15	ACTGGGACTC	3028	5302
OPG 16	AGCGTCCTCC	2964	6015
OPG 17	ACGACCGACA	3006	4894

Table 2 - Primer OPG 20 Operon Kit (molecules for life) used

The primer sequences have been designed, using the software Primer3 (http://www.genome.wi.mit.edu/cgibin/primer/primer3_www.cgi) and in agreement with the mtDNA sequence from sheep (AF010406.1 Genbank. *Ovis aries*), are: Forward primer (5'-ACCCGGAGCATGAATTGTAG-3 '), reverse primer (5'-GGGGGAAGCGTGTTAAAAAT-3').
RAPD-PCR was performed in 50 µl reaction containing 1U Ampli-Taq DNA polymerase (Qiagen Quality Top Taq), 1 × PCR buffer II (10 mM Tris-HCl pH 8.3, 50 mM KCl), 0.25 mM each dATP, dCTP, dGTP and dTTP (Life Technologies, Gibco BRL), 0.83 µM each primer (OPG Kit

20 Operon Primers Molecules for Life) and 5 µl template DNA. A final concentration of 1 mM $MgCl_2$ proved to be most suitable. Amplifications have been run on a Biometra T Personal thermal cycler. Several protocols have been assessed. The most successful combination has been the following: an initial denaturation at 94°C for 1 min, followed by three cycles of 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1 min. Additional 42 cycles of 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 1 min, have been then run, followed by a final extension at 72°C for 5 min. Samples have been finally cooled to 4°C. In each amplification set, two negative PCR controls have been processed in order to verify that no contamination had occurred. All PCR products have been visualized under ultraviolet light in 0.5 µg/ml ethidium bromide in 3% agarose in 1X TAE buffer gel. The PCR reaction for the mitochondrial region has been performed in a total volume of 50 µl containing 0.5 U Ampli-Taq DNA polymerase (Perkin Elmer), 1X PCR buffer II (10 mM Tris-HCl pH 8.3, 50 mM KCl), 0.25 mM each dATP, dCTP, dGTP and dTTP (Life Technologies, Gibco BRL), 1 µM each primer, 1.5 mM MgCl2 and 5 µl of DNA or RAPD-PCR products. The following procedures were employed: an initial denaturation at 94°C for 1 min, followed by 30 cycles of 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min. A final extension at 72°C for 5 min then followed, and samples have been cooled to 4°C. Amplicons for DNA sequencing have been purified using Microspin™ sephacryl columns (Amersham Pharmacia Biotech) according to the manufacturer's

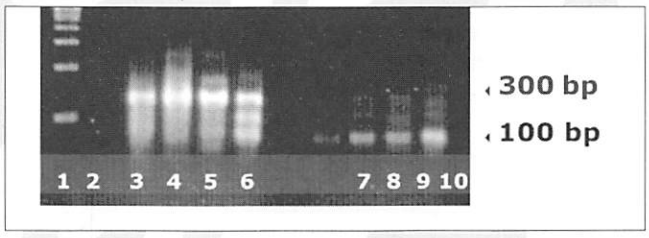

Figure 3. RAPD amplification obtained with the primer OPG 17. 1 Marker 100 bp Low Ladder Sigma-Aldrich, 2 negative control, 3 sample S1, 4 sample K2, 5 sample S2, 6 sample S3, 7 samples V3, 8 samples A2, 9 samples A1. 10 negative control

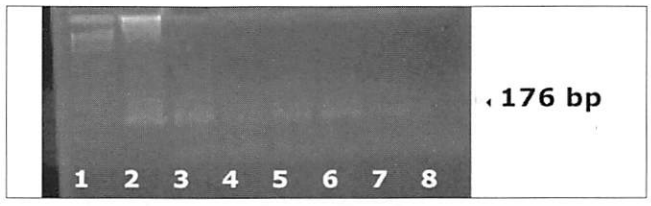

Figure 4. PCR products obtained with primers for the mitochondrial D-loop region. 1 Marker 100 bp Low Ladder Sigma-Aldrich, 2 sample S1, 3 sample K2, 4 sample S2, 5 sample S3, 6 samples V3, 7 samples A2, 8 negative control

directions. The sequencing has been performed by BMR Genomics.

The sequences have been matched with those from a BLAST search of the National Center for Biotechnology Information (NCBI) database (http:/www.ncb.nlm.nih.gov). Multiple sequence alignments, with the reference sequences downloaded from the EMLB (http://www.ebi.ac.uk/embl) and GeneBank, have been performed through the Clustal-W program.

Results

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CLUSTAL 2.1 multiple sequence alignment

A1          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 72
A2          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 72
A3          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 72
V3          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 84
B1          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 76
B2          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 76
P1          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 76
V1          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 84
V2          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 70
G3          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTTATATATTCTTTT 67
P3          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 67
K1          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 82
K2          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 82
K3          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 82
G1          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 77
G2          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 77
S3          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 67
S2          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 67
S1          CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 67
AF010406.1  CATAATATAATTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 300
P2          ----------TTAATGGTCACAGGACATATCTGCTGTATCGTGCATTTATATATTCTTTT 50
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A1          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGCTTCCC------------- 119
A2          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGCTTCCC------------- 119
A3          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGCTTCCC------------- 119
V3          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGC------------------ 126
B1          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGC------------------ 118
B2          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGC------------------ 118
P1          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGC------------------ 118
V1          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGCCATCCTCATAATGG---- 140
V2          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGC------------------ 112
G3          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACG------------------- 108
P3          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACG------------------- 108
K1          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGCTTCC-------------- 128
K2          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGCTTCC-------------- 128
K3          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGCTTCC-------------- 128
G1          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGCTTCC-------------- 123
G2          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGCTTCC-------------- 123
S3          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGCTTCC-------------- 113
S2          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGCTTCC-------------- 113
S1          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGCTTCC-------------- 113
AF010406.1  TTCCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGCTTCCCCCCTAGATATTAA 360
P2          T-CCCCCCTTCCCCTTAAATATTTATCACCATTTTTAACACGCTTCCCCCCA---------- 100
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Table 3 - All sequences were aligned using the software package Sequencher™ European Bioinformatics Institute 2011 (ClustalW2 - Multiple Sequence Alignment).AF010406.1 Genbank. Ovis aries sequences

21 out of 24 archaeological bone samples have been successful amplified. The samples analyzed by the RAPD technique showed several bands (Fig. 3). This outcome indicates that even small amounts of finely dispersed and highly fragmented DNA can be amplified by this technique. The RAPD products have been amplified by PCR with the primer pair designed in the D-loop region. The samples S1, K2, A3, S2, S3, V3, A2, gave an amplicon of 176 bp. The sequences have been compared with the mitochondrial sequence of modern sheep, allowing the identification of the species on the basis of nucleotide similarities. All sequences have been aligned by using the software package Sequencher™ European Bioinformatics Institute 2011 (Clustal-W - Multiple Sequence Alignment, Table 3). The sequencing confirmed that the amplified DNA is mtDNA of sheep (Fig. 4). In order to guarantee the accuracy of the method all samples were analyzed in triplicate. No differences have been found between RAPD sequences and those directly amplified by PCR. In addition, these results demonstrate

that the samples examined have not been contaminated by bacteria and other microorganisms.

Discussion

RAPD PCR is not the most popular technique employed in ancient DNA studies; nevertheless, it has revealed a method that allows the amplification of degraded DNA samples, in spite of the poor amount of DNA. DNA degradation was indeed mostly observed in bone remains, which are more susceptible to the environmental conditions. Dental DNA is less exposed to degradation because it is housed in the pulp chamber that acts as a protective envelope. Results obtained with the RAPD PCR technique have enabled further PCR amplification cycles. The sequencing of DNA amplicons obtained with the double amplification technique did not show substantial differences compared to the samples amplified directly, without the use of RAPD. The combination of the two techniques, RAPD and classical PCR, allowed us to amplify those remains which previously had not given results. Therefore, our studies can also be focused on those areas where the conditions of archaeological remains particularly damaged by environmental factors, such as soil conditions and the antiquity of the biological specimens.

Acknowledgments

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