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Matthew C. Dulik
University of Pennsylvania, dulik@sas.upenn.edu

Joseph G. Lorenz

Theodore G. Schurr
University of Pennsylvania, tgschurr@sas.upenn.edu

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Hasanlu IVB: An Ancient DNA Pilot Project

Matthew C. Dulik, Joseph G. Lorenz, and Theodore G. Schurr

Introduction

Throughout the periods of Hasanlu's history, patterns emerge which suggest both a persistence of cultural elements (architectural features, ceramic types, etc.) and, at times, a discordance marking the boundaries of historical periods (Dyson 1989). This discontinuity, especially as it exists with the spatial changes between Periods IV and III, indicates a possible change in the community residing at the site. This change may have resulted in the complete displacement of an earlier population by a new group of individuals, or merely a shift in the authority presiding over the region.

Evidence of such a change (or lack of change) in populations may be observed in the genetic material of the site's inhabitants. In fact, previous morphological analysis of a portion of the human remains exhumed from the site indicated the presence of at least two distinct populations (Rathbun 1972). However, this study relied mostly on a few nonmetric traits. A better understanding of the population's history may be possible by examining the DNA of these individuals. In addition, the carnage left behind from the Period IVB battle provides an opportunity to examine the inhabitants of Hasanlu, as well as their invaders, who ultimately brought an end to the major Iron Age II occupation.

In order to understand the population history of the site, we extracted genetic material from the ancient inhabitants' remains to determine whether DNA exists in them and, if so, whether differences in the genetic material could aid in understanding the dynamics of these populations. This process is made possible by means of the polymerase chain reaction (PCR). PCR allows the rapid replication of DNA sequences such that low amounts of DNA can be amplified exponentially. Since its conception in the mid-1980s, this simple, yet efficient technique has been fundamental to molecular genetics and has been utilized for innumerable applications.

If any ancient DNA (aDNA) is present in a sample, then the best chance of amplifying DNA is to target the mitochondrial DNA (mtDNA). mtDNA is a preferable source of genetic information from human remains because it is present in higher copy number than nuclear DNA genes. Also, mtDNA is informative as a marker because it does not recombine and is passed down from mother to child. In this manner, one can use it to determine the maternal genetic history of a population. In addition, this molecule has a fair degree of discriminatory power such that, in many cases, contamination from modern sources can be easily recognized.

One segment of the mtDNA is particularly valuable. This segment, called the hypervariable region 1

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(HVR1), has a higher mutation rate than the rest of the molecule. Accordingly, mutations (changes in the DNA sequence) accumulate rapidly within this fragment, thereby allowing for an increase in differences between individuals over time. These differences provide the data necessary for understanding relationships between individuals, the structure and history of a population, and the extent of interactions between these populations.

aDNA obtained from human remains provides unique challenges that require specialized labs and techniques to overcome the problematic characteristics of these molecules. Oftentimes, the DNA is degraded, resulting in the presence of only short fragments no larger than about 200 base pairs in length. Multiple amplifications of overlapping fragments must be performed in order to reconstruct longer sequences. In addition, because aDNA is usually degraded, the introduction of a modern contaminant is the greatest hazard associated with this work (Yang and Watt 2005). DNA from inadequately cleaned equipment, benches, or disposable supplies, or cells from anyone who came in contact with the samples (excavators, osteologists, geneticists) can be easily shed from the modern source to the sample. These modern contaminants usually outcompete the aDNA when amplified because the modern DNA is not as damaged as the ancient form. Even a single contaminating cell can provide thousands of copies of intact mtDNA, as compared to far fewer generated from damaged aDNA molecules.

In addition to the problems of contamination, PCR inhibitors can be co-extracted with aDNA. These inhibitors are often found in the soil from which the remains were excavated and can be absorbed into the bone (including tannins, humic acid, and Maillard products). The presence of some proteins found in living organisms (for example, collagen) can also inhibit the PCR (Wilson 1997). Removal of these inhibitors is critical for the characterization of aDNA.

The purpose of this initial study was to determine the feasibility of extracting aDNA from human teeth of several individuals excavated at Hasanlu, Iran. If it was present, then the larger scope of this research project would involve examining the population affinities of the human remains, dating to the Iron Age (Periods III, IV, and V), found on the Citadel Mound and the Lower Mound (cemetery) at the site.

Materials and Methods

Samples

Six teeth were chosen from individuals previously analyzed using craniometric methods to address issues of population affinity (Dulik 2005). As a group, these individuals (58-4-96; 58-4-100; 59-4-105; 61-5-341; 63-5-314; 65-31-734) were excavated in both the lower and upper mounds of the site and dated to Period IV.[1] The teeth were chosen because of their completeness and lack of pathology. In addition, these teeth were still in their respective alveolar crypts, which is beneficial because it reduces the potential for contamination from researchers who handled the remains during past studies.

Pre-PCR Lab Conditions

Control of contamination is critical for aDNA analyses (Yang and Watt 2005). All sample handling and preparations were carried out in a pre-PCR clean lab dedicated to ancient DNA analysis. No modern DNA samples were extracted or amplified in this space. Lab coats, masks, disposable gloves, and sleeves were used in this lab to prevent contamination of the ancient samples with DNA from the researchers. The hood and equipment were cleaned with full-strength bleach before and after use of the lab and in between each sample extraction. Anything entering the pre-PCR lab was wiped down with bleach. In addition, the hood surface and accessories inside the hood were exposed to UV irradiation at 254 nm to make any potential DNA in the hood unamplifiable (Ou, Moore, and Schochetman 1991).

Sample Preparation

After photo-documentation of the teeth, the samples were transported to the pre-PCR Ancient DNA Lab at the Coriell Institute for Medical Research in Camden, NJ, where they were subsequently processed. The surfaces of the tooth roots were scraped with sterile scalpels, and the samples were UV irradiated for 30 minutes per exposed side. A 0.125 inch borer attachment that fits a Dremel™ drill was used to slowly to enter the pulp cavity to avoid spreading bone powder or overheating the sample, as these incidents

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could aide in the cross-contamination of samples and destruction of any authentic aDNA inside the tooth, respectively.

DNA Extraction

Powdered samples were incubated in 2 ml of 0.5 M EDTA, with 100 µl of Proteinase K and 100 µl of 0.1 M N-phenacylthiazolium* bromide (PTB) and were placed on a shaker at 37°C overnight. Additional Proteinase K and PTB were added the following day and left at 37°C. An extraction buffer containing Tris, $NaCl_2$, 10% SDS, and ddH_2O was used to lyse the cells, freeing the DNA for extraction. Throughout the entire extraction process, open and closed negative controls were used to detect the presence of systematic contamination of samples. These controls were prepared in the same manner as the ancient samples, except that no DNA source was purposely added. Thus, a positive amplification from these controls would indicate that contaminating DNA was present during the extraction of the samples.

The aDNA was obtained by phenol/chloroform extraction of the organic materials in solution. Equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1) were added to the aqueous portion of the bone powder/EDTA mixture and spun at 13,000 rpm for 10 minutes. This step was repeated once. The aqueous phase containing nucleic acids was aliquoted to a new tube and mixed with an equal volume of chloroform: isoamyl alcohol (24:1). After re-spinning the sample, the aqueous phase was removed to a fresh tube. The DNA was then precipitated with an equal volume of cold 95% ethanol and then washed with 70% ethanol. The samples were air-dried in the pre-PCR hood and resuspended with 1X TE (10mM Tris; 1 mM EDTA, pH7.8).

DNA Amplification

PCR amplifications were carried out with 5 µl of DNA, 1X PCR Buffer, 2.5 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM primers, 10mg/ml bovine serum albumin, and 2.5 Units of Taq polymerase in a 25 µl total volume. DNase I and its buffer were added to each 0.2 ml reaction tube prior to adding the primers, Taq polymerase and DNA. The tubes were incubated at room temperature for 15 minutes followed by incubation at 65°C to deactivate the DNase I enzyme (Eshleman and Smith 2001). The DNase I enzyme was added to destroy any DNA in the PCR reaction tube before the primers, Taq polymerase, and sample were added.

The PCR conditions had an initial 5- to 10-minute hold at 95°C followed by 40 cycles of 1-minute holds at 95°C (denaturing step), 54°C (annealing step; temperature varied depending on the primers used), and 72°C (extension step), with a final extension of 10 minutes at 72°C. PCR products were run through 1% polyacrylamide gels, stained with ethidium bromide, and visualized under a UV transilluminator. Negative controls were used for each PCR amplification. These were prepared identically to aDNA samples, except that no DNA was added to the tubes. Positive controls were prepared in the post-PCR lab and amplified to test the efficiency of each PCR test.

Multiple approaches were employed to determine the presence of authentic ancient DNA and to identify potential modern contamination. The presence of inhibitors was checked by attempting amplification of samples, quantitating DNA concentration by spectrophotometry using a NanoDrop ND-1000 Spectrophotometer, and spiking known positive controls with the ancient DNA samples. Inhibition of the PCR amplification process was also examined through PCR amplification of both stock samples as well as diluted DNA samples.

Results

After DNA extraction, PCR amplification of a short fragment of mtDNA was attempted. This amplification failed, indicating that either there was no DNA present in the sample or that PCR inhibitors were co-extracted with the aDNA. The presence of PCR inhibitors was suggested by the lack of initial amplification and peaks at a wavelength of 260 nm in the spectrophotometer readings for these samples.

The presence of an inhibitor was confirmed through a spiked control sample test. For this test, modern positive control samples that successfully amplified in previous PCRs were mixed with aliquots of ancient DNA. Attempts to amplify these spiked modern samples failed, suggesting that PCR inhibitors were still present in the aDNA stock solutions and interfered with the PCR reaction for the modern samples. A back-extraction using equal amounts of

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chloroform: isoamyl alcohol (24:1) was performed to remove any residual phenol that might still be present from the initial organic extraction. After this step, the spectrophotometer readings indicated a reduction in the 260 nm peak but also in the overall concentration of DNA (from about 13 ng/µl to 3 ng/µl or lower).

To obtain a more accurate assessment of the DNA concentrations, we used a real-time PCR assay that employs a 5-prime fluorogenic assay (Lorenz et al. 2002). This assay specifically targets the human mitochondrial gene, cytochrome B, and can accurately estimate the total concentration of a sample's mtDNA that is less than a pictogram per microliter. The Real-Time PCR consisted of 2 µl aDNA, 1X RT PCR buffer, 1.75 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM primers, 0.05 µM cytB probe, and 1 Unit Taq polymerase, and was run on an ABI 7900 Real-Time Thermocycler. This analysis indicated that DNA was present and amplifiable.

To determine whether the DNA was authentic aDNA or instead a modern contaminant, the HVR1 segment of the mtDNA was amplified and sequenced. We used three pairs of primers to generate overlapping fragments that covered the entire HVR1 segment. For those samples that amplified for HVR1, QIAquick PCR Purification kits (Qiagen) were used to remove excess single stranded DNA and primers. Samples were sequenced using BigDye Terminator kits v.3.0 (Applied Biosystems) and cleaned up using Dye-Ex Spin columns (Qiagen). Samples were dried down and resuspended in 10 µl of Hi-Di Formamide and read using an ABI 3700 Gene Analyzer. The resulting sequences were compared to the revised Cambridge Reference Sequence (Anderson et al. 1981; Andrews et al. 1999) with Sequencher v.3.0 (Gene Codes).

The results of the HVR1 sequencing were inconsistent (Table 8.1). Different mutations were scored from separate amplification and sequencing events for several of the samples. These mutations are presented in Table 8.1 along with the range of base pairs sequenced. Three of the samples (58-4-96, 58-4-100, and 63-5-314) never produced readable sequences. Sample 59-4-105 produced two different sequences that overlapped in coverage. However, the presence/absence of the 16209 polymorphism in both was not confirmed. Sample 61-5-341 was successfully amplified and sequenced only once. Variable nucleotides 16193 and 16278 are commonly found together in mtDNA haplogroup J2, although two other mutations (16069 and 16126) should have been found as well. Sample 65-31-734 provided three distinctive sequences, none of which could be confirmed as authentic aDNA. Extraction blanks and negative PCR controls amplified occasionally, but never consistently.

Table 8.1. Sequence Results for Hasanlu Ancient DNA Samples

Sample	HVS1	Range Sequenced
58-4-96	N/A	N/A
58-4-100	N/A	N/A
16126-16187-16189	16000-16236	
	16189-16209	16189-16346
61-5-341	16193-16278	16112-16345
63-5-314	N/A	N/A
16189-16209	16000-16237	
	16176-16184	16111-16375
	16223	16112-16345
Extract Blank1	16189	16000-16189
	16189-16209	16189-16346
Extract Blank2	16223	16111-16375
PCR Negative1	16189	16000-16189
PCR Negative2	16223	16112-16345

Conclusions

The most likely reason for this is amplification of a random contaminant. These contaminants can come from clothes, bacteria, lab coats, or "sterile" disposables (Hummel 2003). DNA profiles of those who either worked in the ancient DNA lab or handled the human remains most recently did not match any of the aDNA results and were never amplified. This finding shows that the proper precautions in extraction and analysis of the samples were successful in avoiding contamination. Occasionally, PCR blanks would amplify and be sequenced to identify the source of contamination, but these results were also inconsistent, indicating a problem with supposedly DNA-free supplies. In addition, the amelogenin gene was also targeted to determine the sex of the individual. However, all attempts failed, most likely because the nuclear DNA was too degraded for amplification.

Ultimately, the results indicate that authentic aDNA was not reproducibly amplifiable. It is possible

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that no aDNA remained in the teeth for extraction. However, each sample potentially went through different taphonomic events, which could affect the viability of obtaining DNA from the human remains. Heat and water have been shown to have damaging effects on the preservation of DNA in human bone, and acidic soil conditions can also have a negative impact on successful DNA extraction (Hummel 2003). It is possible that other remains from Hasanlu could contain aDNA, but its presence is entirely dependent on the conditions for each burial, which make it difficult to predict which samples could be successfully extracted. In addition, because the results of HVR1 sequencing were inconsistent, contamination occurred irregularly and never in a systematic fashion. Therefore, the procedures implemented in the aDNA extraction and analysis was effective in not introducing new contaminants. While these results are not conclusive with respect to the population history of these remains, new DNA extractions of the samples are necessary for verifying the presence of aDNA. If successful, analysis of an expanded set of samples would permit the examination of larger questions concerning population replacements. In addition, this project does confirm that the series of procedures used in this study are effective in validating or discrediting the authenticity of aDNA from human remains.

NOTE

1. Samples:

UPM NO.	HAS SKELETON NO.	CONTEXT
58-4-96	SK 3	Cemetery (OP VI)
58-4-100	SK 18	Cemetery (OP VI)
59-4-105	SK 37	BB IW Rm 9
61-5-341	SK 127	BB II Rm 5
63-5-314	SK 209	BB VIII Rm 2
65-31-734	SK 480	Cemetery (OP VIF)

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