Full Length Research Article
Advancements in Life Sciences – International Quarterly Journal of Biological Sciences

ARTICLE INFO

Date Received: 15/09/2020;
Date Revised: 08/01/2021;
Date Published Online: 25/07/2021;

Authors' Affiliation:
1. Department of Genetics Hazara University Mansehra - Pakistan
2. Centre for OMIC Sciences, Islamia college University Peshawar - Pakistan

***Corresponding Author:**
Habib Ahmad
Email: nociralee@gmail.com

How to Cite:
Ali N, Ilyas M, Ahmad H, Khan S, Shafique W, Rehman G, Jamil M, Akbar N, Ilah S, Qayum I, Rahman G. Extraction and Amplification of mtDNA HVR1 from Old and Degraded Samples of Human Skeletal Collected from Khyber Pakhtunkhwa province of Pakistan. Adv. Life Sci. 8(3): 246-250.

Keywords:
Ancient DNA; Degraded samples; mtDNA; HVS1; Pakistan

Open Access

Extraction and Amplification of mtDNA HVR1 from Old and Degraded Samples of Human Skeletal Collected from Khyber Pakhtunkhwa province of Pakistan

Nasir Ali[1], Muhammad Ilyas[2], Habib Ahmad[1]*, Shaker Khan[1], Wajiha Shafique[1], Ghani ur Rehman[1], Muhammad Jamil[1], Nazia Akbar[1], Shkair ul llah[1], Ikram Qayum[1], Gohar Rahman[1]

Abstract

Background: The study of ancient DNA enables the examination of genetic associations between past and present individuals and populations. The recovery and analysis of human DNA from degraded samples precisely has become a central research tool in various scientific fields' ranges from ancient DNA to forensics and medical sciences.

Methods: In the current study, remains of human bones are analyzed that dates back to 1000-1200 BC from different burial sites of Khyber Pakhtunkhwa province of Pakistan. Hypervariable region 1 (HVR1) as a genetic marker was selected. Multiple extractions, qubit assays quantification and cloning of PCR products were performed to get the sequences from the hypervariable region 1 of mtDNA. In this study, we also evaluate the possibility of extracting DNA from degraded human tissues (bones and teeth) such as remains buried in archaeological sites and remains of dead bodies buried for many decades.

Results: Mitochondrial DNA HVR1 of 2000 years old degraded bone specimens were successfully determined by analyzing haplotype, defining polymorphisms in the hypervariable region 1. The prominent haplogroup found was eastern European R (63%) that was also found in present day population by previous studies.

Conclusion: The protocol developed in this study could be used for the extraction of DNA from old degraded and ancient bone samples.

SCAN ME

Introduction

After death due to the lack of DNA repair mechanism, the DNA is degraded rapidly into small fragments through many external challenges like hydrolysis, oxidative nucleotide modifications, the action of cellular nucleases and other hydrolytic enzymes and microbes attack [1,2]. The environmental factors have a major contribution in the degradation of DNA between samples [3]. The real challenge was to overcome these environmental factors and extract PCR amplifiable DNA from degraded samples. The reclamation and analysis of human skeletal tissues like bone and teeth has become a central research tool for scientific fields ranging from ancient DNA to forensics and medical sciences [4]. The optimization of protocol by the previous researchers took more than a year and was applied on different bones of different ages (1000BC to 3500BC). Due to the unique value of ancient skeletons for the purpose of physical anthropology, archaeology, isotopic studies and exhibition, it is clear that a little amount of genetic material is recovered [5-7]. The skeletal materials were recovered under careful and controlled circumstances with the collaboration of archaeologists to minimize the risk of contamination. In the general methods of the DNA isolation, the skeletal samples are ground to form fine powder and decalcified that in turn allows proteolytic digestion of cellular components.

In recent years the Single Nucleotide Polymorphisms (SNPs) have attracted more the attention of scientists for numerous applications ranging from evolutionary study to population and medical genetics [8,9].

SNPs in the non-recombinant part of the mitochondrial genome are particularly valuable because they have a single (maternal) heritage.

In this study, we used human skeletal remains. These specimens were recovered during 2009-2017 from different archaeological expeditions carried out by researchers from Archeology laboratory of the Department of Archeology, Hazara University Manshera Pakistan and by the researchers of Human Genetics Laboratory of Centre for Human Genetics Hazara University, Mansehra, Pakistan from three historical districts of Khyber Pakhtunkhwa. A protocol was drafted so that the excavation and recovering of the bone specimen are collected accordingly to avoid any kind of modern contamination. The approximate locations of these sites are shown in Figure 1. A total of 22 well-preserved samples recovered from Swat (n=12), Dir (n=3), Mansehra (n=2) and Abbottabad (n=5) were selected for DNA analysis. The bone specimens were separately packed in sterilized zip bags and transported in airtight boxes to Centre for Human genetics laboratory where they were stored at -30 °C. A list of all samples used in the study is presented in Table 1.

Methods

Ethical approval of Biosafety conditions

The institutional Bioethical Committee (IBC) of Hazara University Mansehra analyzed the study design against the ethical measures and approved the study. Permission from the Directorate of Archaeology and Museums, Government of Khyber Pakhtunkhwa was also taken for collecting and using human bone specimens from different archaeological sites in the province.

All the steps were carried out laboratories that were physically separated to minimize the risk of any contamination. All the instruments and the surfaces were sterilized by the treatment of chloride bleach and UV radiation were used to sterilized plastic ware and solutions.

Sampling from Archaeological sites and Museums

In the current study, human skeletal remains were used. These remains were recovered by different archaeological expeditions by the archaeologists of Department of Archaeology, Hazara University Mansehra, Pakistan and the researchers of Center for Human Genetics, Hazara University Mansehra, Pakistan during 2009-2017 from four historical districts of Khyber Pakhtunkhwa. The samples were collected according to standard procedure to avoid contamination. The approximate locations of these sites are shown in Figure 1. A total of 22 well-preserved samples i.e. Swat (n=12), Dir (n=3), Mansehra (n=2) and Abbottabad (n=5), where these burials were intact and well preserved were selected for DNA analysis. The bone specimen after light brushing (different brush was used for each bone specimen) for removal of extra soil and mud were used and were packed in sterilized zip bags. The samples were transported in airtight boxes to Centre for Human Genetics laboratory where they were stored at -30^{o}C. in Table 1, the list of samples used in this study are presented.

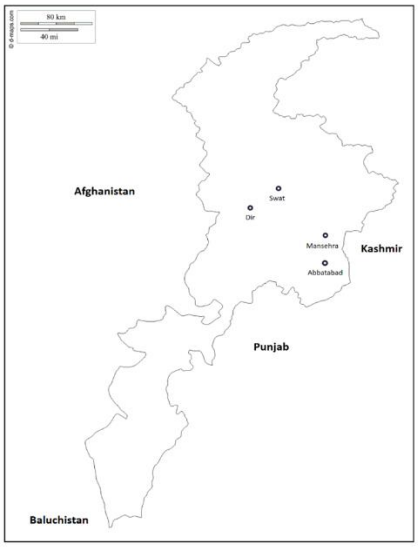

Figure 1: Khyber Pakhtunkhwa (North West province of Pakistan) map with sampling areas highlighted (District Dir, Swat, Abbottabad and Mansehra).

Bone specimen preparation

Before samples processing, the exterior surface was exposed to UV radiation for 20 minutes, followed by brief wiping with 2% bleach (sodium hypochlorite) to avoid

contamination. The outer surface was removed by a saw, file and forceps. After this the bone specimen was washed with HPLC grade water to remove the dust and other particles. The surface layer of bone specimens was removed using Dremel carbon cutting disc and file. About 2-3mm outer surface was removed in order to avoid all possible contamination and a 0.5cm piece was cut off using Dremel tool and file.

Extraction of DNA

Before conducting the study, standard guidelines were followed for the study of ancient DNA and to control DNA contamination. Physically separated two laboratories were used to extract DNA form the samples. The cleaning and dryness of the bone samples were carried out in clean laboratory, making sure no other experiment is going on side by. The DNA was extracted using protocol developed by Centre for Human Genetics laboratory. All the equipment was washed with 15% bleach and with ethanol if necessary and then exposed to UV rays for 20 min before each use. Contaminant free equipment were used in the two laboratories where laboratory was limited to only three researchers who were working in the same laboratory on the same samples. The DNA profiles of the researchers were obtained to overcome the risk of modern contamination. The cut bone specimen were powdered with mortar and pestle in the presence of liquid nitrogen (-200^{o}C). Separate mortar and pestle used for each bone specimen, only one bone specimen was powdered at a time and bone powder were stored at -20^{o}C until further used. About 0.1 to 0.2g of bone powder was used for DNA extraction experiments. A negative control extraction blank was included to avoid contamination.

Amplification and Sequencing

The PCR was carried out in a total 25ml containing Green Taq Ready Mix (Thermo-Fisher) equipped with Taq DNA polymerase, Mgcl2, dNTPs, Taq buffer and primers for Hypervariable Region 1 (HVR1; nucleotide positions 16128–16348) of mtDNA, as per the revised Cambridge reference sequence (rCRSs) [10], were amplified and sequenced. Table no 2 shows primers used that were previously reported [11].

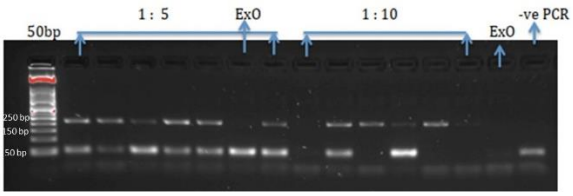

Figure 2: Agarose gel picture of the successfully amplified HVR1 region of mtDNA.

Polymerase chain reaction (PCR) amplification was performed using a minimum of two fragment length size 221bp for HVR1. The 25ml total volume contain ready mix, template DNA, PCR grade water, forward and reverse primers. The PCR conditions were optimized according to the primers and DNA concentration using nano-drop. Initial denaturation was at 95°C/10min, 95°C/30sec, Primer annealing temperature was 60°C/45sec and elongation temperature was 72°C/1min and 72°C/7min respectively. The Amplification was performed on 2720 thermal cycler of applied biosystem and results were visualized on 1% agarose gel. Amplification and sequencing were performed using same primers. Sanger sequencing was performed to identify the polymorphisms in mtDNA HVSI region of the samples. The sequencing was carried out on ABI DNA Sequencer (model 3730) commercially.

Forward primer sequence 5`>3`	Reverse primer sequence 5`>3`	PCR position	Size	Target marker
GGTACCATAAATACTT GACCACCT	GACTGTAATGTG CTATGTACGGTAA A	16153 & 16322	221bp	HVS1

Table 1: Primer sequences and their relative information used in this study.

Sequence analysis using bioinformatics tools

Basic Local Alignment Search Tool (BLAST) were used to make sure the uniqueness of our sequences and avoid the similarity of our sequences with other species. The 221-base pair (bp) hypervariable region 1 (HVR1) sequence was edited and aligned against the rCRSs using the BioEdit software. To confidently assign mtDNA to the relevant haplogroups, 22 haplogroup-diagnostic single nucleotide polymorphisms (SNPs) that define major haplogroups found in South Asian and Western Asian populations. The FASTA sequences were then used to find out exact variants and haplotypes using MITOMASTER [12], HaploGrep (http://haplogrep.uibk.ac.at) and with PhyloTree Build 17 (http://www.phylotree.org).

Results

In the current study, we investigated DNA sequences from a relatively large number of skeletal remains of substantial antiquity (about 1000 years old), excavated, and handled by archeologists with precautions to avoid contamination. Starting with more than 50 bone specimens, we recovered reliable mtDNA sequences from 22 samples from the historical places and museums in Swat, Dir, Mansehra and Abbottabad.

It is also to be keep in view that it is not necessary that the individuals were living in same time and might have separate by several hundreds of years. The current study represents the DNA sample-set of the past population that lived in North-western Pakistan.

A 221bp mtDNA-HVR1 segment between positions 16128 to 16348 was sequenced from the old degraded human bones collected from Khyber Pakhtunkhwa, Pakistan. Within 22 samples, 12 different haplotypes were identified and defined by 44 variable positions. Haplotype prediction was done using MitoMaster [12] and HaploGrep (http://haplogrep.uibk.ac.at). The result obtained from haplotype prediction shows that 15 samples were belonging to macrohaplogroup R, four samples to macrohaplogroup U, two samples belongs to N and only one sample was found that belongs to M haplogroup. In Swat the most prominent haplogroup were found eastern European haplogroup R, which counts 63% of all the mitochondrial samples of current

ID	Area	Mega hg	Possible time of origin	Predicted hg	Origin	Variants
DR13	Dir	U	60,000 years ago	U7	South Asia	A16182AC, A16309G, A16318T
DR14	Dir	M	60,000 years ago	M18	South Asia	A16183C,C16223T, A16309G, A16318T
DR15	Dir	R	70,000 years ago	H2a	Eastern Europe	A16180C, C16256T
SW3	Swat	R	70,000 years ago	H2a	Eastern Europe	T16178C, C16256T
SW4	Swat	U	60,000 years ago	K	West Asia	T16224C, T16311C
SW6	Swat	N	75,000 years ago	W	West Asia	C16223T, C16292T
SW7	Swat	U	60,000 years ago	U4a	Northern Asia	C16223T
SW9	Swat	N	75,000 years ago	I1c	Central and Eastern Europe	C16223T, C16264T, C16291T, T16311C, G16319A
IS5	Swat	R	70,000 years ago	H42a	Undetermined	A16206d, C16294T
IS6	Swat	R	70,000 years ago	H1a	Eastern Europe	C16218T,A16219T, A16340T
IS7	Swat	R	70,000 years ago	H2a	Eastern Europe	A16202d,A16206d, A16219d
IS8	Swat	R	70,000 years ago	H2a	Eastern Europe	A16202d, A16206d
IS9	Swat	R	70,000 years ago	F2i	Southwestern China	G251T,A259C, A263G, G275A
KK1	Swat	R	70,000 years ago	H42a	Undetermined	A16202d, A16206d, A16216T,A16226d, C16294T
KK2	Swat	R	70,000 years ago	T2	European	A16254d, C16294T, C16296T, A16338T
MS10	Mansehra	R	70,000 years ago	H1b	Eastern Europe, Central Asia and North Asia	A16219d, A16220C, C16339T
MS13	Mansehra	U	60,000 years ago	U4a	Northern Asia	A16202d, C16223T
AD2	Abbottabad	R	70,000 years ago	H1a	Eastern Europe	A200G, A263G
AD3	Abbottabad	R	70,000 years ago	H1a	Eastern Europe	T131A, TT138d, C141A, T142A, A200G, A263G
AD4	Abbottabad	R	70,000 years ago	H2a	Eastern Europe	A200d, A263G
AD5	Abbottabad	R	70,000 years ago	H1a	Eastern Europe	TC139d, A200G, A263G
AD6	Abbottabad	R	70,000 years ago	H3a	north-west Europe	T157d, A175d, T179d, A181C, C182T, A211d

Table 2: Complete samples details of bone and teeth samples collected from across the Pakistan (for degraded DNA analysis). Predicted haplogroups and mega haplogroups are also shown.

study. The haplogroup U is identified in samples from Swat and Mansehra. U is ancestral clade of the haplogroup R, it seems to originate from the west Asia around 46000 years ago.

Because of the sensitivity of this protocol, the issue of detection and quantitation of external contamination is important. Careful laboratory practices designed to completely overcome the problem of contaminations are essential in a forensic laboratory and cannot be overstressed. However, in cases where low-level contaminations found, the experiments discussed herein suggest that with careful quantification, correct typing results can be achieved when the ratio of sample to contaminant is greater.

Discussion

DNA from all bone samples was successfully extracted and amplified. The use of the current primers enabled us to successfully amplify mtDNA HVSI region and also the sequencing and assignment of mtDNA haplogroups. The short length of the amplified product makes these primers suitable for highly old and ancient samples. The PCR conditions described in this paper worked successfully for the primers given in table 1. Besides, these primers could be used with a large range of hums bone specimens. Hence, this can be a valuable tool for studying population genetics of human kind.

In the current study, the eastern European haplogroup R was found most prevalent. The eastern European lineage in KP population is also reported in previous studies of modern population [13, 14]. This shows the relation of ancient samples with the current population. studies of modern population [13, 14]. This shows the relation of ancient samples with the current population. The molecular anthropology data in current study may not give simple and explicit answer to the interaction of Khyber Pakhtunkhwa individuals with migrant populations, but provide indications that will help to design a comprehensive and more defined genetic research to recognize features of historical and demographic variations of the populations involved. Moreover, to understand the genetic contribution of the old population on modern population, the number of Old skeletal remains of a wider geographical area will be greater than before, as proposed by the historians.

Acknowledgement

We are thankful to the TRGP Higher Education Commission for financial support. We are also grateful to the Directorate of Archaeology and Museums Peshawar for facilitation and providing us access to the ancient bone samples in different archaeological sites and museums. Special thanks to the people who helped us in exploring different Archeological sites in Khyber Pakhtunkhwa.

Funding

This work was financially supported by the Higher Education Commission of Pakistan. Grant No. (D/Acad/SS&H/TRGP/2016/2010)

Compliance with Ethical Standards

Competing interests

The authors declare that they have no conflict of interests in this work.

Ethics approval

The institutional Bioethical Committee (IBC) of Hazara University Mansehra analyzed the study design against the ethical measures and approved the study.

Author Contributions

NA, MI and HA designed the experiments and supervised the research. NA, SK, WS, GR and MJ performed the DNA isolation and amplification. NA, IQ, SU and NA collected the samples. NA, NA and MI participated in the analyses and provided consultation. NA, MI and HA wrote the paper. All authors have read and approved the manuscript.

References

1. Höss M, Jaruga P, Zastawny TH, Dizdaroglu M, Paabo S. DNA damage and DNA sequence retrieval from ancient tissues. Nucleic acids research. (1996);24(7):1304-7.
2. Pääbo S. Ancient DNA: extraction, characterization, molecular cloning, and enzymatic amplification. Proceedings of the National Academy of Sciences. (1989);86(6):1939-43.
3. Dobberstein RC, Huppertz J, von Wurmb-Schwark N, Ritz-Timme S. Degradation of biomolecules in artificially and naturally aged teeth: implications for age estimation based on aspartic acid racemization and DNA analysis. Forensic science international. (2008);179(2-3):181-91.
4. Adler CJ, Haak W, Donlon D, Cooper A, Genographic Consortium. Survival and recovery of DNA from ancient teeth and bones. Journal of Archaeological Science. (2011);38(5):956-64.
5. Hanihara T. Morphological variation of major human populations based on nonmetric dental traits. American Journal of Physical Anthropology: The Official Publication of the American Association of Physical Anthropologists. (2008);136(2):169-82.
6. Hillson SW. Diet and dental disease. World archaeology. (1979);11(2):147-62.
7. Bently DE, Hatch CT. Shaft levitation made simple. Turbomachinery International. (2006);47(5):30-2.
8. Lai E. Application of SNP technologies in medicine: lessons learned and future challenges. Genome Research. (2001);11(6):927-9.
9. Morin PA, Luikart G, Wayne RK. the SNP workshop group, 2004. SNPs in ecology, evolution and conservation. Trends Ecol. Evol. (2004);19:208-16.
10. Andrews RM, Kubacka I, Chinnery PF, Lightowlers RN, Turnbull DM, Howell N. Reanalysis and revision of the Cambridge reference sequence for human mitochondrial DNA. Nature genetics. (1999);23(2):147-.
11. Kjellström A, Edlund H, Lembring M, Ahlgren V, Allen M. An analysis of the alleged skeletal remains of Carin Göring. PLoS One. (2012);7(12):e44366.
12. Lott MT, Leipzig JN, Derbeneva O, Xie HM, Chalkia D, Sarmady M, Procaccio V, Wallace DC. mtDNA variation and analysis using MITOMAP and MITOMASTER. Current protocols in bioinformatics. (2013);44(1):1-23.
13. Ullah, I, Ahmad, H, Hemphill, BE, Nadeem, MS., Tariq, M, Tabassum, S. Mitochondrial genetic characterization of Gujar population living in the Northwest areas of Pakistan. Advancements in Life Sciences. (2017); 4(3): 84-91.
14. Akbar, N, Ahmad, H, Nadeem, MS, Hemphill, BE, Muhammad, K, Ahmad, W, Ilyas, M. HVSI polymorphism indicates multiple origins of mtDNA in the Hazarewal population of Northern Pakistan. Genetics and Molecular Research. (2016); 15(2): 15027167.